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Phosphorylation of the Hepatitis C Virus NS5A Protein In Vitro and In Vivo: Properties of the NS5A-Associated Kinase

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NS5A derived from a hepatitis C virus (HCV) genotype 1b isolate has previously been shown to undergo phosphorylation on serine residues (T. Kaneko, Y. Tanji, S. Satoh, M. Hijikata, S. Asabe, K. Kimura, and K. Shimotohno, Biochem. Biophys. Res. Commun. 205:320–326, 1994). In this report, phosphorylation of NS5A derived from HCV isolates of the 1a and distantly related 2a genotypes is demonstrated. Phosphoamino acid analysis of NS5A from the 1a isolate indicated that phosphorylation occurs predominantly on serine, with a minor fraction of threonine residues also being phosphorylated. NS5A phosphorylation was observed in diverse cell types, including COS-1, BHK-21, HeLa, and the hepatoma cell line HuH-7. Phosphorylation of a glutathione *S*-transferase (GST)/HCV-H NS5A fusion protein was also demonstrated in an in vitro kinase assay. This activity seemed to be highest when the pH of the reaction was neutral or slightly alkaline and displayed a preference for Mn^{2+} over Mg^{2+}, with an optimum concentration of approximately 10 mM Mn^{2+}. Somewhat surprisingly, in vitro phosphorylation of NS5A was inhibited by the addition of ≥0.25 mM Ca^{2+} to reaction buffer containing Mn^{2+} and/or Mg^{2+}. Comparison of phosphopeptide maps of NS5A phosphorylated in vitro and in cultured cells showed that most of the phosphopeptides comigrated, suggesting that one or more kinases involved in NS5A phosphorylation in vivo and in vitro are the same. The effects of various kinase inhibitors on NS5A phosphorylation were consistent with a kinase activity belonging to the CMGC group of serine-threonine kinases. The development of an in vitro kinase assay for NS5A phosphorylation should facilitate identification of kinase(s) responsible for its phosphorylation and of phosphorylation sites which may influence the function of NS5A in HCV propagation.

An estimated 1.4% of the general U.S. population is seropositive for hepatitis C virus (HCV), which corresponds to approximately 3.5 million people (5). The incidence of HCV infection in other countries varies but is at least 2% in Japan, certain Middle Eastern countries, and South America, as estimated from surveys of healthy individuals and/or blood donors (5, 34). HCV establishes chronic infections in a large percentage of these individuals and is associated with progressive liver pathology, including cirrhosis and hepatocellular carcinoma (reviewed in reference 43).

HCV has been classified in its own genus in the family *Flaviviridae*, which also includes the *Flavivirus* and *Pestivirus* genera. All members of this family have enveloped virions containing a positive-sense, single-stranded RNA genome with one long open reading frame. In the case of HCV, this genome is approximately 9.5 kb in length and encodes a polyprotein of just over 3,000 amino acids (aa) that is processed by a combination of host and viral proteases into at least 10 individual proteins. In order from the N terminus of the polyprotein, these proteins are C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. C is the putative capsid protein, E1 and E2 are thought to be envelope glycoproteins, p7 is a polypeptide of unknown function that is inefficiently cleaved from E2, and NS2 through NS5B are presumed nonstructural proteins which may be involved in replication of the viral genome.

Labeling experiments with [^{32}P]orthophosphate and phosphoamino acid analysis (PAA) of NS5A from the genotype 1b isolate HCV-J expressed transiently in COS-1 cells have shown that NS5A is phosphorylated on serine residues (24). Phosphorylation has been observed in the absence of other viral proteins (44), suggesting either that NS5A catalyzes its own phosphorylation or, more likely, that one or more cellular kinases are responsible. Deletion mapping and mutagenesis experiments have suggested that three serines in the central region (Ser-2197, Ser-2201, and Ser-2204) and additional unidentified residues in the C-terminal region of NS5A are sites for phosphorylation (44); however, none of the sites has been definitively identified by amino acid sequencing. Although a 56-kDa form of phosphorylated NS5A is produced in the absence of other nonstructural proteins, interaction of the N-terminal region of HCV-J NS5A with NS4A has been shown to stimulate production of a 58-kDa phosphorylated form (6). Deletions in the N-terminal region of NS5A have also been observed to enhance production of the 58-kDa form (6).

Previous studies of NS5A phosphorylation have focused on mapping regions in NS5A and NS4A of the HCV-J isolate that may be involved in phosphorylation, but NS5A phosphorylation has not been reported for other HCV genotypes. Analyses of NS5A phosphorylation in other genotypes are especially important given that the amino acid sequence identity between NS5A from the genotype 1b isolate HCV-J and the closely related, genotype 1a isolate HCV-H is approximately 80% and drops to 60% in comparison to the distantly related, genotype 2a isolate HCV-J6. Furthermore, little or no information is available concerning the activity(ies) responsible for NS5A phosphorylation. Therefore, the phosphorylation of NS5A from the HCV-H and -J6 isolates was analyzed to determine whether NS5A phosphorylation is a feature conserved among HCV isolates or unique to the HCV-J isolate. Also, to facilitate efforts to identify the kinase(s) responsible for NS5A phosphorylation and characterize its activity, an in vitro assay for phosphorylation of HCV-H NS5A was developed and analyzed for the effects of various reaction parameters, including

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pH, divalent cation concentration, temperature, length of incubation, and various kinase inhibitors on the observed phosphorylation. The relevance of this in vitro assay to phosphorylation of NS5A in cell culture was investigated by comparing phosphopeptide maps and kinase inhibitor profiles of NS5A phosphorylated in vitro or in intact cells.

MATERIALS AND METHODS

Plasmid constructs. Unless otherwise indicated, all HCV-coding regions were derived from cDNA clones of the HCV-H strain (17). Plasmids pTM3/HCV 827-3011, pTM3/HCV1712-2420, pBRTM/HCV1973-3011, and pTM3/SINnsP3 have been described elsewhere (17, 29, 31). pTM3/HCV-H 5A was obtained by replacing the *Xba*I-*Sse*8387I fragment of pTM3/HCV1712-2420 with the *Xba*I-*Sse*8387I fragment of pBRTM/HCV1973-3011 (32). In all of these constructs, the region of interest is expressed under the control of a promoter for the bacteriophage T7 RNA polymerase.

Three changes in the NS5A sequence of pTM3/HCV-H 5A, $P_{2341}S$, $M_{2365}T$, and $F_{2400}L$, were made by replacing its *Spl*I-*Xho*I fragment with the *Spl*I-*Xho*I fragment of a PCR product amplified from pMTL/HCV 5923-9340/9 (26) with the primers corresponding to the N- and C-terminal regions of NS5A (BRL 313 [5′-AATCCATGGGGATTCCCTTTGTGTCC-3′] and CMBL 452810 [5′-GGC TCGAGCTAGCAGCACACGACATC-3′]). These changes were introduced to create a match with the consensus NS5A sequence recently determined for the HCV-H strain (27). pTM3/HCV-J6 5A was constructed by inserting the *Nco*I-*Sal*I fragment of a PCR product amplified from clone ϕ8 of the HCV-J6 isolate (39) with primers corresponding to the N- and C-terminal regions of NS5A (BRL 336 [5′-CACCATGGCAAGCGGCTCGTGGCTC-3′] and BRL 338 [5′-TAGT CGACCTAGCAGCACACGACGGA -3′]) into the *Nco*I-*Sal*I site of pTM3 (37).

To construct pGEX-3x/HCV-H 5A, a plasmid containing the last 16 aa of NS4B and the entire NS5A region fused N terminally to glutathione *S*-transferase (GST), pTM3/HCV 1712-2420 was digested with *Stu*I and *Bsu*36I, the 5′ overhang created by *Bsu*36I was filled in by T4 DNA polymerase, and the resulting blunt-ended fragment was subcloned into the *Escherichia coli* expression vector pGEX-3x (Pharmacia), which had been linearized with *Eco*RI and treated with T4 DNA polymerase. To construct pTM3/GST/HCV-H 5A, the *Afl*III-*Eae*I fragment of a PCR product amplified from pGEX-3x/HCV-H 5A with primers corresponding to the N terminus of GST (BRL 306 [5′-TATACA TGTCCCCTATACTAGGTTATTG-3′]) and the C terminus of NS4B (CMBL 487525 [5′-CGCTCGAGCTAGCATGGAGTGGTACA-3′]) and the *Eae*I-*Sse*8387I fragment of pGEX-3X/HCV-H 5A were ligated into pTM3/HCV-H 5A that had been linearized with *Sse*8387I and partially digested with *Nco*I. To construct pTM3/GST, the *Afl*III-*Eae*I GST fragment described above and the *Eae*I-*Bam*HI fragment of pGEX-3x were ligated into the *Nco*I-*Bam*HI site of pTM3.

pGEX-3x/HCV-H 2179-2420 was constructed by linearizing pTM3/HCV 2179-2420 with *Stu*I, partially digesting it with *Nco*I, removing the 5′ overhang by treatment with mung bean nuclease, and ligating the resulting blunt-ended fragment into the *Sma*I site of pGEX-3x. pTM3/HCV-H 2179-2420 was constructed by replacing the *Nco*I-*Sst*I fragment of pTM3/HCV-H 5A with the *Nco*I-*Sst*I fragment of a PCR product amplified with primers BRL 314 (5′-TCACCATG GCATCCCATATAACAGCA-3′) and CMBL 452810.

