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The N-Terminal Region of Hepatitis C Virus-Encoded NS5A Is Important for NS4A-Dependent Phosphorylation

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We previously showed that two proteins, a 56-kDa protein (p56) and a 58-kDa protein (p58), are produced from the hepatitis C virus (HCV) nonstructural 5A region (NS5A) and that the production of p58 is enhanced by the presence of NS4A (T. Kaneko, Y. Tanji, S. Satoh, M. Hijikata, S. Asabe, K. Kimura, and K. Shimotohno, Biochem. Biophys. Res. Commun. 205:320–326, 1994). Both proteins have phosphorylated serine residues, some of which are located in the C-terminal region. In p58, phosphorylation of serine residues in the central region of HCV NS5A is important for production of p58 in an NS4A-dependent manner. To clarify the mechanism of NS5A phosphorylation, in particular phosphorylation in the central region, phosphorylation of deleted and mutated forms of NS5A was analyzed using a transient protein production system in cultured cells in the presence or absence of NS4A. Association of the NS5A region from amino acids 2135 to 2139 with NS4A was important for NS4A-dependent phosphorylation of NS5A.

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is a positive-stranded RNA virus of about 9.5 kb (3, 9, 16). The HCV genome includes a single large open reading frame that encodes proteins of 3,010 to 3,033 amino acid (aa) residues (3, 9–11, 16, 17). This precursor polyprotein undergoes proteolytic processing and generates a putative core protein (C), three putative envelope proteins (E1, E2 type A, and E2 type B), and six nonstructural proteins (NS). The order of these proteins on the genome is NH_2-C-E1-E2 (type A or type B/p7)-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (5).

Two proteins, a faster-migrating form of the 56-kDa protein (p56) and a slower-migrating form of the 58-kDa protein (p58) are produced from the NS5A region (6, 7, 18–20), and both forms are phosphoproteins (7). Recent findings indicate that both proteins are phosphorylated at serine residues in the C-terminal region of NS5A and that p58 is additionally phosphorylated at serine residues in the central region of aa 2197 to 2204 (21). When only the NS5A region of the HCV genome is expressed in cultured cells, most of the product is p56 and a trace amount is p58. In the presence of NS4A, however, the production of p58 is augmented strongly and both forms of NS5A are produced. The physiological role of NS5A phosphorylation remains to be clarified. Recently, NS5 of dengue virus type 2 (DEN-2) was shown to be phosphorylated (8). In DEN-2, the degree of NS5 phosphorylation affected its subcellular localization and affinity for NS3. Although both forms of HCV NS5A are mainly located in the perinuclear membrane fraction (21), regulation of the phosphorylation of DEN-2 NS5 and HCV NS5A may play similar roles in viral replication.

With the intention of understanding the phosphorylation mechanisms of NS5A of HCV, we analyzed the phosphorylation of a variety of N-terminally deleted and internally deleted forms of NS5A by means of electrophoretic mobility. We clarified that one region in the N-terminal portion of NS5A had an important role in the phosphorylation of the central region of NS5A. When that region was deleted, phosphorylation of the central region of NS5A was no longer augmented by NS4A. Furthermore, we found for the first time that NS5A associated with NS4A. The region from aa 2135 to 2139 of NS5A was involved both in the association of NS5A with NS4A and in phosphorylation in the NS5A central region.

Identification of the region in NS5A that is important for regulation of phosphorylation at serine residues in the central region of NS5A. We reported that pCMV/N1973-2250D, which corresponded to the peptide produced by aa 1973 to 2250 of the HCV precursor polyprotein fused to *Escherichia coli* dihydrofolate reductase (DHFR), produced two distinct proteins in the presence of NS4A (21). However, when proteins N2197-2204D and N2174-2223D, which cover regions of NS5A from aa 2197 to 2204 and from aa 2174 to 2223, respectively, fused with DHFR, were produced in COS-1 cells, production of only a single form of each DHFR-fused protein was observed with or without NS4A (unpublished data). This suggested the possibility that a sequence which regulates phosphorylation in the central region of NS5A is present in the N-terminal half of NS5A, if at all. To examine the presence of such a region, we prepared plasmids that produced proteins which were serially deleted from the N terminus of NS5A and looked at the NS4A-dependent phosphorylation of those products (primers used in the PCR construction of those plasmids are described in Tables 1 and 2). The type 1b strain HCV polypeptide regions expressed in COS-1 cells for this study are shown in Fig. 1A. DNA transfections and Western blot (immunoblot) analysis were performed as described previously (4). The antibodies used were anti-NS5A antibody (α-NS5A), a generous gift from A. Takamizawa, Osaka University, Osaka, Japan, and anti-E-tag antibody (α-E-tag) (Pharmacia).

