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Human VAP-B Is Involved in Hepatitis C Virus Replication through Interaction with NS5A and NS5B

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The hepatitis C virus (HCV) nonstructural protein (NS) 5A is a phosphoprotein that associates with various cellular proteins and participates in the replication of the HCV genome. Human vesicle-associated membrane protein-associated protein (VAP) subtype A (VAP-A) is known to be a host factor essential for HCV replication by binding to both NS5A and NS5B. To obtain more information on the NS5A protein in HCV replication, we screened human brain and liver libraries by a yeast two-hybrid system using NS5A as bait and identified VAP-B as an NS5A-binding protein. Immunoprecipitation and mutation analyses revealed that VAP-B binds to both NS5A and NS5B in mammalian cells and forms homo- and heterodimers with VAP-A. VAP-A interacts with VAP-B through the transmembrane domain. NS5A interacts with the coiled-coil domain of VAP-B via 70 residues in the N-terminal and 341 to 344 amino acids in the C-terminal polyproline cluster region. NS5A was colocalized with VAP-B in the endoplasmic reticulum and Golgi apparatus. The specific antibody to VAP-B suppressed HCV RNA replication in a cell-free assay. Overexpression of VAP-B, but not of a mutant lacking its transmembrane domain, enhanced the expression of NS5A and NS5B and the replication of HCV RNA in Huh-7 cells harboring a subgenomic replicon. In the HCV replicon cells, the knockdown of endogenous VAP-B by small interfering RNA decreased expression of NS5B, but not of NS5A. These results suggest that VAP-B, in addition to VAP-A, plays an important role in the replication of the HCV genome.

Hepatitis C virus (HCV) infects 170 million people worldwide and frequently leads to cirrhosis or hepatocellular carcinoma (6, 29). HCV is classified in the family *Flaviviridae* and possesses a single-stranded positive-sense RNA with a length of 9.6 kb. The HCV genome encodes a single large precursor polyprotein composed of about 3,000 amino acids (aa) that is processed by cellular and viral proteases, resulting in at least 10 structural and nonstructural (NS) proteins (29). Details of HCV's replication cycle are unknown because of the low viral load in the sera of HCV-infected individuals and the lack of a reliable and robust cell culture system to support HCV infection and replication. The development of HCV RNA replicons in which a synthetic HCV genomic or subgenomic RNA replicates efficiently in the human hepatocarcinoma cell line Huh-7 has enabled the study of viral RNA replication in cell culture (4, 20, 24). The HCV RNA replication complex, composed of the viral NS proteins and host cellular proteins, replicates the viral RNA genome at the intracellular membrane. Thus far, the HCV replicon system has greatly contributed to the understanding of HCV replication and pathogenesis associated with the expression of viral NS proteins. Replication of positive-strand RNA viruses generally involves certain intracellular membrane structures, including the endoplasmic reticulum (ER), Golgi apparatus, endosome, and lysosome (39). Recently, several groups have succeeded in demonstrating cell-free replication activities of replication complexes in crude membrane fractions of HCV subgenomic replicon cells (2, 3, 14, 53). These cell-free systems provide semi-intact polymerase assays for biochemical dissection of HCV RNA replication and are a useful source for the isolation of HCV replication complexes. Replication complexes were detected in detergent-resistant membrane structures, most likely lipid raft structures (2, 14). Although HCV NS proteins presumably form a membrane-associated RNA replication complex with host proteins, the precise components and mechanisms for replication are poorly understood.

HCV NS5A is a phosphoprotein that appears to possess multiple and diverse functions in viral replication, interferon resistance, and pathogenesis (26, 35). Cell culture-adaptive mutations have been shown to cluster in the central portion of NS5A in subgenomic HCV replicons, indicating that NS5A is involved in the viral replication process either directly or by interacting with host cellular proteins (4, 55). This observation, together with the modulation of NS5A hyperphosphorylation by NS3, NS4A, and NS4B and physical interaction with other viral NS proteins, strongly supports the notion that NS5A is an essential component of the HCV replication complex (21, 30, 36). NS5A has been shown to be associated with a range of cellular proteins involved in cellular signaling pathways, such as interferon-induced kinase PKR (11), growth factor receptor-binding protein 2 (Grb2) (45), p53 (27, 37), phosphoinositide-3-kinase p85 subunit (15), and proteins in protein trafficking and membrane morphology, such as karyopherin β3 (8),