To construct pOP13/CMV/HCV-H 5A, which expresses HCV-H NS5A under the control of a human cytomegalovirus promoter recognized by the transcription machinery of mammalian cells, pTM3/HCV-H 5A was linearized with *Xho*I, partially digested with *Nco*I, and treated with T4 DNA polymerase; the resulting blunt-ended fragment was inserted into pOP13/CMV (Stratagene) (2) that had been linearized with *Not*I and treated with T4 DNA polymerase to fill in the cohesive ends.

Transient expression. For transient expression using the vaccinia virus-T7 hybrid system, slightly subconfluent cell monolayers were infected with vTF7-3 (15) at a multiplicity of infection of 10 PFU/cell in phosphate-buffered saline (PBS) containing 1% fetal bovine serum (FBS) (200 μl per 35-mm-diameter well) for 30 min at room temperature and then transfected with 5 μl of Lipofectamine (GIBCO-BRL) and 1 to 2 μg of plasmid DNA in 500 μl of minimal essential medium (MEM) for 2 h at 37°C. For transient expression in the absence of vTF7-3 infection, subconfluent cells were transfected with 5 μl of Lipofectamine and 2 μg of plasmid DNA per 35-mm-diameter of dish in 500 μl of MEM for 4 h, followed by incubation for 20 h in MEM–5% FBS at 37°C. For analysis of expression and/or phosphorylation in vivo, cells transfected by either method were then incubated for 3 to 4 h in Expre^{35}S^{35}S (50 to 80 μCi/ml; NEN) in MEM containing 1/50 the normal concentration of methionine supplemented with 3% dialyzed FBS or [^{32}P]orthophosphate (100 to 400 μCi/ml; ICN) in MEM lacking phosphate supplemented with 3% dialyzed FBS. For analysis of phosphorylation in vitro, cells that had been infected with vTF7-3 and transfected with the indicated plasmids were incubated in MEM–5% FBS for 4 to 6 h at 37°C.

RIP. Cells were washed twice with cold PBS and harvested in 300 μl of sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-Cl [pH 7.5], 0.5% SDS, 1 mM EDTA, 20 μg of phenylmethylsulfonyl fluoride [PMSF] per ml); cellular DNA was sheared by repeated passage through a 27.5-gauge needle. Lysates were stored at −80°C or analyzed immediately by immunoprecipitation and SDS-polyacrylamide gel electrophoresis (PAGE). For analytical-scale radioimmunoprecipitation (RIP), lysates were heated at 75°C for 10 min, 200 μl of TNA (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 0.67% bovine serum albumin, 1 mM EDTA, 0.33% Triton X-100, 20 μg of PMSF per ml) was mixed with 50 μl of lysate, 5 μl of WU123 (17) was added, and immune complexes were allowed to form by overnight incubation at 4°C with rocking. Immune complexes were collected by adding 50 μl of prewashed Pansorbin cells (Calbiochem) and incubation 1 to 2 h at 4°C with rocking. The resulting immunoprecipitates were washed three times with TNAS (TNA containing 0.5% SDS) and once with TNE (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 20 μg of PMSF per ml), solubilized by heating at 75°C for 10 min in protein sample buffer, and analyzed by SDS-PAGE. For preparative-scale RIP, the following modifications were made to the foregoing procedure: 1.2 ml of TNAS was mixed with 0.3 ml of lysate and incubated with 15 μl of WU123, and immune complexes were collected by incubation with 50 μl of protein A-agarose that had been prewashed and resuspended in TNAS at a 1:1 ratio.

PAA and PPM. ^{32}P-labeled HCV-H NS5A and GST/HCV-H NS5A fusions were isolated by SDS-PAGE (8% gel) of preparative-scale RIPs from cultured cells or of samples phosphorylated in vitro, semidry electrotransfer to Immobilon-P (Millipore), and excision of the appropriate radiolabeled bands. Two-dimensional PAA and phosphopeptide mapping (PPM) were performed according to established protocols (46). For PAA, strips containing the phosphoproteins of interest were cut into small bits, wetted briefly with methanol followed by water, and hydrolyzed by incubation in a screw-cap tube containing 6 N HCl for 1 h at 110°C. After hydrolysis, samples were lyophilized, dissolved in pH 1.9 buffer (2.2% formic acid, 7.8% glacial acetic acid), and mixed with 2 μg each of nonradiolabeled phosphoserine, phosphothreonine, and phosphotyrosine per ml. Samples were spotted on 20- by 20-cm glass-backed plates coated with a 100-μm layer of cellulose. Conditions for electrophoresis were 20 min at 1.5 kV in pH 1.9 buffer in the first dimension and 16 min at 1.3 kV in pH 3.5 buffer (0.5% pyridine, 5% glacial acetic acid) in the second dimension. Plates were then air dried and heated 10 min at 65°C after the application of 0.2% ninhydrin in ethanol to allow visualization of the nonradiolabeled phosphoamino acid standards. Radiolabeled phosphoamino acids were detected by autoradiography with an intensifying screen.

For PPM, excised strips containing the phosphoprotein of interest were wetted briefly in methanol followed by water, incubated in 0.5% polyvinylpyrrolidone-360–100 mM acetic acid for 30 min at 37°C to block nonspecific adsorption of proteases, washed five times with water, and washed twice with freshly prepared 50 mM NH_4HCO_3. The samples were digested with 10 μg of trypsin for 3 h at 37°C; another 10 μg of trypsin was added; and incubation was continued overnight at 37°C, followed by the addition of 10 μg of chymotrypsin and incubation for another 3 h at 37°C. Soluble peptides released into the buffer by protease digestion were then transferred to another tube and pooled with the wash solution obtained by vortexing the remaining bits of Immobilon in 400 μl of water. The resulting mixture was lyophilized, dissolved in 50 μl of freshly prepared performic acid, and incubated for 0.5 to 1 h on ice to ensure that the peptides were fully oxidized and thus eliminate differences in migration due to the presence of various oxidation-state isomers. The performic acid solution was prepared by mixing 9 parts of 98% formic acid and 1 part of 30% hydrogen peroxide, followed by incubation at room temperature for 0.5 to 1 h. The oxidation step was terminated by the addition of 400 μl of water and freezing on dry ice. Frozen samples were lyophilized and dissolved in 10 μl of pH 1.9 buffer, and the amount of radioactivity in 0.5 μl was determined by scintillation counting. Samples were spotted on thin-layer cellulose plates similar to those used for PAA and separated in two dimensions by electrophoresis in pH 1.9 buffer for 25 min at 1.5 kV, followed by ascending chromatography in 7.5% glacial acetic acid–25% pyridine–37.5% *n*-butanol.

In vitro kinase assay. Thirty-five-millimeter-diameter wells of cells expressing GST or GST/HCV-H NS5A were lysed in NETN (120 mM NaCl, 1 mM EDTA, 50 mM Tris-Cl [pH 7.5], 0.5% Nonidet P-40) containing 5 mM dithiothreitol (DTT) and the protease inhibitors aprotinin (1 μg/ml), leupeptin (1 μg/ml), and PMSF (20 μg/ml). The lysates were clarified by centrifugation at 4°C for 15 min at 16,000 × *g*; the supernatants were pooled, and equal portions were incubated with 50 μl of a 1:1 suspension of glutathione-agarose in NETN containing 5 mM DTT and protease inhibitors for 0.5 to 1 h at 4°C with rocking. The glutathione-bound complexes were washed once with NETN containing 5 mM DTT and protease inhibitors and twice with NETN containing protease inhibitors. In standard reactions, the glutathione-bound complexes were then washed once with KWB (50 mM Tris-Cl [pH 7.5], 5 mM $MnCl_2$, 5 mM DTT) and resuspended in 25 μl of the same solution supplemented with 50 mM NaF and 10 μCi of [γ-^{32}P]ATP (Amersham) (KRB). Reaction mixtures were incubated for 10 to 15 min at 37°C, and reactions were terminated by the addition of 25 μl of 3× protein sample buffer.

Kinase inhibitors. Bisindolylmaleimide I-HCl, olomoucine, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB), staurosporine, and hypericin were purchased from Calbiochem; *N*-[2-(*p*-bromocinnamyl-amino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89) and *N*-(2-aminoethyl)-5-chloronaphthalene-1-sulfonamide (A-3) were purchased from ICN.