While intact NS5A (designated N1973-2419) and NS5A with the N-terminal 27 aa residues deleted from it (N2000-2419) showed production of two distinct forms of NS5A in an NS4A-dependent manner (Fig. 2A, lanes 1 to 4), little or no production of the slower-migrating form occurred in the absence of NS4A. Derivatives with progressive deletions of up to 162 aa residues produced both slower- and faster-migrating forms even without NS4A (Fig. 2A, lanes 5 to 14). Previously we

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TABLE 1. PCR oligonucleotide primers used to construct pCMV plasmids[a]

Primer	Sequence	Position(s)
F1	5′-CCGCTGCAGCCATGCTGCCGAAATTGCCG-3′	2000–2004
F2	5′-CCGCTGCAGCCATGGCGCCGAACTATTCC-3′	2075–2079
F3	5′-CCGCTGCAGCCATGCACTACGTGACGGGT-3′	2100–2104
F4	5′-CCGCTGCAGCCATGTTGGACGGGGTGCGG-3′	2125–2129
F5	5′-CCGCTGCAGCCATGGGGCTCAACCAATAC-3′	2150–2154
F6	5′-CCGCTGCAGCCATGCTCACCGACCCCTCC-3′	2175–2179
F7	5′-CCGCTGCAGCCACATGTTGCACAGGTACGCT-3′	2130–2134
F8	5′-CCGCTGCAGCCACATGCCGGCGTGCAGACCT-3′	2135–2139
F9	5′-CCGCTGCAGCCACATGCTCCTACGGGAGGAT-3′	2140–2144
F10	5′-GTGGGGGACTTCTTGGACGGGGTGCGGTTG-3′	2096–2099,2125–2130
F11	5′-TTCTTCACTGAAGGGCTCAACCAATACCTG-3′	2121–2124,2150–2155
F12	5′-GACGGGGTGCGGCCGGCGTGCACACCTCTC-3′	2126–2129,2135–2140
F13	5′-CACAGGTACGCTCGGGAGGATGTCACATTC-3′	2131–2134,2142–2147
F14	5′-ACCCAAGCTTCACCATGTCCGGCTCGTGGCTAAGG-3′	1973–1978
F15	5′-TGCACTGCAGCCATGAGCACCTGGGTGCTG-3′	1658–1662
R1	5′-ACAGCGCCCTGGAATAGTTCG-3′	2080–2076
R2	5′-AGCGTACCTGTGCAACCGCAC-3′	2134–2128
R3	5′-CTAGGAATTCGCAGCAGACGATGTCGTC-3′	2419–2414
R4	5′-ATGAGGTCAGCGTCCGGGGAGTCATGGTG-3′	2226–2218
R5	5′-CACCCCGTCCAAGAAGTCCCCCACCCGCGT-3′	2128–2125,2099–2094
R6	5′-TTGGTTGAGCCCTTCAGTGAAGAATTCGGG-3′	2153–2150,2124–2119
R7	5′-TCTGCACGCCGGCCGCACCCCGTCCAATTC-3′	2138–2135,2129–2124
R8	5′-GACATCCTCCCGAGCGTACCTGTGCAACCG-3′	2146–2143,2134–2129
R9	5′-TCGAATTCGCACGCTTCCATTTC-3′	1707–1711

[a] Sequences complementary to the HCV genome are underlined. Numbering corresponds to positions of amino acid residues in the HCV precursor polyprotein. F primers are positive stranded; R primers are negative stranded.

showed that SSS2200/1/2/AAA, an NS5A-derived protein in which the serine residues at aa 2200, 2201, and 2202 had been mutated to alanine residues, produced significantly less of the slower-migrating form (p58) of NS5A (21). To test whether the production of two distinct forms from these N-terminally deleted forms was affected by those mutations, these three mutations were introduced into N2000-2419, N2075-2419, and N2100-2419. These forms produced only the faster-migrating form of NS5A (Fig. 3, lanes 3, 4, 7, 8, 11, and 12). This indicated that the slower migration of the deleted forms in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was the result of phosphorylation in the central region of NS5A.

