Phosphorylation of Nonstructural 5A Protein of Hepatitis C Virus: HCV Group-Specific Hyperphosphorylation

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We previously showed that two proteins with molecular weights of 56 and 58 kDa are produced from nonstructural protein 5A (NS5A) derived from hepatitis C virus (HCV)-1b genotype. The 56-kDa protein is phosphorylated at serine residues in NS5A, including those located in the C-terminal region of NS5A, while the 58-kDa protein, the hyperphosphorylated form of the 56-kDa protein, is phosphorylated at serine residues in the central region. This hyperphosphorylation is dependent on the presence of HCV NS4A protein. To clarify whether NS4A-dependent phosphorylation also occurs in other HCV genotypes, phosphorylation of NS5A was analyzed by two-dimensional gel electrophoresis. Here, we report that NS5A from the HCV-2a genotype was phosphorylated. However, hyperphosphorylation of NS5A occurs in the HCV-1b genotype but not in the -2a genotype. This result suggests that modification of NS5A phosphorylation reflects the virological features of HCV and that there are physiological differences in the roles of differently phosphorylated NS5A between HCV genotypes.

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of bloodborne chronic hepatitis as well as sporadic non-A, non-B-type chronic hepatitis. HCV is a positive-sense, single-stranded RNA virus belonging to a newly classified genus in Flaviviridae (Choo et al., 1991; Kato et al., 1990; Takamizawa et al., 1991). The virus precursor polyprotein is produced from the largest open reading frame of the virus genome, and individual virus proteins are produced by cleavage of the polyprotein cotranslationally as well as posttranslationally by cellular and viral proteases (Eckart et al., 1993; Grakoui et al., 1993; Hijikata et al., 1991, 1993a,b; Tanji et al., 1994a,b; Tomei et al., 1993). Some of the virus proteins are further modified after polyprotein processing. For example, nonstructural 5A (NS5A) protein is a major phosphoprotein among the HCV proteins (Kaneko et al., 1994). This protein is phosphorylated mainly at serine residues located in the C-terminal and central regions of the molecule (Tanji et al., 1995). Previously, we detected at least two types of NS5A protein, one which shows slow migration and another which migrates faster on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Kaneko et al., 1994). The slower-migrating form is the hyperphosphorylated NS5A produced when the other HCV protein, NS4A, is coproduced. This NS4A-dependent phosphorylation occurs on serine residues in the central region of NS5A (Tanji et al., 1995). Phosphorylation of NS5A of other HCV genotypes has also been reported (Reed et al., 1997). However, it remains to be clarified whether NS4A-dependent phosphorylation also occurs in these cases. Here, we analyzed phosphorylation of NS5A derived from the HCV-2a genotype, which is the genotype most distant from HCV-1b according to phylogenetic classification (Simmonds et al., 1994; Okamoto et al., 1992). We used two-dimensional gel electrophoresis, with isoelectric focusing for the first dimension and size fractionation for the second dimension, to analyze NS5A by increasing the resolution of the phosphorylation state of the protein. We found that hyperphosphorylation of NS5A occurred in the HCV-1b genotype but not in the -2a genotype.

RESULTS

NS4A-dependent hyperphosphorylation of NS5A derived from HCV-1b but not from HCV-2a

In the absence of NS4A, HCV-1b NS5A is phosphorylated at various serine residues including those in the C-terminal region of the molecule. The phosphorylated
molecule migrates to the position corresponding to a molecular mass of 56 kDa on SDS–PAGE. When NS4A is coproduced, some NS5A molecules are hyperphosphorylated and migrate to a position corresponding to 58 kDa. Thus, in the presence of NS4A, HCV-1b NS5A can be resolved to at least two bands, p56 and p58 (Kaneko et al., 1994; Tanji et al., 1995). Phosphorylation and hyperphosphorylation can also be observed in E-tag-fused HCV-1b NS5A (Fig. 1A, lanes 1 and 2). We used the E-tag-fused NS5A constructs throughout this study because the amino acid sequence of the major epitope of NS5A is different between HCV genotypes. In HCV-1b, the radioactivities of the faster and the slower migrating NS5A disappeared after treatment with bacterial alkaline phosphatase, although the faster migrating form was detectable by Western blot (Fig. 1A, lane 3). This indicated that NS5A products were phosphorylated and the slower migrating form was more sensitive to phosphatase treatment than the faster migrating form. This is consistent with our previous observations (Kaneko et al., 1994) and suggested that the production of the slower migrating form of NS5A was a result of additional phosphorylation to the faster migrating form.