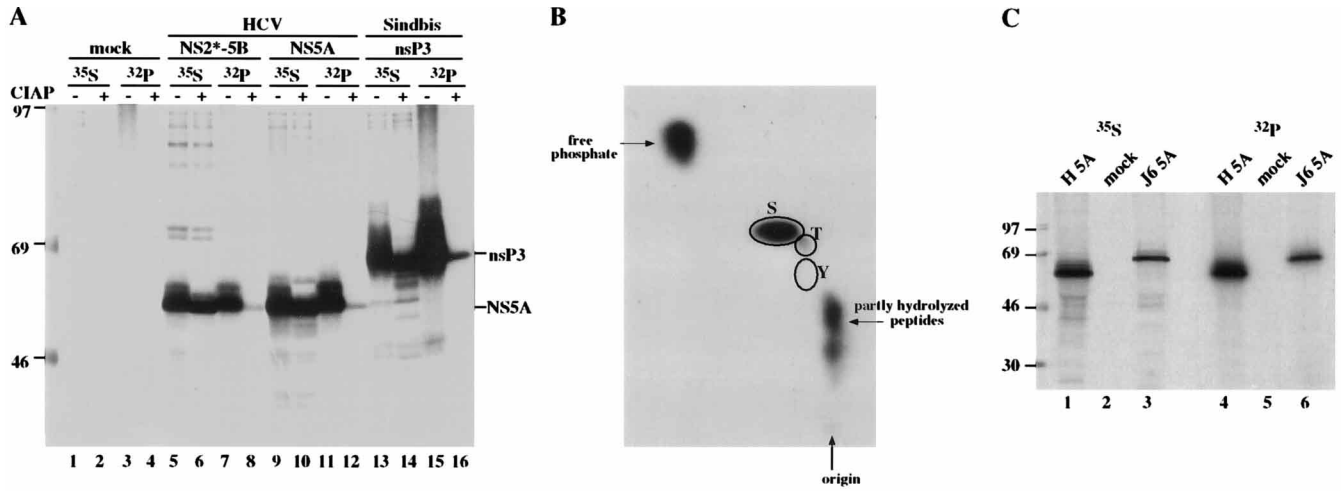

FIG. 1. Phosphorylation of NS5A from the HCV-H and -J6 isolates in cultured cells. (A) Phosphorylation of HCV-H NS5A in the presence or absence of other nonstructural proteins. BHK-21 cells previously infected with vTF7-3 were transfected with pTM3 (lanes 1 to 4), pTM3/HCV 827-3011 (lanes 5 to 8), pTM3/HCV-H 5A (lanes 9 to 12), and pTM3/SINnsP3 (lanes 13 to 16) for 2 h at 37°C and labeled for 3.5 to 4 h with ^{35}S protein-labeling mix or [^{32}P]orthophosphate as indicated. Lysates were prepared; HCV NS5A and Sindbis virus nsP3 were immunoprecipitated with specific antisera (17, 29) and separated by SDS-PAGE (8% gel). The even-numbered lanes were treated with 20 U of calf intestinal alkaline phosphatase (CIAP) for 1 h at 37°C prior to SDS-PAGE. Mock phosphatase treatments were performed on samples in the odd-numbered lanes. (B) PAA of HCV-H NS5A. ^{32}P-labeled NS5A, immunoprecipitated by the preparative method, was subjected to PAA as described in Materials and Methods. The positions of unlabeled phosphoamino acid standards are indicated. (C) Comparison of HCV-H and -J6 NS5A phosphorylation. The experiment was conducted as described for panel A except that cells were transfected with pTM3/HCV-H 5A or pTM3/HCV-J6 5A, as indicated. Sizes are indicated in kilodaltons.

RESULTS

Seryl phosphorylation of NS5A is a conserved feature among divergent HCV isolates. Phosphorylation of NS5A on serine residues has been reported for the HCV-J isolate, a member of the 1b genotype (24). However, NS5A phosphorylation has not been reported for other HCV genotypes, and so the question remains whether this feature is conserved among the various HCV genotypes. To address this issue, NS5A proteins derived from HCV isolates belonging to the 1a subtype (HCV-H), which is closely related to the 1b subtype, and genotype 2a (HCV-J6), which is distantly related to both the 1a and 1b subtypes, were analyzed for phosphorylation. The nsP3 protein of Sindbis virus, an alphavirus classified in the family *Togaviridae*, which is another family of viruses with single-stranded, positive-sense RNA genomes, was included in this analysis as a positive control, since nsP3 has been previously shown to undergo efficient phosphorylation (30). As shown in Fig. 1A, both Sindbis virus nsP3 and HCV-H NS5A were labeled efficiently with [^{32}P]orthophosphate. At least two phosphorylated forms of HCV-H NS5A were observed, and little or no difference was detectable in the phosphorylation of HCV-H NS5A expressed individually or within the context of the nonstructural polyprotein, at least in this one-dimensional SDS-PAGE analysis (compare lanes 7 and 11). Reduction of the ^{32}P signal and the conversion of slower-migrating ^{35}S-labeled forms to faster-migrating forms by treatment with alkaline phosphatase confirmed that the observed ^{32}P labeling of HCV-H NS5A and Sindbis virus nsP3 was due to phosphorylation. Phosphoamino acid analysis showed that HCV-H NS5A is phosphorylated mostly on serine residues, like HCV-J NS5A, but unlike HCV-J NS5A also contained a small amount of phosphothreonine (Fig. 1B). Phosphorylation of NS5A was also observed for the HCV-J6 isolate, as demonstrated by [^{32}P]orthophosphate labeling (Fig. 1C), indicating that NS5A phosphorylation is conserved among distantly related HCV isolates. The slight disparity in the electrophoretic mobilities of the HCV-J6 and HCV-H NS5A proteins is probably due to differences in the lengths of their polypeptide chains (466 versus 448 aa) or in the composition of amino acids at nonidentical positions, which, as mentioned in the introduction, comprise about 40% of the total.

NS5A can be phosphorylated in vitro. To facilitate the characterization and identification of one or more kinases that phosphorylate HCV NS5A, attempts were made to develop an assay for its phosphorylation in vitro. For convenience, kinase(s) responsible for NS5A phosphorylation may be referred to in singular terms; however, the number of kinases that catalyze NS5A phosphorylation is unknown, and this usage should not be construed as a suggestion that only one kinase is involved. Several different approaches were tried before successful in vitro phosphorylation was observed in assays using a fusion protein consisting of a 26-kDa fragment of the GST protein from *Schistosoma japonicum* linked to the N terminus of HCV-H NS5A (Fig. 2A). Previous analysis of this fusion protein in BHK-21 cells indicated that the GST moiety does not interfere with NS5A phosphorylation in vivo (Fig. 2B). In the standard in vitro assay, this GST/HCV-H NS5A fusion protein was transiently expressed in a baby hamster kidney (BHK-21) or human hepatoma (HuH-7) cell line (38), using the vaccinia virus-T7 hybrid system (15). Cells expressing the fusion protein were lysed with a buffer containing a nonionic detergent such as Nonidet-P 40, and then GST/HCV-H NS5A and any associated proteins were partially purified by incubation with glutathione-agarose followed by several cycles of washing to remove nonspecifically bound proteins. The partially purified samples were incubated in a buffer containing [γ-^{32}P]ATP to allow phosphorylation of GST/HCV-H NS5A by kinases that may have remained associated with it through the isolation process.

As shown in Fig. 2A, GST/HCV-H NS5A was phosphorylated efficiently in this in vitro assay. The kinase responsible for this phosphorylation required divalent cations (lane 10) and preferred Mn^{2+} to Mg^{2+} under the conditions tested (compare lane 4 to lane 2). Surprisingly, the addition of 1 mM $CaCl_2$ to