In either the presence or absence of NS4A, proteins N2075-2419, N2100-2419, N2125-2419, N2130-2419, and N2135-2419 yielded two products. However, slower-migrating products increased when NS4A was coproduced (Fig. 2A, lanes 5 to 14). Irrespective of NS4A production, N2140-2419, N2150-2419, and N2175-2419 generated only a single product (Fig. 2A, lanes 15 to 20), suggesting that the 5 aa residues 2135 to 2139 had an important role in the generation of the slower-migrating form. Protein 5AΔ2100-2124 (with 25 internal aa deleted from NS5A in positions 2100 to 2124) and protein 5AΔ2130-2134 (with aa 2130 to 2134 deleted) showed a small amount of each slower-migrating form in the absence of NS4A; this production of the slower-migrating form was augmented when NS4A was coproduced (Fig. 2C, lanes 3, 4, 7, and 8). On the other hand, the internally deleted proteins 5AΔ2125-2149 and 5AΔ2135-2141, which also yielded the slower-migrating form irrespective of NS4A production, were not augmented by the presence of NS4A (Fig. 2C, lanes 5, 6, 9, and 10). From internal deletion analysis of NS5A constructs, we concluded that the 7 aa residues 2135 to 2141 were involved in the augmentation of the production of the slower-migrating form of NS5A by NS4A. Deletion of this region severely impaired production of the slower-migrating form even in the presence of NS4A.

Physical association of NS5A with NS4A. To clarify the regulatory mechanism of NS4A on NS5A phosphorylation, we looked for a possible interaction between NS4A and NS5A. When the region of NS5A alone was expressed, NS5A was not precipitated by α-E-tag (Fig. 4A, lane 2). When N1973-2419, N2000-2419, N2130-2419, or N2135-2419 was produced with NS4AE, α-E-tag precipitated NS5A and its deleted forms as well as NS4AE (Fig. 4A and B, lanes 4, 6, 8, and 10). To a lesser extent, α-NS5A could precipitate NS4AE (Fig. 4B, lanes

TABLE 2. Combinations of primers used to construct pCMV plasmids

PCR product	Primers
PP1	F1 + R1
PP2	F2 + R2
PP3	F3 + R2
PP4	F4 + R3
PP5	F5 + R3
PP6	F6 + R3
PP7	F7 + R3
PP8	F8 + R3
PP9	F9 + R3
PP10	F10 + R4
PP11	F11 + R4
PP12	F12 + R4
PP13	F13 + R4
PP14	F14 + R5
PP15	F14 + R6
PP16	F14 + R7
PP17	F14 + R8
PP18	PP10 + PP14
PP19	PP11 + PP15
PP20	PP12 + PP16
PP21	PP13 + PP17
PP22	F15 + R9