NS5A of HCV-2a is 19 amino acid residues larger than that of HCV-1b and thus runs more slowly on the gel (Fig. 1A, lanes 4–6). NS5A of HCV-2a was also metabolically labeled by $[^{32}P]$orthophosphate. Radioactivity of HCV-2a NS5A disappeared after treatment with bacterial alkaline phosphatase (Fig. 1A, lane 6). This indicated that phosphorylation of HCV-2a NS5A occurred like that of HCV-1b NS5A. When HCV-2a NS5A was produced together with HCV-2a NS4A, however, NS5A product was not separated into two bands by SDS–PAGE as was seen in the case of HCV-1b NS5A (Fig. 1A, lanes 2 and 5). These results suggested that the major band of HCV-2a NS5A corresponds to the faster migrating form of HCV-1b NS5A and was not phosphorylated in an NS4A-dependent manner.

HCV-2a NS5A is not associated with HCV-2a NS4A

We previously showed that association of HCV-1b NS5A with HCV-1b NS4A is required for NS4A-dependent phosphorylation of NS5A (Asabe et al., 1997). The lack of hyperphosphorylation of HCV-2a NS5A might have been caused by the lack of association of NS5A with NS4A. Then, the association of these two proteins was analyzed by coimmunoprecipitation using anti-E-tag antibody for precipitation of NS5A or using anti-FLAG-tag antibody to precipitate NS4A protein. In this experiment, we used NS4A with a FLAG-tag fused to the C-terminus of the molecule. Cells producing both NS4A and NS5A derived from the two HCV genotypes were prepared and the cell lysates were analyzed for association of these proteins. In the case of HCV-1b, the anti-E-tag antibody coprecipitated both NS5A and NS4A (Fig. 1B, lane 5), the anti-FLAG-tag antibody also coprecipitated NS4A and NS5A (Fig. 1B, lane 12). In the case of HCV-2a, however, the anti-E-tag antibody precipitated only NS5A (Fig. 1B, lane 11) and the anti-FLAG-tag antibody precipitated only NS4A (Fig. 1B, lane 12). This result indicated that HCV-2a NS5A did not associate with NS4A derived from HCV-2a. Together with our previous report (Asabe et al., 1997) it is shown that NS4A-dependent hyperphosphorylation of HCV NS5A.
phorylation of NS5A is correlated with the association of NS5A with NS4A.

Analysis of phosphorylated NS5A by two-dimensional gel electrophoresis

The failure to detect NS4A-dependent phosphorylation of HCV-2a NS5A by the one-dimensional SDS–PAGE suggested that the hyperphosphorylated form of NS5A might comigrate to the same position as the faster migrating protein since phosphorylation does not always result in changes in migration. Thus, we analyzed phosphorylation state of NS5A by two-dimensional gel electrophoresis, i.e., IEF for the first run and SDS–PAGE for the second run. E-tagged NS5A derived from genotypes 1b and 2a was produced in COS-1 cells as described under Materials and Methods. The cell lysates were prepared and separated by two-dimensional gel electrophoresis. After electrophoresis, Western blotting was performed using an anti-E-tag antibody. Unexpectedly, HCV-1b NS5A derived from genotypes 1b and 2a was produced in COS-1 cells as described under Materials and Methods. The cell lysates were prepared and separated by two-dimensional gel electrophoresis. After electrophoresis, Western blotting was performed using an anti-E-tag antibody. Unexpectedly, HCV-1b NS5A protein corresponding to 56 kDa by one-dimensional SDS–PAGE was split into several spots with different pI values, from 4.5 to 5.0 (Fig. 2A). In cells coproducing HCV-1b NS4A, several additional spots corresponding to 58 kDa were observed with pI values from 4.5 to 4.4 (Fig. 2B). HCV-2a NS5A was distributed in the region from pI 4.4 to 5.0 with fewer spots than HCV-1b NS5A (Fig. 2C). In the presence of HCV-2a NS4A, the additional spot derived from HCV-2a NS5A was not observed (Fig. 2D). Thus, we concluded that HCV-2a NS5A was not hyperphosphorylated by HCV-2a NS4A.