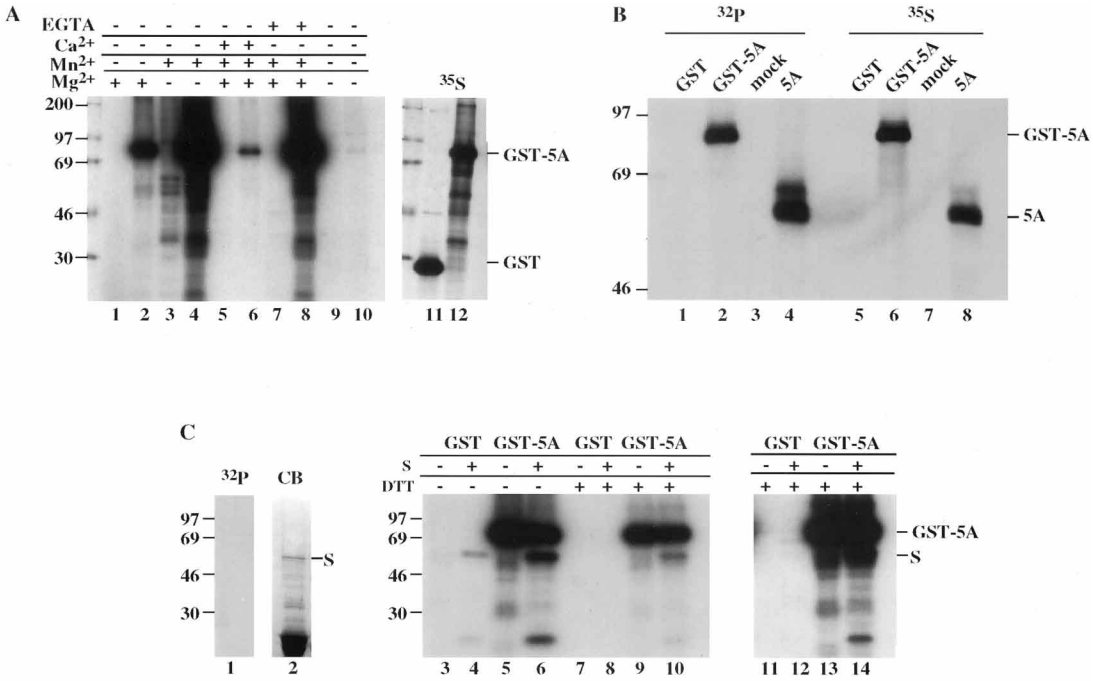

FIG. 2. In vitro phosphorylation of HCV NS5A. (A) Efficient phosphorylation of a GST/HCV-H NS5A fusion protein in vitro. Wells (35-mm diameter) of BHK-21 cells previously infected with vTF7-3 were transfected with pTM3/GST (odd-numbered lanes) or pTM3/GST/HCV-H 5A (even-numbered lanes) for 2 h at 37°C. After transfection, the cells were incubated for 4 h at 37°C in MEM–5% FBS (lanes 1 to 10) or labeled with ^{35}S protein-labeling mix (lanes 11 and 12) and harvested, and GST- or GST/HCV-H NS5A-associated cellular proteins were isolated on glutathione-agarose. Samples labeled intracellularly with ^{35}S were eluted immediately with protein sample buffer, whereas in vitro kinase reactions were performed on samples from unlabeled cells prior to elution with protein sample buffer. Both types of samples were then analyzed by SDS-PAGE (8% gel). The in vitro kinase reaction mixtures contained 5 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and/or 150 μM EGTA as indicated. (B) Effect of the GST moiety on phosphorylation of NS5A in vivo. BHK-21 cells previously infected with vTF7-3 were transfected with the indicated plasmids, labeled for 3.5 h with [^{32}P]orthophosphate or ^{35}S protein-labeling mix, and harvested; NS5A-containing proteins were immunoprecipitated with WU123 and analyzed by SDS-PAGE (8% gel). (C) Specificity of the association between NS5A and its kinase. pTM3/GST or pTM3/GST/HCV-H 5A was expressed in BHK-21 cells and purified as described for panel A. Prior to the in vitro kinase reaction, samples in lanes 4, 6, 8, and 10 were mixed with a GST-truncated NS5A substrate (S) purified from *E. coli* transformed with pGEX-3x/HCV-H 2179-2420 as described previously (23), with minor modifications. Lanes 11 to 14 are longer exposures of lanes 7 to 10. In lanes 1 and 2, an in vitro kinase assay was performed with S in the absence of any mammalian cell-derived proteins. The autoradiograph of the reaction is shown in lane 1; lane 2 contains the same sample stained with Coomassie brilliant blue. The regions of lanes 3 to 10 corresponding to the migration of S were quantitated on a phosphorimager (Bio-Rad), and the degree of specificity was calculated by using the following formulas: 1 − [(lane 4 − lane 3)/(lane 6 − lane 5)] and 1 − [(lane 8 − lane 7)/(lane 10 − lane 9)]. Sizes are indicated in kilodaltons.

a buffer containing Mn^{2+} and Mg^{2+} strongly inhibited the in vitro phosphorylation (lane 6). Consistent with this finding, the addition of EGTA, which selectively chelates Ca^{2+}, had no effect on in vitro phosphorylation of NS5A (lane 8).

The kinase activity responsible for NS5A phosphorylation associates specifically with NS5A. GST itself, although expressed at a level similar to that of the GST/HCV-H NS5A fusion protein (Fig. 2A, lanes 11 and 12) was not phosphorylated by the kinase in vitro (lanes 1, 3, 5, and 7). However, the observation that GST was not a substrate for the kinase in this assay did not exclude the possibility that the kinase responsible for NS5A phosphorylation in vitro associated with GST rather than NS5A. To investigate this possibility, a glutathione-agarose-bound truncated GST/HCV-H NS5A substrate isolated from *E. coli* (designated S in Fig. 2C) was mixed with partially purified, glutathione-agarose-bound GST or GST/HCV-H NS5A expressed in BHK-21 cells prior to the kinase reaction. The *E. coli*-expressed substrate was truncated to distinguish it from the full-length GST/HCV-H NS5A fusion protein expressed in BHK-21 cells, which is also a substrate for in vitro phosphorylation, and the ability of this truncated polypeptide to undergo phosphorylation in vivo was verified before it was used as a substrate in vitro (data not shown). This truncated substrate was also negative for autophosphorylation or phosphorylation by associated *E. coli* proteins (Fig. 2C, lane 1) and therefore suitable for the specificity analysis. The corresponding Coomassie blue-stained substrate is shown in lane 2. (The intensely stained polypeptides at the bottom of the lane are most likely GST-containing degradation products of the substrate fusion protein.)

Phosphorylation of the *E. coli*-expressed substrate by kinase(s) captured on glutathione-agarose from BHK-21 cells expressing GST/HCV-H NS5A but not GST would indicate that the activity responsible for phosphorylation of this substrate associates specifically with NS5A rather than the GST moiety or the affinity matrix. As shown in Fig. 2C, some kinase activity was associated nonspecifically with GST-glutathione agarose complexes when DTT was omitted from the lysis and wash buffers, as evidenced by phosphorylation of the *E. coli*-expressed substrate in lane 4. However, most of the kinase activity responsible for phosphorylation of the *E. coli*-expressed substrate seemed to associate specifically with NS5A, since the phosphorylation level of this substrate was nearly four times higher when the GST/HCV-H NS5A protein, rather than GST, expressed in BHK-21 cells was used for kinase capture (lane 6). The degree of specific association between NS5A and this kinase activity increased from approximately 74% to 93% when 5 mM DTT was included in the buffers used for lysis and the first wash cycle, as determined by phosphorimager quantitation and supported by visual inspection of long autoradio-

graphic exposures (lanes 11 to 14). Given its specific association with NS5A and activity in the presence of Mn^{2+}, the transacting kinase activity may well be the same enzyme as that responsible for phosphorylation of mammalian-expressed GST/HCV-H NS5A.

Comparison of NS5A phosphorylation patterns in vitro and in vivo. As shown in Fig. 2A, in vitro phosphorylation of NS5A occurs more efficiently in the presence of Mn^{2+} than Mg^{2+}. Since Mn^{2+} preference is more typical of tyrosine kinases than serine/threonine kinases, PAA was performed to determine the nature of the NS5A phosphate acceptor sites in vitro. Phosphorylation of NS5A in vitro occurred mostly on serine, irrespective of the presence of Mn^{2+} or Mg^{2+}, although a small amount of phosphothreonine was detected in the presence of Mn^{2+} but not Mg^{2+}, as determined from Fig. 3A and long exposures of the same experiment (data not shown). These results are very similar to those obtained from PAA of NS5A phosphorylated in vivo (Fig. 1B and reference 24), indicating that NS5A phosphorylation in both cases was catalyzed by one or more serine/threonine kinases.

Once serine had been confirmed as the major phosphate acceptor residue in vitro, phosphopeptide maps of NS5A phosphorylated in vitro and in vivo were compared to determine if the locations of these phosphorylation sites were also similar. Since most NS5A phosphorylation experiments presented in this report used cells infected with a vaccinia virus recombinant encoding the bacteriophage T7 RNA polymerase for expression of NS5A, phosphopeptide maps of NS5A expressed in noninfected cells were also generated to ensure that NS5A phosphorylation was not influenced by vaccinia virus-encoded kinases or kinase inhibitors. The human hepatoma cell line HuH-7 was selected for this experiment rather than BHK-21 to determine simultaneously whether the pattern of NS5A phosphorylation observed in BHK-21 cells is similar to the pattern obtained in cells derived from liver tissue, which is thought to be the major site of HCV replication in vivo. As seen by comparison of the phosphopeptide maps in Fig. 3B and D, the most intensely labeled phosphopeptides, a to e, were present in both maps. Phosphopeptides f to o were also present in both maps, as seen more clearly in longer exposures of Fig. 3B (data not shown), although phosphopeptides f, h, i, and o were somewhat more intense in the HuH-7-derived samples. Phosphopeptides a to i, k, and o were also present in the map of NS5A phosphorylated in vitro (compare Fig. 3B or D with Fig. 3C, or note the comigration of these phosphopeptides in Fig. 3E). Phosphopeptides l and n were the only ones present in the in vitro map that were absent in the in vivo map. Phosphopeptides a and b, which seem to contain the most efficiently utilized phosphorylation sites in vivo, were also phosphorylated in vitro, although the signal corresponding to these phosphopeptides was much weaker. The less efficient utilization of these sites in vitro may be due to the prior addition of nonradiolabeled phosphate groups during the expression period in vivo. The modest differences observed in the patterns of NS5A phosphorylation in vitro and in cultured cells cannot be attributed to the influence of the GST moiety, since phosphopeptide maps of GST/HCV-H NS5A and HCV-H NS5A phosphorylated in vivo were practically identical (data not shown). In any case, the overall similarity of the in vitro and intracellular NS5A phosphorylation patterns indicates that mutual phosphorylation sites are utilized in vitro and in vivo and suggests that one or more kinases responsible for phosphorylation at these sites in vitro and in vivo are also the same.

