Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity

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Hepatitis C virus (HCV) NS5A is RNA-dependent RNA polymerase (RdRP), the essential catalytic enzyme for HCV replication. Recently, NS5A has been reported to be important for the establishment of HCV replication in vitro by the adaptive mutations, although its role in viral replication remains uncertain. Here we report that purified bacterial recombinant NS5A and NS5B directly interact with each other in vitro, detected by glutathione S-transferase (GST) pull-down assay. Furthermore, complex formation of these proteins transiently coexpressed in mammalian cells was detected by coprecipitation. Using terminally and internally truncated NS5A, two discontinuous regions of NS5A (amino acids 105–162 and 277–334) outside of the adaptive mutations were identified to be independently essential for the binding both in vivo and in vitro (Yamashita, T., Kaneko, S., Shirōta, Y., Qin, W., Nomura, T., Kobayashi, K., and Mkyrakami, S. (1998) J. Biol. Chem. 273, 15479–15486). We previously examined the effect of His-NS5A on RdRP activity of the soluble recombinant NS5B in vitro (see Yamashita et al. above). Wild NS5A weakly stimulated at first (when less than 0.1 molar ratio to NS5B) and then inhibited the NS5B RdRP activity in a dose-dependent manner. The internal deletion mutants defective in NS5B binding exhibited no inhibitory effect, indicating that the NS5B binding is necessary for the inhibition. Taken together, our results support the idea that NS5A modulates HCV replication as a component of replication complex.

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† The abbreviations used are: HCV, hepatitis C virus; RdRP, RNA-dependent RNA polymerase; DTT, dithiothreitol; PBS, phosphate-buffered saline; aa, amino acid(s); GST, glutathione S-transferase; ISDR, interferon sensitivity-determining region.
the role and molecular mechanism of NS5A in viral replication remains uncertain. Recently, NS5A has come to be highlight since the adaptive mutations of NS5A have been reported in selectable self-replicating RNAs in tissue culture cell systems, strongly suggesting that NS5A is important for HCV replication (30, 31). To gain further insight into its function in viral replication, we examined protein-protein interaction between NS5A and the NS5B RdRP, which is the central catalytic enzyme in HCV replicase, and studied the effect of NS5A on the RdRP activity of NS5B, based on the concept that NS5A might be one component of the replicase.

MATERIALS AND METHODS

Plasmid Constructions—A plasmid derived from pGENK1, pGENKS (23, 36), was used to express the recombinant glutathione S-transferase (GST)-fused proteins in *Escherichia coli*, which contains multiple cloning sites. The bacterial His-tagged ex-portion of pSG5UTPL (33), which was inserted into EcoRI, KpnI, and XmaI, downstream of large cloning sites, was inserted into EcoRI, KpnI, and XmaI, downstream of large cloning sites, and d335–411 (lane 4) were separated by SDS-10% PAGE and detected by silver staining. Approximately 0.2 μg of FLAG-NS5A wild type and mutants were mixed with 1 μg of GST-NS5Bt and pulled down with GST resin after preblocking by 1% bovine serum albumin. After washing with PBST, each bound protein was fractionated by SDS-10% PAGE and subjected to Coomassie Brilliant Blue staining. A NS5A.

FIG. 1. Detection of HCV NS5A and NS5B complexity *in vitro* by GST pull-down assay and mapping of NS5B binding regions on NS5A. A, partially purified bacterial recombinant protein GST (lane 1) and GST-NS5Bt (lane 2) were separated by SDS-10% PAGE and detected by Coomassie Brilliant Blue staining. B, partially purified bacterial recombinant protein FLAG-NS5A wild type (lane 1) and internally deleted mutants, FLAG-NS5A d105–162 (lane 2), d277–334 (lane 3), and d335–411 (lane 4) were separated by SDS-10% PAGE and detected by silver staining. Approximately 0.2 μg of FLAG-NS5A wild type and mutants were mixed with 1 μg of GST-NS5Bt and pulled down with GST resin after preblocking by 1% bovine serum albumin. After washing with PBST, each bound protein was fractionated by SDS-10% PAGE and subjected to Western blot analysis with anti-FLAG monoclonal antibody (C, lanes 2–5). 0.2 μg of FLAG-NS5A wild type was mixed with 1 μg of GST after preblocking and then pulled down, fractionated, and detected as GST-NS5Bt (C, lane 1).
the EcoRI and BamHI sites. The wild type or mutant NS5A DNA fragment was inserted into the SacI and BamHI sites.