Deletion of the C-terminal region of HCV-1b NS5A does not show significant effect on NS4A-dependent hyperphosphorylation

NS4A-independent phosphorylation (basal phosphorylation) occurs on serine residues including those located in the C-terminal region of NS5A. Compared with HCV-1b NS5A, HCV-2a NS5A has an insertion of 19 amino acid residues in the C-terminal region of the molecule. To clarify whether the C-terminal region of NS5A plays some role in regulating NS4A-dependent hyperphosphorylation, the 69 amino acid residues from the C-terminal of HCV-1b NS5A were deleted and this C-terminal-deleted NS5A (5ADC-1b) was examined for hyperphosphorylation by HCV-1b NS4A. 5ADC-1b was detected mainly as a single band by one-dimensional SDS–PAGE (data not shown). On two-dimensional gel electrophoresis, products of 5ADC-1b were scattered into several spots in the region from pI 5.6 to 6.2 with the same mobility, but the total number of 5ADC-1b spots was reduced compared with those seen with intact NS5A (Fig. 3A vs Fig. 2A). In the presence of HCV-1b NS4A, slower migrating spots appeared and these were distributed in the region from pI 5.0 to 5.5 (Fig. 3B). Thus, the C-terminal region of NS5A was not essential for NS4A-dependent hyperphosphorylation. In the case of 5ADC-2a, in which 92 amino acid residues were deleted from the C-terminal region, several spots of 5ADC-2a with pI from 5.5 to 6.2 were observed (Fig. 3C). When HCV-2a NS4A was coproduced, the pattern of 5ADC-2a products on two-dimensional gel electrophoresis was unchanged in

FIG. 2. Analysis of phosphorylated NS5A by two-dimensional gel electrophoresis. Plasmids expressing HCV-1b NS5A, 5A-1b (A and B), or HCV-2a NS5A, 5A-2a (C and D) were transfected together with the plasmid expressing NS4A corresponding to the same genotype of NS5A, respectively (B and D), or with the empty vector (A and C). Lysates of these cells were prepared as described under Materials and Methods and were subjected to two-dimensional gel electrophoresis followed by Western blotting. Electrophoresis in the second dimension was performed through SDS–8% polyacrylamide gels. pI values are shown at the top. The positions of NS5A, 5A-1b and 5A-2a, are indicated with arrows plus —. Spots migrating faster than NS5A in the second run of electrophoresis were unidentified but were different from NS5A derivatives.
This confirmed that HCV-2a NS5A was not hyperphosphorylated by HCV-2a NS4A. Increase of the spot of 5ADC-2a with the highest pI value was observed when NS4A-2a was coproduced (Figs. 3C and 3D). We do not know the reason. Since this spot is the least phosphorylated or the unphosphorylated form of the NS5A derivative, it may be possible that the basal phosphorylation of 5ADC-2a was affected by the presence of NS4A. However, since we did not observe such suppressive effect by NS4A in other derivatives of NS5A, it is assumed that this suppressive effect, if any, might be an artifact to this particular type of NS5A derivative.