Characterization of in vitro kinase activity. Once similarities between NS5A phosphorylation in vitro and in vivo had been established, parameters of the in vitro reaction, such as divalent cation concentration, pH, temperature, and length of incubation, were analyzed in greater detail. Based on pilot experiments, 5 mM $MnCl_2$ and 50 mM Tris-Cl (pH 7.5) were established as components of the standard reaction buffer, along with 5 mM DTT, 50 mM serine/threonine phosphatase inhibitor NaF, and 10 μCi of [γ-^{32}P]ATP per reaction. As indicated in Fig. 4A, the optimal divalent cation concentration for in vitro phosphorylation of NS5A was approximately 10 mM in the case of $MnCl_2$ or 2.5 mM in the case of $MgCl_2$, although the overall level of phosphorylation was much higher in the presence of Mn^{2+} than Mg^{2+}. The addition of $CaCl_2$ at concentrations of ≥0.25 mM to reaction buffer containing 5 mM $MnCl_2$ dramatically inhibited NS5A phosphorylation (Fig. 4B). The activity was reduced by at least 90% within a fairly narrow range of $CaCl_2$ concentration (0.1 to 0.5 mM). However, this concentration is still at least 100-fold higher than the typical intracellular Ca^{2+} concentration, even when elevated in response to extracellular stimuli (4).

The optimal pH of the reaction was approximately 7.2 (Fig. 4C). This optimum was determined by measuring the in vitro phosphorylation of NS5A in buffers spanning a broad pH range (5.5 to 9) in 0.5-U increments (data not shown), followed by analysis of 0.2-U increments within a narrower pH range (6.8 to 8.2) encompassing the optimum (Fig. 4C). Although most of the in vitro kinase reactions were performed in the presence of Tris-Cl, HEPES was selected as the buffer for the analysis shown in Fig. 4C because its pK_a (7.5) is centered in the range from pH 6.8 to 8.2, while the pK_a of Tris-Cl (8.1) is located at the upper end of this range. However, similar levels of activity were obtained in both buffers, at least at pH 7.4 (Fig. 4C).

In vitro phosphorylation of NS5A was impaired by preincubation of samples at temperatures of ≥45°C prior to the addition of [γ-^{32}P]ATP; however, the kinase(s) responsible for NS5A phosphorylation seemed to be quite active under most conditions, as evidenced by the detection of NS5A phosphorylation during incubation of the reaction mixture on ice for periods as short as 10 min (data not shown). Peak levels of NS5A phosphorylation were observed after approximately 2 h of incubation at 37°C or 4 h of incubation at 30°C (data not shown), although NS5A phosphorylation was easily detected by incubation of reaction mixtures for 10 min at 30 or 37°C, followed by SDS-PAGE and a 10-min autoradiographic exposure at −80°C with an intensifying screen (e.g., Fig. 5A).

Effects of kinase inhibitors. In an attempt to narrow the search for the kinase that phosphorylates NS5A and further characterize its activity, several kinase inhibitors were analyzed for their effects on NS5A phosphorylation in vitro and in vivo. These included bisindolylmaleimide I-HCl, a protein kinase C (PKC)-specific inhibitor; olomoucine, which is thought to be specific for proline-directed kinases; H-89, which preferentially inhibits cyclic nucleotide-dependent kinases; DRB, often described as a specific inhibitor of casein kinase II (CKII) but also reported to inhibit the cyclin-dependent kinase (CDK)-activating kinase (CAK) (50); hypericin, a photosensitive inhibitor of retrovirus replication that reduces the activity of mitogen-activated protein kinase (MAPK), CKII, and PKC; and the broad kinase inhibitors A-3 and staurosporine. Phosphorylation of NS5A was reduced approximately 33 or 67% by the addition of 10 or 100 μM DRB, respectively, to the in vitro assay (Fig. 5A, lane 16 or 17). Since the reported 50% inhibitory concentration (IC_{50}) of DRB for CKII in vitro is 6 μM (51), the degree of inhibition by DRB observed in Fig. 5A is somewhat lower than expected for CKII. The lack of substantial inhibition by 10 μM hypericin or 200 μM A-3, a concentration more than 1,600 or 40 times higher, respectively, than

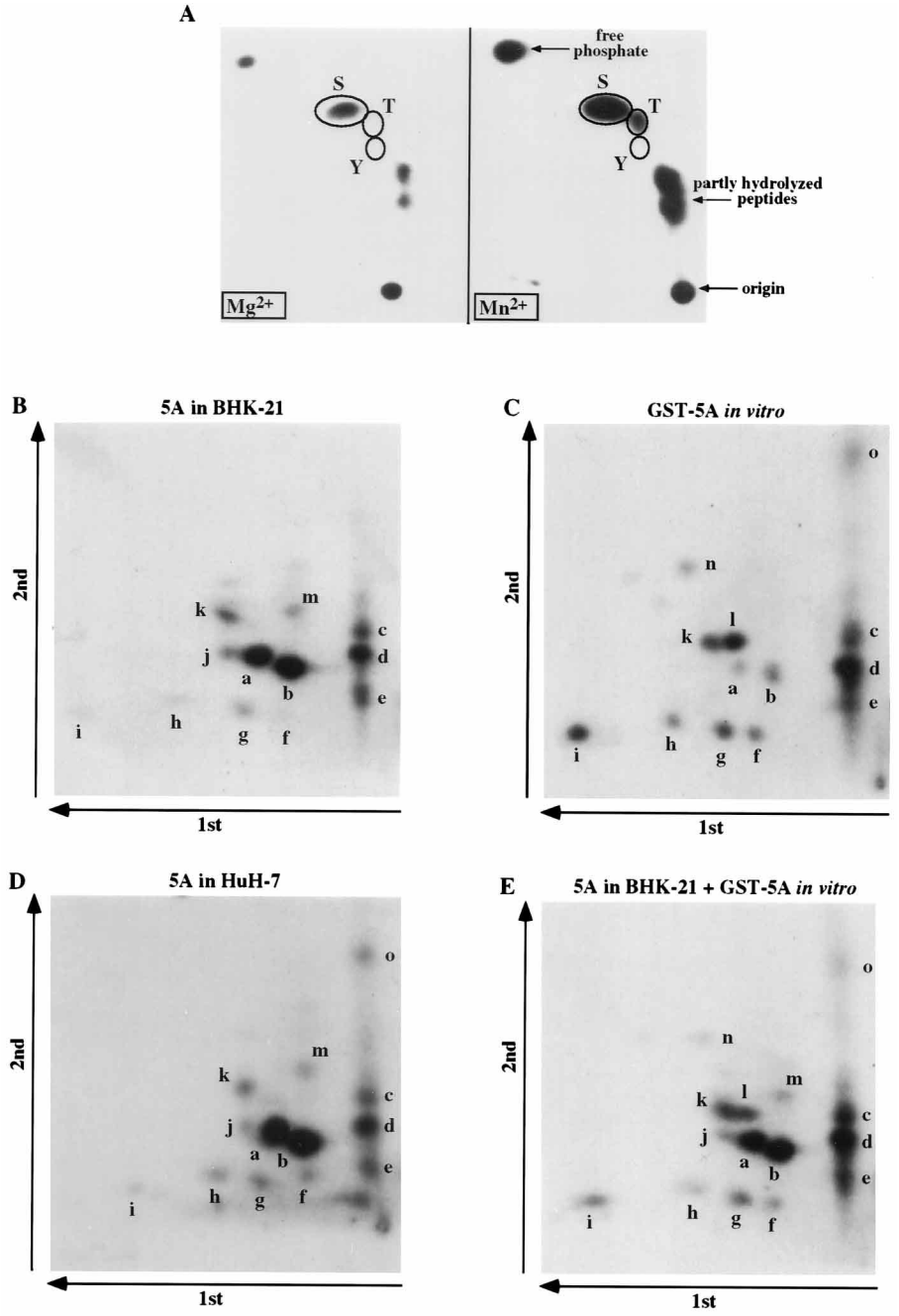

FIG. 3. Comparison of intracellular and in vitro NS5A phosphorylation. (A) PAA of GST/HCV-H NS5A phosphorylated in vitro in the presence of 5 mM $MnCl_2$ or 5 mM $MgCl_2$; (B) PPM of HCV-H NS5A phosphorylated in intact BHK-21 cells; (C) PPM of GST/HCV-H NS5A phosphorylated in vitro; (D) PPM of HCV-H NS5A phosphorylated in intact HuH-7 cells. (E) Mixture of approximately equal amounts of samples shown in panels B and C. Samples in panels B and C were obtained by transfecting vTF7-3-infected BHK-21 cells for 2 h at 37°C with pTM3/HCV-H 5A or pTM3/GST/HCV-H 5A as indicated. In panel D, non-vTF7-3-infected HuH-7 cells were transfected with pOP13CMV/HCV-H 5A for 20 h at 37°C. At the end of the transfection period, the cells were labeled for 4 h with 200 (B) or 400 (D) μCi of [^{32}P]orthophosphate per ml or incubated for 4 h in MEM–5% FBS (C). NS5A proteins were immunoprecipitated from cell lysates with WU123 by the preparative method (B and D), or standard in vitro kinase reactions were performed (C), followed by elution with protein sample buffer, separation by SDS-PAGE (8% gel), and PPM. Individual phosphopeptides are indicated by lowercase letters.