All of the mammalian expression vectors were derived from pSG5UTPL (33, 36). The pNKFLAG vector (33), which was constructed by replacement of Not-I-BamHI site of pSG5UTPL with a fragment composed of an artificial initiation codon and sequences encoding FLAG epitope and an artificial stop codon containing BamHI and BglII sites at 5’- and 3’-ends, respectively. The pNKGST vector, the GST-fused protein expression vector, was constructed by replacement of the Not-I-BamHI site by a fragment encoding the GST protein, threonin digestion site, and multiple cloning sites (EcoRI, SacI, KpnI, XmaI, Sall, and BamHI) derived from pGENKS.

HCV JK-1 cDNA (34) harboring NS5A and NS5B was subcloned by PCR using the sets of primers, NS5A For (representing forward) and NS5A Rev (representing reverse) and NS5B For and NS5B Rev, respectively (Table I). NS5A For and NS5B For contain artificial initiation codons downstream of the SacI site. NS5A Rev and NS5B Rev contain BamHI sites. Amino-terminally truncated mutants of NS5A were subcloned by PCR using different For primers with NS5A Rev (Table I). NS5A carboxy-terminally truncated mutants were subcloned using NS5A For and different Rev primers (Table I). All For and Rev primers contain SacI and BamHI sites, respectively. NS5A 105–447 For, 110–444 For, and 335–447 For primers included artificial initiation codons downstream of the SacI site, and the other For primers contained methionine initiation sites. Internal deleted mutants of NS5A were constructed by PCR using sets of For and Rev primers composed of upstream and downstream sequences of deleted sites (Table I).

All cDNA of NS5A and NS5B wild type and mutants were inserted in the SacI and BamHI sites of the vectors, and the sequences of all of the constructions were confirmed by the dideoxy sequence method.

Expression and Purification of Baculoviral Recombinant NS5A and NS5B Proteins—GST-fused HCV NS5B protein was expressed and purified as described previously (23). GST-NS5A and GST-NS5B 335–447 proteins were expressed in BL21 plysS (DE3) by induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside at 30 °C for 8 h. Cells were harvested and sonicated in buffer A (phosphate-buffered saline containing 1 mM DTT and 1% Triton X-100). The supernatants after centrifugation were mixed with glutathione-Sepharose 4B beads (GST resin) (Amersham Biosciences, Inc.), and GST-fused protein was allowed to absorb to the beads for 1 h at 4 °C. The beads were washed with buffer A and then with 50 mM Tris-HCl, pH 8.0, containing 1 mM DTT.

FLAG-tagged NS5A and NS5A mutants were expressed in BL21 and harvested in buffer A as GST-NS5A. FLAG-tagged proteins were purified by incubating the sonication supernatants with anti-FLAG M2 resin (Kodak Scientific Imaging System), followed by several washes with buffer A and elution with buffer containing the FLAG peptide (0.1 mg of FLAG peptide per ml, 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT). The protein concentration was measured by the Bradford method or Coomassie Brilliant Blue staining or silver staining with bovine serum albumin as a standard.

E. coli strain BL21 plysS (DE3) transformed with wild or an internal deletion mutant of pYUT/NS5A was grown in the presence of 100 μg/ml ampicillin at 37 °C and then induced with 0.7 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 27 °C. The recovered and then washed cell pellet was resuspended in pre cleared buffer (50 mM sodium phosphate, 10 mM β-mercaptoethanol, 10 mM mercaptoethanol, 1% Triton X-100). The suspension was sonicated on ice and centrifuged at 15,000 g for 15 min. The pellet was suspended in denaturing binding buffer (100 mM sodium phosphate, 10 mM Tris-HCl, 8 mM urea) with the pH value adjusted to 8.0 by HCI. The lysate was centrifuged at 15,000 g for 5 min, and the cleared lysate was subjected to affinity binding to Ni2+-ni trotetraacetic acid-Sepharose (Qiagen) preequilibrated with denaturing binding buffer. The resin was washed once with denaturing binding buffer and then washed several times with denaturating buffer mixed with increasing amounts of bridge buffer (100 mM sodium phosphate, 10 mM Tris-HCl, 2 mM DTT, 1 mM NaCl, 2% Triton X-100) to renature the protein and then finally washed with bridge buffer. The bound His-NS5A was eluted with native elution buffer (50 mM sodium phosphate (pH 8.0), 500 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, 1% Triton X-100). The suspension was sonicated on ice and centrifuged at 15,000 g for 15 min. The supernatant was mixed with glutathione-Sepharose 4B beads (GST resin) and GST-fused protein was allowed to absorb to the beads for 1 h at 4 °C. The washed beads were then used for Western blot analysis with anti-FLAG antibody (output). Signal intensities of input and output in Western blot analysis were quantified by NIH Image version 1.62 software, and percentages of recovered proteins were calculated. We defined that NS5As interact with NS5B at more than 1% recovery. C, the nitrocellulose membranes used for Western blot analysis of coprecipitants with anti-FLAG antibody were reprobed with anti-GST monoclonal antibody.