**The N-terminal region of NS5A suppresses NS4A-dependent hyperphosphorylation**

The amino acid sequence of NS5A derived from HCV-1b and -2a is relatively highly conserved in the central region, whereas it differs to some extent in the C-terminal and N-terminal regions. We were interested in clarifying whether the N-terminal region of NS5A has some function in regulating NS4A-dependent hyperphosphorylation. Previously, we showed that the association of HCV-1b NS4A with NS5A is important for hyperphosphorylation of NS5A (Asabe et al., 1997). Moreover, deletion of the N-terminal region stimulated hyperphosphorylation even in the absence of NS4A (Asabe et al., 1997). This suggested that in addition to association of the N-terminal region of HCV-1b NS5A with NS4A, the N-terminal region of HCV-1b NS5A has a suppressive function on NS4A-dependent hyperphosphorylation. In contrast to the case of HCV-1b NS5A hyperphosphorylation, NS4A-dependent hyperphosphorylation of HCV-2a NS5A was not observed. Then, we examined whether deletion of the N-terminal region of HCV-2a NS5A had any effect on NS4A-dependent hyperphosphorylation.

Deletion of 129 amino acid residues from the N-terminal region of HCV-1b NS5A (5ADN-1b) resulted in production of the hyperphosphorylated form even in the absence of HCV-1b NS4A. The faster migrating 5ADN-1b was distributed in the region of pI values from 4.1 to 4.6, whereas the slower migrating forms were split from 3.8 to 4.0 (Fig. 4A). pI values of these products were not changed even when NS4A was coproduced but the amount of slower migrating forms was increased (Fig. 4B). Products of HCV-2a NS5A with deletion of the N-terminal 127 amino acid residues (5ADN-2a) corresponding to the faster migrating form were separated from those with pI 4.3 to 4.4 (Fig. 4C). Production of 5ADN-2a gave a few spots, including a major spot at pI 4.4, by two-dimensional gel electrophoresis. However, coproduction of HCV-2a NS4A with 5ADN-2a resulted in accumulation of NS5A with a pI value of 4.3. Production of the more acidic form of NS5A was not detected (Fig. 4D). These results indicated that although the intact form of HCV-2a NS5A was not hyperphosphorylated by HCV-2a NS4A, hyperphosphorylation occurred when the N-terminal region was deleted. Thus, it is likely that the N-terminal region of NS5A of both genotypes has a suppressive function on NS4A-dependent hyperphosphorylation. Interaction of 5ADN-2a with HCV-2a NS4A examined by coprecipitation analysis was not observed (data not shown).
DISCUSSION

Previously, we showed the presence of two forms of HCV-1b NS5A with different degrees of phosphorylation at serine residues in the molecule (Kaneko et al., 1994; Tanji et al., 1995). In this study, we observed that HCV-2a NS5A was phosphorylated, but not hyperphosphorylated, by HCV-2a NS4A. HCV-2a NS5A did not associate with HCV-2a NS4A, unlike the association observed in HCV-1b. Although these results supported our previous observation that NS4A-dependent hyperphosphorylation of NS5A was correlated with the association of NS5A with NS4A, we failed to observe the interaction of the N-terminal-deleted NS5A of HCV-2a, 5ADN-2a, with HCV-2a NS4A. 5ADN-2a could be hyperphosphorylated when HCV-2a NS4A was coproduced, although the degree of the hyperphosphorylation was not as great as that observed in case of HCV-1b NS5A (Figs. 4C and 4D). It may be possible that HCV-2a NS4A interacts with 5ADN-2a so weakly that such an interaction cannot be detected by the coimmunoprecipitation experiment used in this study. Alternatively, NS4A may have an additional function, such as stimulation of a kinase which is responsible for phosphorylation of NS5A, and such function might not be highlighted in our previous work because of its weakness.

We revealed the presence of multiple forms of NS5A molecule differing in degree of phosphorylation by two-dimensional gel electrophoresis. Both slower and faster migrating forms of HCV-1b NS5A were resolved into several spots with different pI values by IEF. All these spots assembled to nearly one spot with a higher pI value of 5.0 after treatment with alkaline phosphatase, indicating that a heterogeneous population of NS5A is produced by different degrees of phosphorylation (data not shown). There are several conserved serine residues distributed in the central and the C-terminal regions of NS5A among HCV genotypes. The amino acid sequences surrounding the putative phosphorylation sites in NS5A suggested that several kinases, including casein kinase II and proline-directed kinases, are involved in phosphorylation. Although there is no direct evidence that NS5A is phosphorylated by these kinases in vivo, it was shown that inhibitors of these kinases suppressed phosphorylation of NS5A (Reed et al., 1997). NS5A was also shown to interact with a double-stranded RNA-activating protein kinase (Gale et al., 1997) or a cellular protein kinase (Ide et al., 1997). The former, however, does not seem to phosphorylate NS5A. A cellular kinase as well as a cAMP-dependent protein kinase A catalytic subunit was shown to phosphorylate NS5A in vitro (Ide et al., 1997).