the reported IC_{50} or K_i for CKII (3, 22), is also inconsistent with CKII-dependent in vitro phosphorylation of NS5A. Therefore, the effect of DRB may be due to the inhibition of CAK, for which the IC_{50} of DRB is 10 to 50 μM (50), or a kinase related to CKII and proline-directed kinases such as CAK, which have been grouped together based on their phylogenetic similarity (18). Consistent with this idea, the proline-directed kinase inhibitor olomoucine reduced NS5A phosphorylation by 73% at a concentration of 1 mM in vitro. This concentration is at least 40 or 100 times higher, respectively, than the reported IC_{50}'s for p44 MAPK (25 μM) or the CDKs Cdc2, CDK2, and CDK5 (3 to 7 μM) but is closer to the IC_{50}

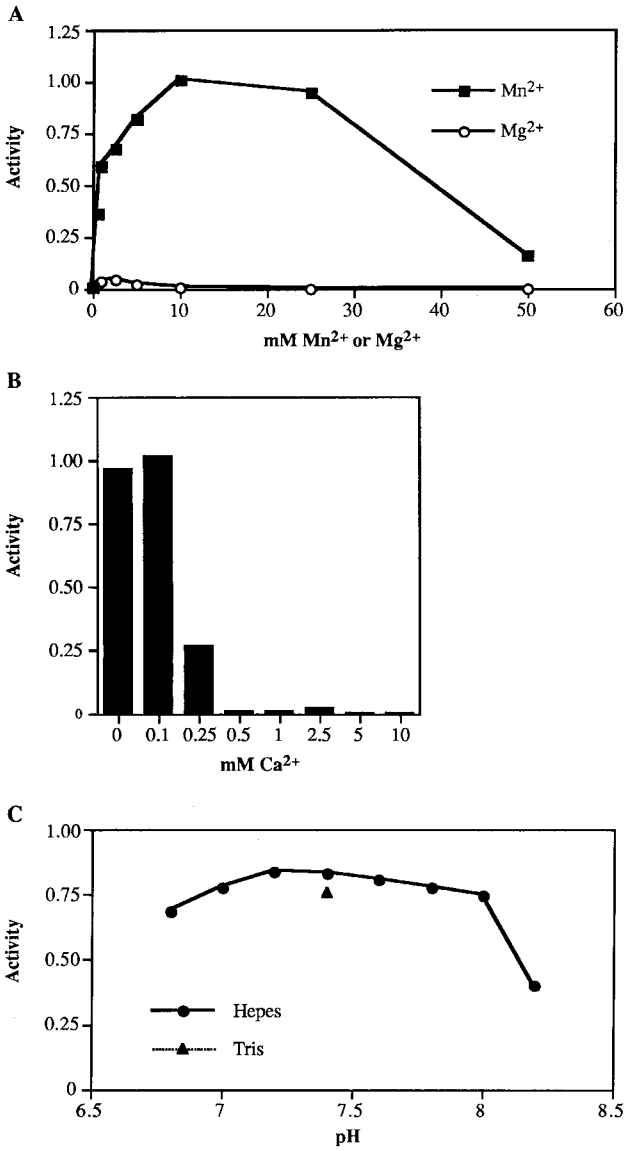

FIG. 4. Effect of divalent cation concentration and pH on in vitro phosphorylation of NS5A. (A) $MnCl_2$ or $MgCl_2$; (B) $CaCl_2$; (C) HEPES, pH 6.8 to 8.2. In all three sets of experiments, 35-mm-diameter wells of vTF7-3-infected BHK-21 cells were transfected with 1 μg of pTM3/GST/HCV-H 5A for 2 h at 37°C, incubated for 3.5 to 4 h at 37°C in MEM–5% FBS, washed once with cold PBS, harvested in NETN containing 5 mM DTT and protease inhibitors, and clarified. The supernatants were pooled and divided into equal aliquots prior to isolation on glutathione-agarose to ensure that all in vitro kinase reaction mixtures contained equal amounts of GST/HCV-H NS5A. Proteins were eluted after the reaction by heating in protein sample buffer and analyzed by SDS-PAGE (8% gel), followed by autoradiography and phosphorimager quantitation. The compositions of the KWB and KRB used in these experiments were standard except for the replacement of 5 mM $MnCl_2$ with the indicated concentrations of $MnCl_2$ or $MgCl_2$ in panel A, the addition of $CaCl_2$ in panel B, or the substitution of 50 mM HEPES, pH 6.8 to 8.2, for 50 mM Tris-Cl, pH 7.5, in panel C. Panel C also shows a parallel reaction performed in the presence of 50 mM Tris-Cl, pH 7.4.

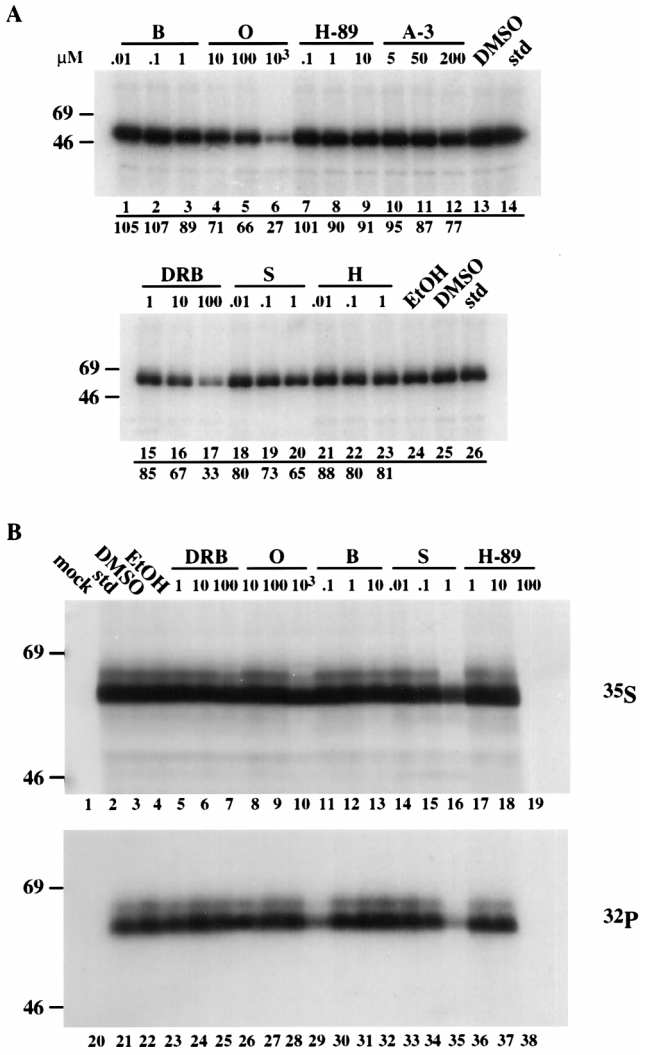

FIG. 5. Effects of protein kinase inhibitors on NS5A phosphorylation in vitro and in vivo. (A) In vitro. Kinase reactions were performed as described in the legend to Fig. 4 except that the KRB contained various concentrations of protein kinase inhibitors as indicated. B, bisindolylmaleimide I-HCl; O, olomoucine; S, staurosporine; H, hypericin. The other kinase inhibitors are designated by their standard abbreviations. As determined by phosphorimager quantitation, the percentages of NS5A phosphorylation relative to the appropriate solvent controls (dimethyl/sulfoxide [DMSO] for B, O, H-89, A-3, S, and H or ethanol [EtOH] for DRB) are given below the lane numbers. (B) In vivo. vTF7-3-infected BHK-21 cells were transfected for 2 h with pTM3 (mock) or pTM3/HCV-H 5A at 37°C, incubated for 30 min in medium containing the designated concentrations of protein kinase inhibitor, labeled for 3 h in medium containing ^{35}S protein-labeling mix or [^{32}P]orthophosphate in addition to the kinase inhibitor as indicated, harvested, immunoprecipitated with WU123 by the analytical method, and analyzed by SDS-PAGE (8% gel) followed by autoradiography. Sizes are indicated in kilodaltons. std, standard reaction.

for other proline-directed kinases, such as CDK6 (≥150 μM) or CDK4 (>1 mM) (47). The IC_{50}s of olomoucine have not been determined for all proline-directed kinases, so CDK6 or a related but less-well characterized kinase may catalyze NS5A phosphorylation in vitro.