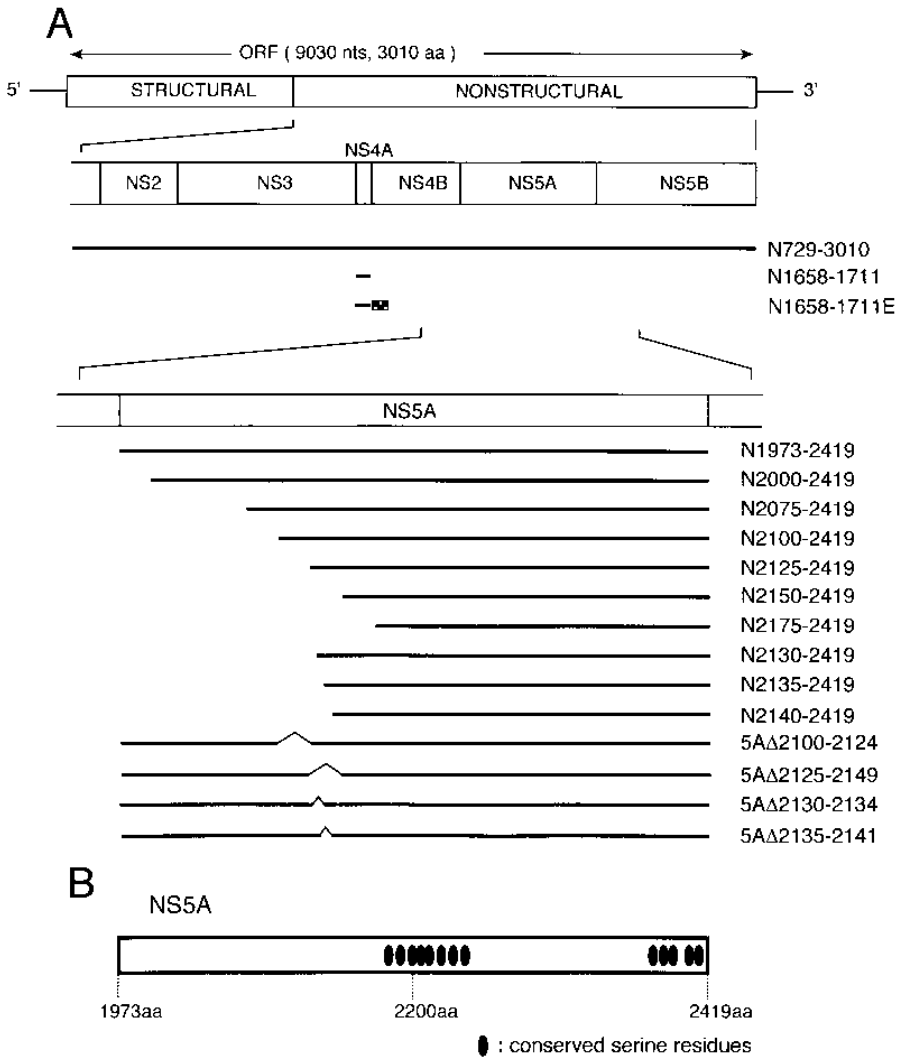

FIG. 1. Schematic representations of HCV polyprotein fragments produced by expression plasmids. (A) The genomic and polyprotein structures of the NS region from NS2 to NS5B are shown enlarged below the HCV open reading frame (ORF). The regions of polypeptides in the HCV precursor polyprotein are shown by thick bars. The designations of HCV polypeptides synthesized in COS-1 cells are shown at the right. Numbers in the peptide designations indicate amino acid positions from the N to the C terminus of the HCV precursor polyprotein (9). The construction of plasmids pKS(+)/CMV, pCMV/N729-3010, pCMV/N1658-1711, pCMV/N1973-2419, and pCMV/SSS2200/1/2AAA has been described previously (5, 7, 21). We designed pCMV/C-Etag to express a protein fused to an E tag at its C terminus in the following way. An oligonucleotide that included the sequences for a *Pst*I site, an *Eco*RI site, 39 nucleotides coding the peptide tag for use in the Recombinant Phage Antibody System (Pharmacia), and a *Hin*dIII site was synthesized. It was digested with *Pst*I and *Hin*dIII and inserted into the *Pst*I and *Hin*dIII sites of pKS(+)/CMV. To introduce deletion mutations into the HCV NS5A region, a series of plasmids were constructed by PCR. The sequences of the synthetic oligonucleotides used as PCR primers are shown in Table 1. The combinations of positive-stranded and negative-stranded primers used for PCR products PP1 to PP22 are shown in Table 2. PP1 was digested with *Pst*I and replaced with the *Pst*I fragment of pCMV/N1973-2419 to obtain pCMV/N2000-2419. PP2 and PP3 were digested with *Pst*I and *Eco*RI and replaced with the *Pst*I and *Eco*RI fragments of pCMV/N1973-2419 to obtain pCMV/N2075-2419 and pCMV/N2100-2419, respectively. PP4 to PP9 were digested with *Pst*I and *Eco*RI and inserted into the *Pst*I and *Eco*RI sites of pCMV/C-Etag to obtain pCMV/N2125-2419E, pCMV/N2150-2419E, pCMV/N2175-2419E, pCMV/N2130-2419E, pCMV/N2135-2419E, and pCMV/N2140-2419E, respectively. The *Nde*I fragments of pCMV/N2125-2419E, pCMV/N2150-2419E, pCMV/N2175-2419E, pCMV/N2130-2419E, pCMV/N2135-2419E, and pCMV/N2140-2419E were replaced with the *Nde*I fragment of pCMV/N1973-2419 to obtain pCMV/N2125-2419, pCMV/N2150-2419, pCMV/N2175-2419, pCMV/N2130-2419, pCMV/N2135-2419, and pCMV/N2140-2419, respectively. Internally deleted forms of protein N1973-2419 were constructed as follows. PCR products PP10 to PP17 were obtained by using primers shown in Table 2. The resultant products were used as primers to obtain second PCR products, PP18 to PP21. PP18 to PP21 were digested with *Mlu*I and *Nhe*I and replaced with the *Mlu*I and *Nhe*I fragments of pCMV/N1973-2419 to obtain pCMV/5AΔ2100-2124, pCMV/5AΔ2125-2149, pCMV/5AΔ2130-2134, and pCMV/5AΔ2135-2141, respectively. PCR product PP22 was digested with *Pst*I and *Eco*RI and inserted into the *Pst*I and *Eco*RI sites of pCMV/C-Etag to obtain pCMV/N1658-1711E, which expressed NS4A fused to an E tag at its C terminus. The checkered box indicates E tag fused in frame at the C-terminal end of the HCV polypeptide (abbreviated E in the peptide designations). nts, nucleotides. (B) Distribution of well-conserved (among HCV genotypes) serine residues on the NS5A protein.