The presence of several isoforms of NS5A differing in degree of phosphorylation suggests that cellular kinases are inefficiently involved in independent or interdependent phosphorylation of the target sites. Previously, we showed that mutation of some serine residues in the central region of HCV-1b NS5A affected hyperphosphorylation by NS4A, although mutation of other serine residues did not (Tanji et al., 1995). This suggested the presence of hierarchical serine residues, phosphorylation of which influences the phosphorylation of those farther down the hierarchy. It is worth mentioning here that NS5A contains a recognition site for casein kinase II which often acts as a primary protein kinase in the
hierarchical phosphorylation process (Roach, 1991). Using two-dimensional gel electrophoresis, we observed that several spots of the hyperphosphorylated form of HCV-1b NS5A shifted to smaller pI values when phosphorylated at many serine residues. This suggested that NS4A-dependent hyperphosphorylation induces multisite phosphorylation by interdependent mechanisms.

There are several groups of HCV according to phylogenetic analysis. Groups 1 and 2 are the most divergent (Simmonds et al., 1994; Okamoto et al., 1992). These two groups can be distinguished serologically by different reactivity to the epitope present in NS4 corresponding to amino acid residues 1676 to 1760 (Tanaka et al., 1994). NS4A-dependent hyperphosphorylation of NS5A can be observed in the HCV-1b genotype corresponding to group 1, but not in HCV-2a in group 2. This result suggests that modification of NS5A phosphorylation reflects different virological features of HCV between the two groups of HCV. Although the roles of NS5A, as well as phosphorylation of this molecule in virus replication, remain to be clarified, our results suggested that NS5A has different physiological roles between these two groups of HCV.

MATERIALS AND METHODS

Construction of plasmids

The plasmids pCMV/5A1-447E, which expresses HCV-1b NS5A tagged with E-tag at the C-terminus, and pCMV/5A130-447E, expressing the N-terminal-deleted HCV-1b NS5A, were generated by replacing the AccI–ClaI fragment of pCMV/N2100-2419 (Asabe et al., 1997). Plasmid pCMV/5A1-378E, expressing the C-terminal-deleted HCV-1b NS5A, was constructed by inserting the PstI–EcoRI fragment of a PCR product amplified using pCMV/N729-3010 (Hijikata et al., 1993a) as a template with a positive-stranded primer, 5'-CCGAAGCT-TCTCGAGATGTCCGGCTCGTGGCTA-3', and a negative-stranded primer, 5'-TTGAATTCCTTAGTAGCCAGCTCGGCC-3', into the PstI–EcoRI site of pCMV/C-E-tag (Asabe et al., 1997). The resultant plasmids, pCMV/5A1-447E, pCMV/5A130-447E, and pCMV/5A1-378E, encode NS5A-derived proteins consisting of 1 to 447 aa, 130 to 447 aa, and 1 to 378 aa of HCV-1b NS5A, respectively, fused to an E-tag sequence at their C-termini (Fig. 5). Names of proteins produced from these plasmids are abbreviated 5A-1b, 5ADN-1b, and 5ADC-1b, respectively, as shown in parentheses in Fig. 5. Constructions of plasmid pCMV/N1658-1711, which encodes HCV-1b NS4A, has been described previously (Kaneko et al., 1994). To obtain expression plasmids of HCV-2a NS4A and NS5A proteins, the cDNA clone encoding the HCV-2a gene (Kohara et al., unpublished) was used. Plasmids pCMV/5A1-466(2a)E and pCMV/5A128-466(2a)E were generated by inserting the PstI–EcoRI fragment of the PCR product amplified using the HCV-2A cDNA clone as a template with positive-stranded primer 5'-TTCCTGCCAGCAGCAGCTGC-3' and negative-stranded primer 5'-TTGAATTCCTTAGAGCCAGCTCGGCC-3', into the PstI–EcoRI site of pCMV/C-E-tag. pCMV/5A1-374(2a)E was constructed by inserting the linkers 5'-CCATTGGCAGAGGCAGGGGATTG-3' and negative-stranded primer 5'-CTGCAGATTTGCAGCACACAGGC-3' and with positive-stranded primer 5'-CATTCTGCAGCCATGTCTACTACATACAGGAGGATTG-3' and negative-stranded primer 5'-CTGAGATTTGCAGCACACAGGC-3', respectively, into the PstI–EcoRI site of pCMV/C-E-tag. pCMV/5A1-374(2a)E was constructed by inserting the linkers 5'-CCATTGGCAGAGGCAGGGGATTG-3' and 5'-AATTCATTGGG-3' into the MscI–EcoRI site of pCMV/5A1-
lysates were boiled and diluted 10-fold with RIPA buffer. To lysis, 1% Triton X-100, and 1% sodium deoxycolate. Cell containing 50 mM Tris–HCl (pH 7.2), 150 mM sodium chloride, and lysed with 0.1 ml of 1% SDS–RIPA buffer containing the primers 5’TGCAGAAGGAGGACATGTTCTG-3’ and 5’-CTAGAATTCTCTTAAGATTCTCACAATA-3’ into the PstI–EcoRI site of pKS(+) CMV.