Little or no inhibition was observed by 1 μM PKC-specific inhibitor bisindolylmaleimide I-HCl. Since in vitro inhibition of PKC by bisindolylmaleimide I-HCl has been reported at concentrations approximately 100-fold lower than 1 μM (45), this observation suggests that PKC does not phosphorylate NS5A in vitro. Consistent with this result, 200 μM A-3, 10 nM staurosporine, and 1 μM hypericin, which are more than three times greater than their reported K_is or IC_{50}s for PKC in vitro (3, 22, 40), only slightly (<25%) inhibited NS5A phosphorylation in vitro (Fig. 5A, lanes 12, 18, and 23). The cyclic AMP-dependent protein kinase (PKA) and cyclic GMP-dependent

TABLE 1. Quantitative analysis of the effects of protein kinase inhibitors on NS5A phosphorylation in vivo

Inhibitor	Concn (μM)	% Expression[a]	% Phosphorylation[b]	% Phosphorylation (normalized)
DRB	1	106	119	112
	10	105	106	101
	100	96	94	98
O	10	106	88	83
	100	107	92	86
	1,000	68	43	64
B	0.1	91	88	97
	1	89	97	110
	10	85	99	116
S	0.01	81	90	111
	0.1	57	73	127
	1	31	24	78
H-89	1	81	77	95
	10	82	74	91
	100	0	0	—[c]

[a] ^{35}S phosphorimager signals for NS5A in the presence of the indicated kinase inhibitor concentrations relative to the dimethyl sulfoxide (O, B, S, or H-89) or ethanol (DRB) control.

[b] ^{32}P phosphorimager signals for NS5A in the presence of the indicated kinase inhibitor concentrations relative to their respective controls, divided by percent expression. O, B, and S are as defined in the legend to Fig. 5.

[c] —, not calculable since percent expression in this case was 0.

protein kinase (PKG) were also excluded as the kinases responsible for the majority of NS5A phosphorylation in vitro, based on the effects of three different inhibitors: H-89, A-3, and staurosporine. The lack of significant inhibition observed by 10 μM H-89, a concentration 200- and 20-fold higher than the in vitro K_is of H-89 for PKA and PKG (10), respectively, suggests that neither of these kinases phosphorylates NS5A in vitro (lane 9). Similarly, the addition of 200 μM A-3 (lane 12), a concentration 50-fold higher than the in vitro K_is of A-3 for PKA and PKG (22), or of 100 nM staurosporine (lane 19), which is at least 10 times higher than its IC_{50}s for these kinases (40), resulted in <30% inhibition of NS5A phosphorylation in vitro. Together, the results of treatment with the broad serine/threonine kinase inhibitors A-3 and staurosporine further suggest that myosin light-chain kinase (MLCK), calcium/calmodulin-dependent kinase II (CaMKII), and casein kinase I (CKI) are not responsible for the majority of NS5A phosphorylation in vitro, since treatment with 200 μM A-3 (lane 12), which is 20 or 2.5 times higher than the K_i for MLCK or CKI (22), respectively, or 100 nM staurosporine (lane 19), which is 100 or 5 times higher than the K_i or IC_{50} for MLCK or CaMKII (40, 49), respectively, resulted in less than 50% inhibition of NS5A phosphorylation in vitro.

The effects of most of these inhibitors on NS5A phosphorylation in vivo were similar to those observed in vitro (Fig. 5B and Table 1), although the effects of A-3 and hypericin were not analyzed in vivo due to a lack of information regarding the concentrations of these compounds necessary for kinase inhibition in intact cells. Bisindolylmaleimide I-HCl at 10 μM (lane 32) had no apparent effect on NS5A phosphorylation in vivo, even though this concentration is 5 to 50 times higher than the reported IC_{50} for PKC in intact cells (45). Treatment of cells with 10 μM H-89, which should inhibit PKA (10), also seemed to have little or no effect on NS5A phosphorylation (lane 37). When the concentration of H-89 was increased to 100 μM, no NS5A phosphorylation was observed in vivo (lane 38), but the block occurred at the level of expression rather than posttranslational modification (lane 19). Similarly, NS5A phosphorylation did not seem to be affected by the inclusion of 10 nM staurosporine in the culture medium (lane 33) but was decreased in the presence of 1 μM staurosporine (lane 35). A significant fraction of this decrease resulted from reduced NS5A expression (lane 16). In intact cells, staurosporine is often used at concentrations of 10 to 500 nM, although the effective concentrations for inhibition of PKC and other kinases have not been well characterized in vivo.

The major difference between NS5A phosphorylation in vitro and in vivo occurred in response to DRB. DRB inhibition is supposed to be similarly potent in vitro and in vivo (51), but 100 μM DRB had little or no effect on the phosphorylation of NS5A in vivo (lane 25), even though it inhibited the majority of NS5A phosphorylation in vitro. This discrepancy may be due to the enrichment of particular kinases during the affinity purification steps in vitro or to the influence of different reaction conditions on kinase activity.

Olomoucine had similar effects on NS5A phosphorylation in vitro and in vivo. The efficiency of olomoucine on specific kinases has not been examined intracellularly, but the IC_{50} for inhibition of various stages of cell growth or maturation ranges from 10 to 100 μM (1, 47). Although 1 mM olomoucine had a moderate effect on the amount of NS5A expression (lane 10), NS5A phosphorylation was significantly reduced by 1 mM olomoucine in vivo (lane 29) and in vitro, indicating that a proline-directed or related kinase may be responsible for the majority of NS5A phosphorylation inside mammalian cells and in the in vitro kinase assay.

DISCUSSION

The results presented in this report have shown that phosphorylation of the NS5A protein is a feature conserved among divergent HCV isolates and that NS5A can be phosphorylated by an associated cellular serine/threonine kinase in vitro with characteristics similar to those observed in vivo. Moreover, analysis of the effects of a panel of kinase inhibitors on NS5A phosphorylation in vivo and in vitro has suggested that the kinase responsible for the majority of NS5A phosphorylation may be a member of the CMGC kinase family, which includes CKII and proline-directed kinases such as the CDKs, MAPKs, and glycogen synthase kinase 3 (GSK3).

Although phosphorylation of HCV-H NS5A was confirmed and shown to occur predominantly on serine, similar to the HCV-J NS5A protein, some minor differences in phosphorylation, such as a low level of phosphorylation on threonine residues in addition to serine, were observed. Also, as noted in Results, the mobilities of phosphorylated NS5A species observed when HCV-H NS5A was expressed in the presence or absence of the other nonstructural proteins appeared to be very similar. This observation appears to conflict with the results previously obtained by Kaneko and coworkers (24), who found that production of a slower-migrating form of phosphorylated HCV-J NS5A was significantly enhanced by coexpression with NS4A or deletion of the NS5A N terminus. The influence of NS4A expression on HCV-J NS5A phosphorylation was observed in COS-1 cells, while most of our analyses of NS5A phosphorylation were performed with BHK-21 cells. However, this discrepancy did not seem to be attributable to cell-type-specific differences, since results similar to those shown in Fig. 1 were also obtained in assays using COS-1, HeLa, and HuH-7 cells (data not shown). Moreover, deletion of the first 32 aa of HCV-H NS5A resulted in the appearance of two discrete phosphorylated forms (data not shown), consistent with results obtained for HCV-J NS5A (6). The explanation for these various observations is not clear but may involve HCV isolate-specific differences.

NS5A phosphorylation is thought to be mediated by a cellular kinase for several reasons: (i) NS5A phosphorylation can occur in the absence of other viral proteins (Fig. 1A), (ii) full-length NS5A produced as a fusion protein with GST in *E. coli* is phosphorylated only in the presence of mammalian cell extracts (unpublished observations), and (iii) NS5A lacks motifs present in the catalytic domains of nearly all eukaryotic kinases. PAA of NS5A from the HCV-H and -J isolates has confirmed that NS5A is phosphorylated by a serine/threonine kinase. Furthermore, the similarity of the phosphopeptide maps of HCV-H NS5A phosphorylated in vitro and in vivo suggests that the kinase responsible for NS5A phosphorylation in vitro may also be involved in its intracellular phosphorylation. Phosphorylation of HCV NS5A in cell lines derived from diverse cell types, including kidney (COS-1 and BHK-21), liver (HuH-7), and cervix (HeLa), or from species as distantly related as humans (HuH-7 and HeLa) and hamsters (BHK-21) indicates that this kinase is broadly distributed and perhaps highly conserved. This may enable HCV to replicate in multiple cell types, e.g., hepatocytes and lymphocytes (reviewed in reference 41), if indeed NS5A phosphorylation and/or its interaction with the kinase is essential.