**Fig. 2.** Interaction between NS5A and NS5B proteins in vivo and mapping the NS5B binding regions on NS5A.

COS1 cells were transiently cotransfected with mammalian expression vectors, cFLAG-NS5As (wild type and amino- or carboxyl-terminally and internally truncated mutants) and pNKGST-NS5B (lanes 1–21) or pNKGST-NS5Bt (lanes 22–25). A, total lysates were fractionated by SDS-10% PAGE and subjected to Western blot analysis with anti-FLAG monoclonal antibody (input). B, coprecipitants by GST resin were washed with PBS containing 0.5% Triton X-100, fractionated by SDS-10% PAGE, and detected by Western blot analysis with anti-FLAG antibody (output). Signal intensities of input and output in Western blot analysis were quantified by NIH Image version 1.62 software, and percentages of recovered proteins were calculated. We defined that NS5As interact with NS5B at more than 1% recovery.
Interaction of HCV NS5A and NS5B

NaOH (pH 8.0), 170 mM KCl, 7.5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 1% Triton X-100 containing 1% bovine serum albumin. After extensively washing with GBT buffer, the bound proteins were fractionated by 10% SDS-PAGE and subjected to Coomassie Brilliant Blue staining. To examine the dose effect of wild type His-NS5A on NS5B binding, 0.25, 0.5, 1, and 10 µg of wild type His-NS5A were incubated with prebound GST-NS5Bt.

Preparation of Cell Extracts, Coprecipitation with GST Resin, and Western Blot Analysis—Transient transfection of COS1 cells was carried out as reported previously (35). The cells were harvested and washed with phosphate-buffered saline (PBS) and sonicated in PBS lysis buffer (PBS containing 240 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, and 1 mM DTT) with 10 µl of aprotinin and leupeptin per ml. Total cell lysates were diluted 5-fold with PBS lysis buffer and mixed with 10 µl of GST resin, and then incubated for 3 h on a rotator at room temperature. After washing with PBS containing 0.5% Triton X-100, the bound proteins were eluted, fractionated by SDS-10% PAGE, transferred onto nitrocellulose membranes, and subjected to Western blot analysis with anti-FLAG monoclonal antibody. The proteins were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences). Signal intensities of Western blot analysis were quantified by NIH Image version 1.62 software, and percentages of recovered proteins were calculated. The nitrocellulose membranes used for Western blot analysis with anti-FLAG monoclonal antibody were reprobed with anti-GST monoclonal antibody (Zymed Laboratories Inc.) according to the manufacturer’s instructions (Amersham Biosciences).

Poly(A)-dependent UMP Incorporation Assay—RdRP activity of GST-NS5Bt was examined by the UMP incorporation assay reported previously (23). 20 ng (10 nmol) of GST-NS5Bt was incubated at 25 °C for 2 h in the reaction solution (20 µl) containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 1 mM DTT, 1 mM EDTA, 40 units of RNase inhibitor, 4 µCi of [α-32P]UTP (800 Ci/mmole), 10 µM UTP, 10 µg/ml poly(A), and 1 µg/ml oligo(U). The reaction was stopped by transferring the reaction solution to DE81 filter (Whatman), which was then washed extensively with 0.5 M Na2HPO4 (pH 7.0) and briefly rinsed with 70% ethanol. The filter-bound radioactivity was measured by a scintillation counter.

Varying amounts of wild type or internal deletion mutant His-NS5A were added to the reaction before the addition of the substrate to examine the effect of NS5A on NS5B RdRP activity.