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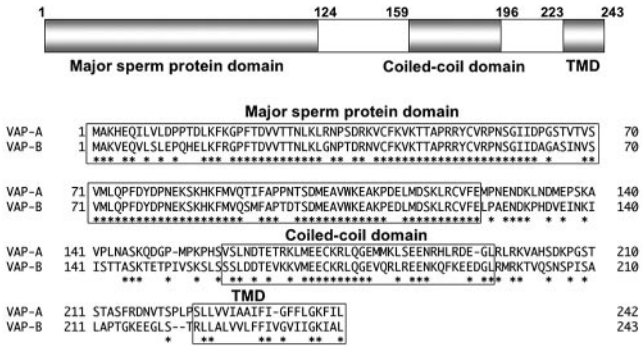

FIG. 1. Schematic representation of VAP-B and alignment of amino acid sequences of VAP-A and VAP-B. The major sperm protein domain, coiled-coil domain, and TMD are indicated. The asterisks indicate identical amino acid residues between VAP-A and VAP-B.

apolipoprotein A1 (40), amphiphysin II (56), and vesicle-associated membrane protein (VAMP)-associated protein (VAP) subtype A (VAP-A), also called VAP-33 (48). Host fatty acids and geranylgeranylation appear to modulate the host and viral proteins involved in HCV RNA replication (19, 49, 54). Gao et al. showed that small interfering RNA (siRNA) or the dominant-negative mutant of VAP-A resulted in relocation of NS5B from detergent-resistant to detergent-sensitive membranes and reduced HCV RNA replication (12). In addition, Evans et al. suggested that NS5A hyperphosphorylation disrupts interaction with VAP-A and negatively regulates HCV RNA replication (9). Like many of the fusion proteins, VAP is a tail-anchored protein with a globular amino-terminal domain followed by a stalk region containing a coiled coil (Fig. 1), and it is ubiquitously expressed in human tissues (7). In humans, there are two isoforms of VAP, VAP-A and VAP-B, encoded by separate genes, and VAP-C is a splicing variant of VAP-B missing the C-terminal two-thirds (23, 32). VAP-B shows 63% amino acid identity to VAP-A (32, 51). The first proposed function for VAP arose from its initial identification as an interactor with the membrane fusion protein synaptobrevin/VAMP in *Aplysia* (43). Since then, it has been shown to be involved in vesicle transport, including the regulation of COP-I vesicle transport in the ER/Golgi pathway (13, 44), VAMP/synaptobrevin-mediated neurotransmitter release (38), and VAMP-2-mediated Glut-4 trafficking at the plasma membrane (10); it is also involved in the interaction between the microtubule network and tight junctions (22). Recently, VAP has been linked to the function of mammalian neurons, where VAP is enriched on microtubules (42), because a mutation in human VAP-B causes familial amyotrophic lateral sclerosis type 8 (32).

To gain a better understanding of the interactions between NS5A and host proteins involved in HCV replication, we screened human libraries by a yeast two-hybrid system using NS5A as bait and identified VAP-B as an NS5A-binding protein. In this study, we examined the biological significance of the interaction between VAP-B and NS proteins in HCV replication and found that VAP-B binds to both NS5A and NS5B in mammalian cells and forms homo- and heterodimers with VAP-A. Immunodepletion of VAP-B suppressed the replication of HCV RNA in a cell-free replication assay, and the knockdown of endogenous VAP-B by siRNA decreased the expression of NS5B but not that of NS5A. These results suggest that VAP-B plays an important role in HCV replication through interaction with NS5A and NS5B.

MATERIALS AND METHODS

Cells. Human embryo kidney 293T, human cervical carcinoma HeLa, and human hepatoma Huh-7 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, Mo) containing 10% fetal calf serum (FCS), while the Huh-9-13 cell line, which possesses an HCV subgenomic replicon (4, 20, 23), was cultured in DMEM supplemented with 10% FCS and 1 mg/ml G418. All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Antibodies. Chicken anti-human VAP-B antibody was prepared by immunization using the synthetic peptides of residues from 188 to 203, KQFKEEDGL RMRKTVQ, of human VAP-B. A mouse monoclonal antibody to human VAP-A was purchased from BD Pharmingen (San Diego, CA). Mouse monoclonal antibodies to giantin, influenza virus hemagglutinin (HA), and GluGlu (EE) tag were from Covance (Richmond, CA). Mouse anti-FLAG antibody M2, horseradish peroxidase-conjugated antibody, and mouse monoclonal anti-beta-actin antibody were from Sigma. A mouse monoclonal antibody to protein disulfide isomerase (PDI) was from Affinity Bioreagents (Golden, CO). Rabbit polyclonal antibody to NS5A was prepared by immunization using peptides of residues from 409 to 422, DVESYSSMPPLEGE. Mouse monoclonal antibody to NS5B was described previously (41).