The observed preference of this kinase for Mn^{2+} and inhibition by Ca^{2+}, at least in vitro, are somewhat unusual. The inhibitory effect of Ca^{2+} concentrations of $\geq$0.25 mM on the in vitro phosphorylation of NS5A, together with the lack of inhibition by EGTA, suggests that the kinase catalyzing this phosphorylation does not require Ca^{2+}. However, the data do not exclude the possibility that Ca^{2+} is required for kinase activity but is already bound to the kinase tightly enough to prevent chelation by EGTA. In the latter case, some mechanism may exist for regulation of kinase activity by Ca^{2+} that results in inhibition of the kinase at higher Ca^{2+} concentrations. For example, such a mechanism has already been postulated for phosphorylase kinase, which is allosterically regulated by Ca^{2+} (reviewed briefly in reference 18 and more extensively in reference 19). Although the properties discussed above are insufficient for kinase identification, this information should be useful for future enzymological studies.

Analysis of the inhibitor profiles of the NS5A-associated kinase in vitro and in vivo did not implicate a particular kinase in NS5A phosphorylation but was useful in excluding well-known members of kinase groups such as PKC isoforms, cyclic nucleotide-dependent protein kinases, and certain calcium/calmodulin-dependent kinases as major participants in NS5A phosphorylation. As described in Results, NS5A phosphorylation was inhibited intracellularly by olomoucine, an inhibitor of proline-directed kinases, and in vitro by both olomoucine and DRB, an inhibitor of CKII and the proline-directed kinase CAK. Interestingly, both CKII and proline-directed kinases have been classified as members of a single kinase family, CMGC, which is an acronym based on the names of its best-characterized members, CDK, MAPK, GSK3, and CKII (18). Unlike most members of other serine/threonine kinase families, which seem to recognize phosphate acceptor sites based on their proximity to basic amino acids, CKII prefers to phosphorylate Ser and, to a lesser extent, Thr residues followed by acidic amino acids. The consensus phosphorylation sites for the CDK and MAPKs are Ser or Thr immediately followed by Pro. MAPK sites usually contain an additional Pro two amino acids upstream of the phosphate acceptor, while CDK sites often contain a basic residue two amino acids downstream of the phosphate acceptor. GSK3 also seems to be a proline-directed kinase, although its substrate recognition requirements are complex and, in some cases, depend on prior phosphorylation events catalyzed by other kinases. The C terminus of NS5A is acidic and contains a large number of Ser and Pro residues, characteristics that are consistent with phosphorylation by a member of the CMGC kinase group.

All of the inhibitors analyzed in this study for their effect on NS5A phosphorylation have been well characterized in vitro, although under conditions somewhat different from those used for in vitro phosphorylation of NS5A. Similarly, conditions used for the treatment of intact cells with these inhibitors were selected based on published descriptions of their activity in vivo. However, such effects are likely to differ somewhat in different cell lines, due to variations in the efficiency of their uptake, half-life in the cells, or modulation by other cell-type-specific factors. Although the kinase inhibitor profiles for NS5A phosphorylation in the in vitro assay and intact BHK-21 cells implicate one or more members of the CMGC family in NS5A phosphorylation, different results might be obtained with other cell types or expression systems. Also, since inhibitor specificity is often determined based on analyses of a fairly small number of well-known kinases, the possibility remains that these inhibitors exert effects on other, less well characterized or unknown kinases.

NS5A is thought to be a component of the RNA replication complex, based on observations that it can be coprecipitated with other nonstructural proteins such as NS2 and the polymerase NS5B (20). The intracellular site of HCV RNA replication has not been established, but several lines of evidence suggest that flavivirus replication occurs on cytoplasmic membranes surrounding the nucleus (reviewed in reference 42), which is consistent with the localization observed for HCV NS5A (21, 33, 44). The conservation of HCV NS5A phosphorylation among divergent isolates may indicate an important regulatory role for this posttranslational modification in HCV RNA replication. Alternatively, NS5A phosphorylation might simply be a by-product of association with a cellular kinase, which is itself an important participant in RNA replication. Further studies will be needed to distinguish among these possibilities.

Interestingly, the NS5 protein of dengue virus type 2 (DEN 2), a member of the related *Flavivirus* genus, is also phosphorylated (25). The flaviviruses produce only one NS5 protein, in contrast to HCV, and although the phosphate acceptor sites might be predicted to lie within the N-terminal region of DEN 2 NS5 by analogy to HCV, their locations have not been determined. The phosphorylation state of DEN 2 NS5 was shown to correlate with its subcellular localization and association with NS3 (25). NS5 proteins with a relatively high phosphate content were enriched in nuclear fractions, while less heavily phosphorylated forms were selectively localized to the cytoplasm and associated with NS3 (25). Since the replication of flavivirus genomes occurs in the cytoplasm, NS5 phosphorylation and nuclear transport may modulate viral RNA replication and/or enable NS5 to perform some unknown function, such as the regulation of host gene expression. Unlike DEN2 NS5, HCV NS5A has not been detected inside the nucleus, although aa 2326 to 2334 can function as a nuclear localization signal when fused to the N terminus of the *E. coli* β-galactosidase protein (21).

A precedent for the regulation of viral replication by phosphorylation of nonstructural proteins has already been established in the case of the negative-stranded, nonsegmented RNA viruses. Phosphorylation of P, a component of the transcription/replication complexes of rhabdoviruses and paramyxoviruses, has been shown to be essential for efficient transcription and ensuing replication of the viral genome (8, 36). Although these viruses seem to utilize similar mechanisms for replication and transcription of their genomes, it is remark-

able that three different, abundant, cellular kinases appear to catalyze the phosphorylation of their P proteins: CKII in the case of vesicular stomatitis virus (7), respiratory syncytial virus (35), and measles virus (11); the zeta isoform of PKC in the case of parainfluenza virus (12); and a proline-directed kinase in the case of Sendai virus (9).

The nsP3 protein of Sindbis virus, another positive-stranded RNA virus, is highly phosphorylated by a cellular serine/threonine kinase with similarities to CKII (30). However, a high level of phosphorylation is not essential for viral replication, since certain deletions in the nonconserved C-terminal region eliminate most nsP3 phosphorylation with only moderate decreases in the yield of infectious virus (28). These results have at least three possible explanations: (i) low-level nsP3 phosphorylation is necessary and sufficient for Sindbis virus replication, (ii) the aforementioned deletions abolish most nsP3 phosphorylation without disrupting its interaction with a kinase important for viral replication, and (iii) neither phosphorylation nor the interaction between nsP3 and its kinase is important for viral replication.

Aside from its putative role in RNA replication, HCV NS5A has also been proposed to modulate the host interferon-stimulated antiviral response. This suggestion is based on observations that the degree of variation within the central region of NS5A (aa 2209 to 2248), termed the interferon sensitivity-determining region (ISDR), appears to correlate with the sensitivity of HCV to interferon treatment in some studies (13, 14). The means by which NS5A affects the host interferon response is not clear, although the recent demonstration that NS5A interacts with the interferon-stimulated, double-stranded RNA-dependent protein kinase (PKR) (16) provides a possible mechanism for regulation of this signalling pathway (reviewed in reference 48). PKR is a major effector of the interferon signal transduction pathway that is activated by double-stranded RNA and inhibits translation of cellular and viral proteins via phosphorylation of the general translational initiation factor eIF2-α. Interaction of PKR with NS5A appears to inhibit its ability to phosphorylate eIF2-α. Moreover, preliminary deletion analyses suggest that the NS5A ISDR may be necessary for its interaction with PKR and the inhibition of eIF-2α phosphorylation (16). Mutations in the ISDR that correlate with interferon sensitivity may disrupt the NS5A-PKR interaction or diminish the ability of NS5A to inhibit PKR function, although such effects have yet to be demonstrated. Interestingly, attempts to demonstrate phosphorylation of NS5A by PKR were unsuccessful (16), suggesting that NS5A may be able to interact with PKR and modulate its activity without being a substrate for phosphorylation by PKR. Consistent with this observation, addition of the PKR activators poly(I · C) or heparin to our NS5A in vitro kinase assay failed to stimulate its phosphorylation, indicating that a kinase other than PKR catalyzes NS5A phosphorylation in vitro (unpublished observations). In fact, 10 U of heparin per ml, an amount which should stimulate PKR, inhibited NS5A phosphorylation in vitro by approximately 70%.

Thus, HCV NS5A appears to associate with at least two distinct kinases, PKR, and another cellular serine/threonine kinase, some properties of which are described in this report. Modulation of NS5A function by phosphorylation, of kinase function by interaction with NS5A, or of both may be a mechanism for regulating one or more aspects of the HCV life cycle. The development of an efficient in vitro assay for NS5A phosphorylation will facilitate future efforts to identify the phosphate acceptor sites and the kinase(s) responsible for NS5A phosphorylation. Such advances should lead to greater understanding of HCV NS5A function and perhaps increased insight into the viral replication process and/or interactions between HCV and its host cells.

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