RESULTS

NS5A Specifically Binds NS5B in Vitro—To elucidate whether NS5A interacts with NS5B directly as a component of HCV replicase, we pursued the in vitro interaction study between bacterial recombinant proteins, FLAG-tagged NS5A, and GST-fused NS5Bt using the GST pull-down assay. NS5Bt is a truncated form of NS5B, from which the membrane anchoring the carboxy-terminal 21 aa had been removed to improve solubility. We have previously shown that NS5Bt retained full RdRP activity (23). Partially purified FLAG-NS5A wild type (WT) (ligands 1B, lane 1) was mixed with comparable amounts of GST and GST-NS5Bt proteins (Fig. 1A, lanes 1 and 2, respectively) and pulled down with GST resin as described under “Materials and Methods.” Pulled down proteins were fractionated by SDS-10% PAGE and subjected to Western blot analysis using anti-FLAG (Fig. 1C). FLAG-NS5A was pulled down with GST-NS5Bt (Fig. 1C, lane 2) but not with GST (lane 1), implying the specificity of direct binding of NS5A and NS5B in vitro.

Interaction between NS5A and NS5B Proteins in Vivo and Mapping the NS5B Binding Region(s) on NS5A—We determined whether the interaction of NS5A and NS5B occurs in vivo by coprecipitation assay and Western blot analysis. Furthermore, mapping of NS5B binding region(s) on NS5A was carried out using a set of plasmids expressing amino-terminally and internally truncated NS5A proteins (Fig. 4). COS1 cells were transiently cotransfected with mammalian expression vectors, cFLAG-NS5A and pNKGST-NS5B, and the cell lysates were precipitated with GST resin. Under conditions in which the expression levels of cFLAG-NS5A proteins (input) were adjusted to be identical (Fig. 2A, lanes 1–21) and the precipitated amount of GST-NS5B protein was even (Fig. 2C, lanes 1–21), the precipitation efficiencies of NS5A proteins (output) were uneven (Fig. 2B, lanes 1–21). The full-length NS5A protein (lane 1) and truncated NS5A proteins up to 105 aa at the amino terminus (lanes 2–4) and up to 46 aa at the carboxyl terminus (lanes 8–10) were efficiently brought down. Internal truncations spanning 163–221 (lane 17), 222–236 (lane 16), 237–276 (lane 15), and 335–411 aa (lane 19) also did not have much effect on the interaction. In contrast, neither of the internally truncated NS5A mutants deleted at 105–162 aa (lane 18) nor 277–334 (lane 21) aa was efficiently coprecipitated. These results are summarized in Fig. 4. Although truncations of more than 46 aa at the carboxyl terminus, NS5A 1–397 (lane 11), 1–355 (lane 12), and 1–334 (lane 13) abolished the NS5B interaction, internal deletion of 335–411 (lane 19) aa retained the ability. These results indicate that this region is actually dispensable. In conclusion, both of the discontinuous regions harboring 105–162 and 277–334 aa seem to be indispensable for the NS5B binding.

We used GST-NS5Bt as a bacterial recombinant protein for an in vitro binding assay. To confirm whether truncation of the membrane anchoring domain of NS5B changes the properties of NS5A binding ability, a coprecipitation assay was carried out using GST-NS5Bt coexpressed with cFLAG-NS5As in COS1 cells. Similar to the results of GST-NS5B wild type, cFLAG-NS5A (lane 24) and cFLAG-NS5A d355–411 (lane 23) proteins were coprecipitated efficiently with GST-NS5Bt protein, whereas cFLAG-NS5A d105–162 (lane 22) and cFLAG-NS5A d277–334 (lane 25) proteins were not (Fig. 2, lanes 18–21). These results show that truncation of the membrane-anchoring domain of NS5B does not affect NS5A binding properties.

Furthermore, to exclude the possibility that tagging by FLAG causes a conformational change of NS5A and effects on binding ability, we pursued the coprecipitation assay using pNKFFLAG-NS5A plasmids encoding NS5As. FLAG-tagged NS5A specifically binds NS5B in vitro (Fig. 3). Tagging of NS5A has no effect on binding properties. COS1 cells were transiently cotransfected with mammalian expression vectors, pNKFFLAG (amino-terminally FLAG-tagged vector)-NS5As (wild type, d105–162, d277–334, d355–411), and pNKGST-NS5B. A. Total lysates were fractionated by SDS-10% PAGE and subjected to Western blot analysis with anti-FLAG monoclonal antibody (input). B, coprecipitants by GST resin were washed with PBS containing 0.5% Triton X-100, fractionated by SDS-10% PAGE, and detected by Western blot analysis with anti-FLAG antibody (output). Signal intensities of input and output in Western blot analysis were quantified by NIH Image version 1.62 software, and percentages of recovered proteins were calculated. C, the nitrocellulose membranes used for Western blot analysis of coprecipitants with anti-FLAG antibody were reprobed with anti-GST antibody.
Interaction of HCV NS5A and NS5B

FIG. 4. Schematic representation of the amino- or carboxyl-terminally and internally truncated NS5A proteins used for coprecipitation and GST pull-down assay and mapping of NS5B binding regions on NS5A. The thin angled lines indicate internally deleted regions. The results of the binding assay shown in Figs. 1–3 are summarized at the right. 4AB, S, and ISDR at the top are putative domains previously reported for the NS4A binding site (28), serine residues essential for hyperphosphorylation (26), and interferon-sensitivity-determining region (28), respectively. The NS5B-binding regions predicted from the results of binding assay both in vivo and in vitro are indicated by shaded boxes at the bottom.