Plasmid pCMV/NS4AFLAG, which expresses FLAG-tagged HCV-1b-derived NS4A, was generated by inserting the PstI–EcoRI fragment of pCMV/N1658-1711E (Asabe et al., 1997) into the PstI–EcoRI site of pCMV/FLAG. pCMV/NS4A(2a)FLAG, which expresses FLAG-tagged HCV-2a NS4A, was constructed by inserting the PstI–EcoRI fragment of pCMV/NS4A(2a) and the linkers 5’-ATGAGGCTTTTGATGAG ATGGAGGAATGTG-3’ and 5’-CTCAGACATGTTCTG-3’ into the PstI–EcoRI site of pCMV/FLAG.

Transfection and protein expression

Approximately 8 × 10^5 COS-1 cells were plated on 35-mm plastic tissue culture dishes. After 1 day of culture, a total of 2 μg of plasmid DNA was transfected using the FuGENE 6 Transfection Reagent (Boehringer). The cells were incubated for 48 h after transfection. Expression of protein in cells was examined by SDS–PAGE followed by Western blotting analysis as described previously (Hijikata et al., 1993a).

Metabolic labeling with [32P]orthophosphate

COS-1 cells were transfected with NS5A expression plasmids for 40 h before metabolic labeling. The cells were incubated with 1 ml of phosphate-free Dulbecco’s modified eagle medium (D-MEM; Gibco BRL) with 10% dialyzed FBS for 1 h and further incubated with 1 ml of phosphate-free D-MEM with 10% dialyzed FBS supplemented with 100 μCi of [32P]orthophosphate (ICN) for 4 h.

Immunoprecipitation

Cells were washed with cold phosphate-buffered saline and lysed with 0.1 ml of 1% SDS–RIPA buffer containing 50 mM Tris–HCl (pH 7.2), 150 mM sodium chloride, 1% Triton X-100, and 1% sodium deoxycolate. Cell lysates were boiled and diluted 10-fold with RIPA buffer. One milliliter of the lysates was preadsorbed with 30 μl of protein G–Sepharose suspension (Pharmacia) for 1 h. After centrifugation, the supernatant was incubated with anti-E-tag antibody for 1 h. The immune complexes were recovered by adsorbing to protein G–Sepharose. The immune complexes were recovered by adsorbing to protein G–Sepharose. The immune complexes were recovered by adsorbing to protein G–Sepharose. The immune complexes were recovered by adsorbing to protein G–Sepharose. The immune complexes were recovered by adsorbing to protein G–Sepharose.