3, 5, 7, and 9). On the other hand, the products of N2140-2419 and N2150-2419 were not coimmunoprecipitated by α-E-tag (Fig. 4A, lanes 12 and 14). NS4AE was not coimmunoprecipitated with N2140-2419 or N2150-2419 (Fig. 4B, lanes 11 and 13). These results indicated that NS5A and NS4AE formed a complex and that the sequence including aa 2135 to 2139 of NS5A was important for the association with NS4A. To confirm that NS5A associates with NS4A and not with the E-tag region of NS4AE, NS5A and NS4A were coexpressed and immunoprecipitated by α-NS5A or α-NS4A in the same way. When NS5A was produced with NS4A, α-NS5A could coprecipitate NS4A as well as NS5A; however, a faint amount of

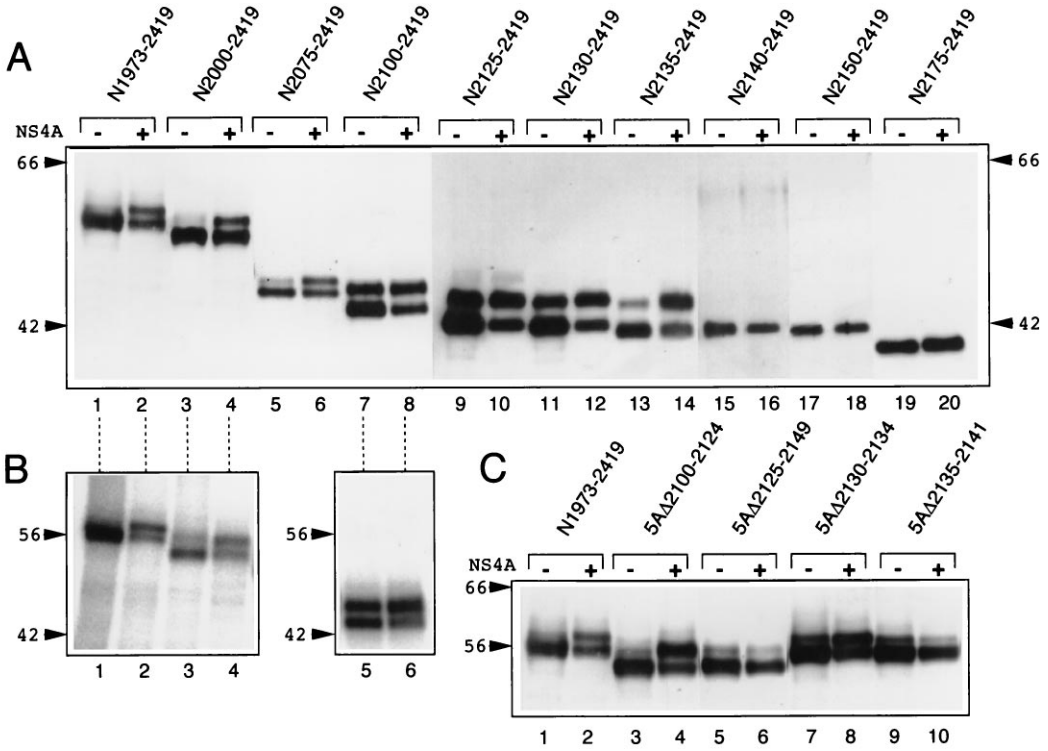

FIG. 2. Identification of the region important for phosphorylation of the slower-migrating protein form. COS-1 cells were transfected with NS5A or its derivative-producing plasmids as denoted above each lane (lanes −) or cotransfected with each of these plasmids plus a plasmid expressing NS4A (lanes +). Cell lysates were fractionated by SDS–8% PAGE, followed by Western blot analysis with α-NS5A. The results with N-terminally deleted forms of NS5A (A) and those with internally deleted forms (C) are shown. Cells transfected with plasmids which produce N1973-2419, N2000-2419, or N2100-2419 were metabolically labeled with [^{32}P]orthophosphate, and lysates were immunoprecipitated with α-NS5A, followed by fractionation by SDS–7% PAGE and autoradiography (B). The positions of molecular mass markers in kilodaltons are shown on the left or right.

NS5A was coprecipitated by α-NS4A (data not shown). This suggested that the binding site of NS4A with NS5A and the epitope of α-NS4A shared the same region, so that α-NS4A could not bind to the NS4A-NS5A complex. The NS4A that was detected by α-NS4A might be in a form that did not associate with NS5A. Although 5AΔ2100-2124, 5AΔ2125-2149, 5AΔ2130-2134, and 5AΔ2135-2141 were coimmunoprecipitated with NS4AE by α-E-tag, less 5AΔ2125-2149 and 5AΔ2135-2141 than 5AΔ2100-2124 and 5AΔ2130-2134 were coprecipitated by α-E-tag (Fig. 4C, lanes 2, 4, 6, and 8). From these data we concluded that the NS5A site that most strongly binds NS4A is from aa 2135 to 2141 and that the amino acids upstream of that site are also implicated in the association with NS4A.