Plasmids. For expression in mammalian cells, a DNA fragment encoding NS5A was generated from HCV genotype 1b strain J1 (1) (GenBank database accession number D89815), and another was generated from genotype 1a strain H77 (52) (GenBank database accession number AF009606) by PCR using *Pfu* turbo DNA polymerase (Stratagene, La Jolla, CA). The fragments were then cloned into the appropriate sites in pEF-FLAG pGBK puro (18) and pEGFP-C3 (Clontech, Palo Alto, CA). The mutations of the NS5A gene were generated by a method known as "splicing by overlapping extension" (16, 17) and cloned into pEF-FLAG pGBK puro. The DNA fragment encoding NS5B of the J1 strain was generated by PCR and cloned into pCAGGs-PUR (33). The DNA fragment encoding human VAP-A was amplified by PCR from a human fetal-brain library (Clontech) and was introduced into pEF-FLAG pGBK puro, pEF-EE hygro (34), pCHA3 (34), and pcDNA3.1-N-HA, in which an HA tag is inserted in the N terminus of the cloning site of pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The cDNAs of human VAP-A and -B were amplified by PCR and cloned into pEF-FLAG pGBK puro, pEF-EE hygro, pcDNA3.1-N-HA, and pEGFP-C3. The genes encoding VAP lacking the transmembrane domain were amplified and cloned into pEF-FLAG pGBK puro. The DNA fragment encoding the human VAP-B protein lacking a coiled-coil region was introduced into pEF-EE hygro. All PCR products were confirmed by sequencing them with an ABI PRSM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Yeast two-hybrid assay and library screening. The NS5A-binding protein was identified by a yeast two-hybrid assay according to the user manual of MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech). The DNA fragment encoding amino acids 1973 to 2419 was amplified from HCV strain J1 by PCR and then was cloned into pGBKT7 (Clontech). The resulting plasmid was designated pGBK T7 HCV NS5A. A human brain library based on pACT2 was purchased from Clontech. The yeast *Saccharomyces cerevisiae* strain AH109, which secretes alpha-galactosidase under the control of MEL1 upstream activation sequence, was grown in yeast extract-peptone-dextrose medium and transformed with the bait and library plasmids. The transformed yeast cells were grown on 2.0% agar plates of dropout medium lacking tryptophan, leucine, histidine, and adenine. The resulting colonies were inoculated on the new dropout plate containing 20 μg/ml X-alpha-Gal (5-bromo-4-chloro-3-indolyl-alpha-*O*-galactopyranoside) and lacking leucine and tryptophan. The total DNA was prepared from all positive clones and then introduced into *Escherichia coli* strain JM109. The prey plasmids of isolated yeast cells were recovered from the clones grown on LB agar plates containing 10 μg/ml ampicillin and then purified. The insert DNA fragments of isolated clones were determined by sequencing. Finally, 48 alpha-galactosidase-positive clones were identified from 2 million clones screened in the fetal-brain library. One of the positive clones contained the complete cDNA of human VAP-B in frame.

Transfection, immunoblotting, and immunoprecipitation. Cells were seeded onto a six-well tissue culture plate 24 h before transfection. The plasmids were transfected into cells by liposome-mediated transfection using Lipofectamine 2000 (Invitrogen). Cells were harvested 36 h posttransfection, washed five times

with 1 ml of ice-cold phosphate-buffered saline (PBS), and suspended in 0.2 ml lysis buffer (20 mM Tris-HCl, pH 7.4, containing 135 mM NaCl and 1% Triton X-100) supplemented with 1 μg/ml leupeptin, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 5 mM $NaVO_4$. Cell lysates were sonicated at 4°C for 5 min, incubated for 30 min at 4°C, and centrifuged at 14,000 × *g* for 5 min at 4°C. The supernatant was immunoprecipitated with 1 μg of antibodies and 10 μl of Protein G-Sepharose 4B Fast Flow beads (Amersham Pharmacia Biotech, Franklin Lakes, NJ). The immunocomplex was precipitated with the beads by centrifugation at 14,000 × *g* for 30 s and then was washed five times with lysis buffer by centrifugation. The proteins binding to the beads were boiled in 30 μl of loading buffer and then subjected to sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and then reacted with primary antibody and secondary horseradish peroxidase-conjugated antibody. The immunocomplexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by using an LAS-3000 image analyzer (Fujifilm, Tokyo, Japan).