FIG. 5. NS5B-binding properties of His-NS5A. A, purification of His-tagged NS5A. Wild type His-NS5A was expressed in E. coli and purified as described under “Materials and Methods.” Samples were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. B, dose-dependent binding ability of wild type His-NS5A to GST-NS5Bt. Varying amounts of wild type His-NS5A were incubated with 1 μg of GST-NS5Bt or 2 μg of GST prebound to glutathione resin. Lane 1, 0.5 μg of wild type His-NS5A input. Lanes 2 and 3, 10 μg of input were incubated with glutathione resin alone and GST bound to the resin, respectively. Lanes 4–7, 0.25, 0.5, 1, and 10 μg of wild type His-NS5A were subjected to GST pull-down assay, respectively. C, NS5B binding ability of internal deletion mutants of His-NS5A. Lanes 1, 2, 4, 6, 8, and 10, input of GST-NS5Bt, His-NS5A/d105–162, d163–221, d222–236, d277–334, and d335–411, respectively. Lanes 3, 5, 7, 9, and 11, recovered His-NS5A/d105–162, d163–221, d222–236, d277–334, and d335–411 by GST pull-down assay, respectively.

but nFLAG-NS5A d105–162 (lane 2) and nFLAG-NS5A d277–334 (lane 3) proteins were not, as the results using cFLAG-NS5As, indicating that the tagging of NS5A does not affect the binding properties.

Mapping of NS5B-binding Regions on NS5A in Vitro—To reaffirm whether the binding properties of NS5A and NS5B shown in the in vivo assay are retained in vitro, we performed the GST pull-down assay using purified bacterial recombinant proteins, FLAG-NS5A mutants, and GST-NS5Bt. Similar to the results in vivo (Figs. 2–4), FLAG-NS5A d105–162 (Fig. 1C, lane 3) and FLAG-NS5A d277–334 (lane 4) proteins were not pulled down with GST-NS5Bt protein, implying that, also in vitro, both of those two discontinuous regions harboring 105–162 and 277–334 aa are indispensable for the NS5B binding.

Effect of NS5A on NS5B RdRP Activity—We have previously characterized the properties of NS5B RdRP activity using soluble bacterial recombinant protein, NS5Bt (23). In this assay, the incorporation of [α-32P]UMP was measured using poly(A) and oligo(U)14 as template and primer, respectively. Since the bacterial recombinant FLAG-NS5A was difficult to purify in large amounts and GST-NS5A preparations contained excess amounts of degradation products, a His tagging method was applied to purify NS5A. His-tagged wild type and four different internal deletion mutants were bacterially expressed and affinity-purified (Fig. 5) (see “Materials and Methods”). These His-tagged proteins were examined for NS5B binding by GST pull-down using GST-NS5Bt. GST-NS5Bt recovered His-tagged wild type and two internal deletion mutants of NS5A (d163–221, d335–441) but not two internal deletion mutants (d105–162, d277–334) (Fig. 5, B and C). The result with the internal deletion mutants is the same as that with the FLAG-NS5A mutant proteins (Fig. 3). The effect of wild type and mutant His-NS5A proteins was examined on NS5Bt RdRP activity by UMP incorporation. Wild type His-NS5A inhibited RdRP activity in a dose-dependent manner, and a 5–10-fold molar excess of His-NS5A almost abolished the RdRP activity (Fig. 6). Two internal deletion mutants defective in NS5B binding (d105–162, d277–334) did not inhibit RdRP activity at all, whereas two internal-deletion mutants having the NS5B binding ability (data with d163–221 not shown) exhibited inhibition activity similar to that of the wild type NS5A (Fig. 6). Therefore, the NS5B binding ability is well correlated to the inhibition activity of His-NS5A on RdRP activity. His-NS5A present at less than 0.1 molar ratio to NS5B weakly stimulated RdRP activity by 20–30%, and this stimulation effect was observed with wild type His-NS5A and the internal deletion mutants having the NS5B binding ability (Fig. 6). Thus, this stimulation effect of His-NS5A may be nonstoichiometric but require the NS5B binding.