Discussion. N-terminally deleted and internally deleted forms of NS5A were analyzed for phosphorylation by detecting the slower-migrating form by SDS-PAGE followed by Western blotting. To confirm that this assay system exactly reflected the phosphorylation state of the protein, NS5A derivatives were metabolically labeled by [^{32}P]orthophosphate and analyzed by radio imaging as previously described (21). The results of the labeling experiments were almost identical to the results from immunoblotting with α-NS5A (Fig. 2B). The fact that treatment of cell lysates with alkaline phosphatase before gel electrophoresis results in the disappearance of the slower-migrating form indicates that the slower-migrating form is a phosphoprotein which is phosphatase labile (7). Thus, the difference in phosphorylation between the faster-migrating form and the slower-migrating form can be analyzed by relative mobility in SDS-PAGE following Western blotting.

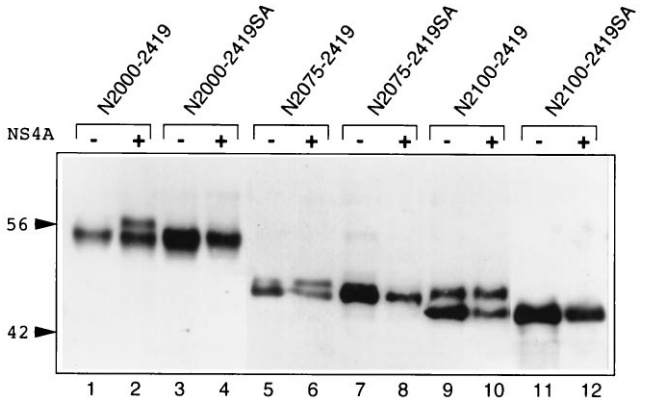

FIG. 3. Effect of triple mutations in serine residues 2200, 2201, and 2202 on NS4A-dependent phosphorylation of N-terminally deleted forms of NS5A. The *Mlu*I and *Nhe*I fragments of pCMV/N2000-2419, pCMV/N2075-2419, and pCMV/N2100-2419 were replaced with the *Mlu*I and *Nhe*I fragments of pCMV/SSS2200/1/2AAA to obtain pCMV/N2000-2419SA, pCMV/N2075-2419SA, and pCMV/N2100-2419SA, respectively. COS-1 cells were transfected with plasmids which produce N-terminal 27-, 102-, and 127-aa-deleted forms of NS5A, N2000-2419, N2075-2419, and N2100-2419, respectively, or with plasmids which produce these peptides with further mutations of serine residues at aa 2200, 2201, and 2202 to alanine residues (abbreviated SA in the peptide designations), respectively, in the absence (−) or presence (+) of NS4A-expressing plasmid. Cell lysates were fractionated by SDS–8% PAGE, followed by Western blot analysis with α-NS5A. The positions of molecular mass markers in kilodaltons are shown on the left.

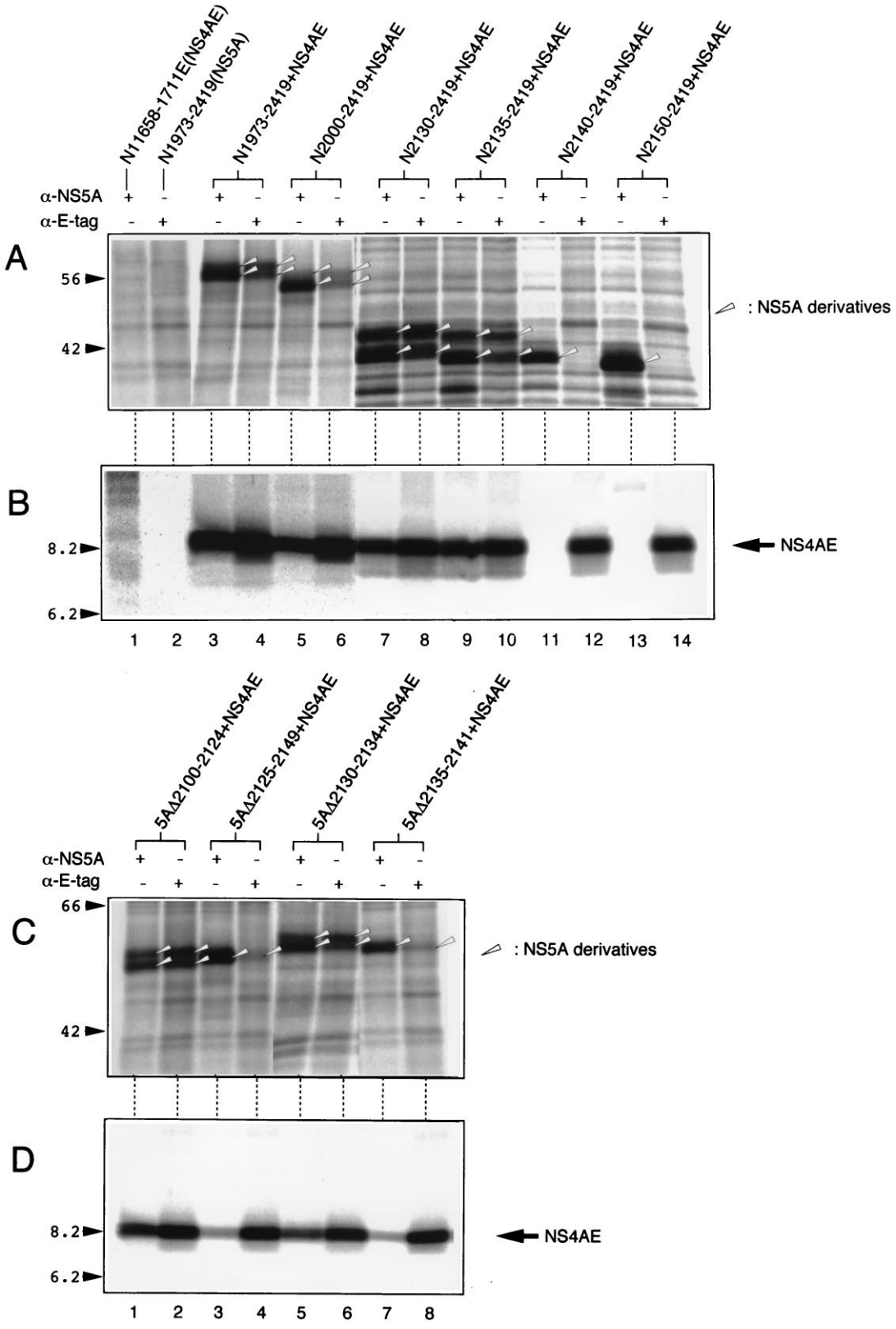
N11658-1711E(NS4AE)
N1973-2419(NS5A)
N1973-2419+NS4AE
N2000-2419+NS4AE
N2130-2419+NS4AE
N2135-2419+NS4AE
N2140-2419+NS4AE
N2150-2419+NS4AE
α-NS5A
α-E-tag
A
56
42
: NS5A derivatives
B
8.2
6.2
NS4AE
1 2 3 4 5 6 7 8 9 10 11 12 13 14
5AΔ2100-2124+NS4AE
5AΔ2125-2149+NS4AE
5AΔ2130-2134+NS4AE
5AΔ2135-2141+NS4AE
C
66
42
: NS5A derivatives
D
8.2
6.2
NS4AE
1 2 3 4 5 6 7 8

FIG. 4. Coprecipitation of NS5A with NS4A. COS-1 cells were cotransfected with NS4AE (NS4A-fused E tag at the C terminus)-expressing plasmid together with NS5A- or N-terminally or internally deleted forms of NS5A-expressing plasmids, as denoted above on each lane. Cells were incubated in 1 ml of methionine-free Eagle's minimal essential medium with 5% dialyzed fetal calf serum supplemented with 100 μCi of [^{35}S]methionine per ml for 4 h. After labeling, the cells were lysed in 1 ml of lysis buffer (0.5% Triton X-100, 120 mM NaCl, 20 mM Tris [pH 7.5], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The cell lysate was centrifuged at 15,000 × *g* for 20 min to remove nuclei and debris, and the supernatant was preadsorbed overnight with 30 μl of protein G-Sepharose suspension. After centrifugation, the clarified supernatant was incubated with α-NS5A or α-E-tag for 2 h. The immune complex was recovered by the addition of 30 μl of protein G-Sepharose and washed three times with lysis buffer and once with NTE buffer (10 mM Tris [pH 7.4]–50 mM NaCl–1 mM EDTA). The immune complex was solubilized in sample buffer and analyzed by SDS–8% PAGE (A and C) or Tricine–SDS–16% PAGE (B and D) (13). The gels were dried and exposed to imaging plates (Fuji Photo Film Co., Ltd.). (A and B) Results with N-terminally 0- to 177-aa-deleted forms of NS5A with NS4AE; (C and D) results with internally deleted forms with NS4AE. A plasmid which produces NS4AE (A and B, lanes 1) or NS5A (N1973-2419) (A and B, lanes 2) was transfected, and the lysate was analyzed by immunoprecipitation with α-NS5A or α-E-tag, respectively, as negative controls. White arrowheads in panels A and C indicate NS5A and its derivatives. The positions of molecular mass markers in kilodaltons are shown on the left.