DISCUSSION

HCV nonstructural (NS) proteins include proteases for sequential processing of polyprotein and the proteins involved in viral replication (12). Among them, NS3/4A is a multifunctional protein of serine protease, helicase, and trinucleotide phosphatase activities (16–20), and NS5B is a core replicating enzyme of RdRP activity (21–23). NS4B and NS5A, which were reported to interact with each other, have been believed to play a role in viral replication (37). We demonstrate in this report the direct interaction of NS5A and NS5B and a positive effect of NS5A on the RdRP activity of NS5B. Recently, we reported...
that four discontinuous sequences of NS5B are essential for NSSA binding (38). On NS5A, two discontinuous regions harboring aa 105–162 and 277–334 on NS5A are deduced to be essential for NS5B binding both in vitro and in vivo. In addition, a sequence, aa 398–411, may have a possible role in NS5B binding, since it is necessary in the truncation analysis but dispensable in NSSAd355–411. At present, the reason for this discrepancy is not clear, but the essential region, aa 277–334, which harbors a long predicted turn at the C terminus, may not function when located at the C terminus. It may require a helix-rich and acidic region (aa 335–397 or 412–447) for the NS5B binding.

Since NS5B directly binds to NSSA/4 (39), NS5A (Qin et al. (23) and this report), and indirectly to NS4B by help of NS5A (37), NS5B is not only the central enzyme for the viral replication but also may be a core protein for assembly of the replication complex. It remains to be determined whether NS5B can contact these NS proteins at the same time or sequentially during different phases of replication. Recently, the important role of NS5A in viral replication has been clearly shown by the adaptive mutations in selective self-replicating RNAs in tissue culture systems. Blight et al. (30) reported that the adaptive mutations were clustered in a region upstream of ISDR, and Lohmann et al. reported that a single mutation of NS5B (R2884G) was mainly responsible to confer cell culture adaptation (31) in addition to several mutations in NS proteins. One of the two adaptive NS5A mutations in the latter report was incompatible to confer colony formations when the NS5B R2884G was introduced. The result strongly suggests a functional interaction of NS5B and NS5A, although the exact mechanism remains obscure. Interestingly, two regions of NS5A essential for the NS5B binding in this report are not overlapped by any other documented regions of NS5A, including the sites of the adaptive mutations (30, 31), three serine residues at the central part necessary for hyperphosphorylation (24), and ISDR (28, 29). Similarly, the basic phosphorylation site (24) is not a prerequisite for the NS5A-NS5B interaction, since the interaction was observed with the bacterial recombinant proteins.

Here we report a dose-dependent inhibitory effect of NS5A on the NS5B RdRP activity in vitro using purified His-NS5A. The analysis with the internal deletion mutants clearly indicates that the NS5B binding ability is necessary for the inhibition. Binding of NS5A to NS5B may be inhibitory for conformational change of NS5B for RdRP activity. We recently found oligomerization of NS5B that is critical for RdRP activity (42). In this context, one interesting hypothesis to be addressed is that the oligomerization of NS5B is the target of NS5A. According to the inhibitory effect, NS5A is a negative regulator of HCV replication by which HCV would be maintained at a low level without inducing host response (30, 31). However, a small amount of His-NS5A (less than 0.1 molar ratio to NS5B) weakly stimulated RdRP activity by 20% at most. This stimulation was observed with wild type and the internal deletion mutants having NS5B binding ability, suggesting that this stimulation effect is nonstoichiometric but requires NS5B binding. The relevance and mechanism of this stimulation effect remain unknown at present. NS5A may have two roles, an inhibitory role in a form complexed with NS5B and a stimulatory role in a nonstoichiometric manner. Phosphorylation of NS5A might be an important step to modulate function(s) of NS5A (24–27), although our experiments in vitro were carried out with the bacterial nonphosphorylated recombinant proteins. It remains to be addressed whether phosphorylated NS5A modifies RdRP activity negatively or positively, since the UMP incorporation assay to detect RdRP activity of NS5B is artificial and may not reflect specific events during HCV replication in which NS5A is involved together with the other HCV NS proteins and putative host factors. Therefore, not only the modulation effects of NS5A on the catalytic activity of NS5B but also other roles of NS5A on HCV replication might be explored in the future.

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Interaction of HCV NS5A and NS5B

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