We found that derivatives with deletions from 102 to 162 aa from the N terminus of NS5A generated the slower-migrating forms even in the absence of NS4A (Fig. 2A, lanes 5, 7, 9, 11, and 13). When N-terminally 102- to 162-aa-deleted forms of NS5A were expressed, production of the slower-migrating form of NS5A was augmented and/or detected more clearly by immunoblotting. Certainly production of each slower-migrating form derived from these N-terminally deleted forms of NS5A was augmented by the presence of NS4A (Fig. 2A, lanes 6, 8, 10, 12, and 14). However, further N-terminal deletion from aa 2140 resulted in production of a single form which appeared to correspond to each faster-migrating form, as judged from the mobility relative to that of the other products. Thus, the 5 aa residues 2135 to 2139 in N2135-2419 seem very important for production of the slower-migrating form of NS5A. Contrary to this observation, the forms with internal deletion of the aforementioned 5 aa residues still produced the slower-migrating NS5A derivatives, although the level of the production was very low and no augmentation of the slower-migrating form by NS4A was observed (Fig. 2C, lanes 5, 6, 9, and 10). This result together with the above observation suggested that the upstream region from aa 2135, as well as the region from aa 2135 to 2139, affected production of the slower-migrating forms of NS5A derivatives. The region from aa 2135 to 2139 seems to be involved in the augmentation of the production of the slower-migrating form of NS5A by NS4A.

Association of NS5A with NS4A through its N-terminal region was clearly demonstrated (Fig. 4). All deleted forms from the N terminus to aa 2134 could bind with NS4A. Further N-terminally deleted forms, however, failed to associate with NS4A. In particular, it is worth mentioning that the NS5A derivative N2140-2419 had impaired association with NS4A. This construct did not produce the slower-migrating form of NS5A, suggesting that physical interaction of NS5A through the region from aa 2135 to 2139 with NS4A is important for the production of the slower-migrating form. The internally truncated forms of NS5A which lacked the region from aa 2135 to 2141 or from aa 2125 to 2149 retained the ability to associate with NS4A. However, the extent of association of these forms with NS4A was lower than that with 5AΔ2100-2124 and 5AΔ2130-2134 (Fig. 4C and D). This result suggested that NS4A interacts through different N-terminal regions of NS5A besides the aa 2135 to 2139 region, but association through these regions may not be sufficient for NS4A-dependent enhancement of the production of the slower-migrating form (Fig. 2C, lanes 6 and 10, and 4C and D, lanes 3, 4, 7, and 8).

The ratio of the slower-migrating form to the faster-migrating form coprecipitated by α-E-tag was almost identical to the ratio in immunoblot analysis (for example: Fig. 2A, lanes 12 and 14, versus 4A, lanes 8 and 10). This suggests that NS4A almost equally associates with the faster-migrating form and the slower-migrating form. The α-NS5A used in this work precipitated the faster-migrating form more efficiently than the slower-migrating form under nondenaturing conditions (for example: Fig. 4A, lanes 3 and 5). Phosphorylation at the central serine residues of NS5A may interfere the association of α-NS5A.

Precisely how NS4A affects phosphorylation in the central region of NS5A remains to be clarified. A cellular serine kinase is involved in phosphorylation of NS5A. Therefore, one role of NS4A may be as a modulator which directly affects the kinase activity. The possibility that some cellular factor is involved in the complex between NS5A and NS4A must be tested. However, the data presented here seems to favor modulation of phosphorylation of NS5A via direct association between NS4A and NS5A. NS4A may facilitate NS5A processing through an interaction that results in revelation of phosphorylation sites to cellular serine kinases. Our data suggested that the intact conformation of NS5A is likely to interfere with the phosphorylation of the central region. However, if the deletion of the N-terminal region changes this conformation or if the association of NS5A with NS4A causes a conformational change in NS5A, then phosphorylation in the central region may occur. The mechanism of this interference remains to be solved.

The physiological role of the phosphorylation of NS5A as well as the function of NS5A in HCV viral replication is not known. We previously showed that most of the nonstructural viral proteins interact and form a complex that associates with microsomal membrane (6), suggesting that NS5A associates with more viral proteins than just NS4A. Replication of the HCV genome is thought to occur at a site that is closely associated with host cell membranes, as is suggested in replication of the flaviviral RNA genome (12, 14).

The P protein of vesicular stomatitis virus, which binds to polymerase protein (L), is phosphorylated at at least two distinct sites (2). The phosphorylation of P protein is necessary for the transcriptional activity of L protein, indicating that it has a regulatory function (1, 2, 15). Similarly, HCV NS5A may be assumed to interact with NS5B, a potential RNA replicase; the phosphorylation state of NS5A therefore may be involved in regulation of RNA replication.

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