Immunofluorescence microscopy. Cells were seeded on an eight-well chamber slide at 2×10^4 per well 24 h before transfection. Transfected cells were washed twice with PBS, fixed with PBS containing 4% paraformaldehyde, and permeabilized with PBS containing 0.5% Triton X-100. The ER and Golgi apparatus of cells were stained with the mouse monoclonal antibody against luminal ER redox enzyme PDI and the rabbit polyclonal antibody against giantin, respectively, in PBS containing 5% bovine serum albumin. Bound primary antibody was revealed with Alexa Fluor 594-conjugated anti-mouse or anti-rabbit antibody. After additional washes with PBS, a coverslip was attached over PBS containing 50% glycerol and observed under an LSM 510 microscope (Carl Zeiss, Tokyo, Japan).

Gene silencing by siRNA. The siRNA target sequence against human VAP-B, 5′-GGUUAUGGAAGAAUGUAAGTT-3′, was synthesized and purified by Ambion (Austin, TX). Negative control siRNA, siCONTROL Non-Targeting siRNA-2, was purchased from Dharmacon (Lafayette, CO). The Huh-7 cells harboring a subgenomic HCV replicon on six-well plates were transfected with 80 nM or 160 nM of siRNA by using siFECTOR (B-Bridge International, Sunnyvale, CA) according to the manufacturer's protocol. Cells were incubated in DMEM supplemented with 10% FCS and harvested at 96 h posttransfection.

RNA replication assay. In vitro RNA replication was determined as previously described with some modification (3). Briefly, the Huh-7 cells harboring a subgenomic HCV replicon grown in a 100-mm dish were treated with lysolecithin (Wako, Osaka, Japan) (250 μg/ml in wash buffer; 150 mM sucrose, 30 mM HEPES [pH 7.4], 33 mM NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate), collected by scraping in 120 μl of incomplete replication buffer (100 mM HEPES [pH 7.4], 50 mM NH_4Cl, 7 mM KCl, and 1 mM spermidine), and centrifuged at 1,600 rpm for 5 min at 4°C. A total of 40 μl of cytoplasmic fraction (supernatant) was treated with 1% Nonidet P-40 (Boehringer Mannheim, Quebec, Canada) at 4°C for 1 h and incubated with antibody for 4 h at 4°C with rotation. Then, samples were incubated with 1 mM of ATP, GTP, and UTP; 10 μM CTP; [α-^{32}P]CTP (1 MBq; 15 TBq/mmol); 10 μg/ml actinomycin D; and 800 U/ml RNase inhibitor (Promega, Madison, WI) for 4 h at 30°C. RNA was extracted from the total mixture by TRI Reagent (Molecular Research Center Inc., Cincinnati, OH). The RNA was precipitated, eluted in 10 μl of RNase-free water, and analyzed by 1% formaldehyde agarose gel electrophoresis.

Real-time PCR. Total RNA was prepared from cell lines by using TRIzol LS (Invitrogen), and first-strand cDNA was synthesized by using a first-strand cDNA synthesis kit (Amersham) with random primers. Each cDNA was estimated by Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) according to the manufacturer's protocol. Fluorescent signals were analyzed with an ABI PRISM 7000 (Applied Biosystems). The HCV NS5A gene was amplified using the primer pairs 5′-AGTCAGTTGTCTGCGCTTTC-3′ and 5′-CGGGGAATTTCCTGGTCTTC-3′. The human beta-actin gene was amplified with the primer pairs 5′-TGGAGTCCTGTGGCATCCACGAAACTACCTTCAACTC-3′ and 5′-CGGACTCGTCATACTCCTGCTTGCTGATCCACATC-3′, which are located at different exons to prevent false-positive amplification from contaminated genomic DNA. The value of the HCV genome was normalized with that of actin mRNA. Each PCR product was found as a single band of the correct size on agarose gel electrophoresis (data not shown).

RESULTS

Isolation of VAP-B as a novel binding partner for HCV NS5A. To examine the protein(s) that interacts with NS5A in more detail, we screened a cDNA library of human fetal brain by a yeast two-hybrid system using a full-length NS5A of genotype 1b as bait. Among the 2 million transformants we screened, we obtained 48 positive clones containing cDNAs that encode proteins interactive with NS5A. A BLAST search against the GenBank database revealed each of two clones that have the cDNA encoding VAP-A and VAP-B in frame. Figure 1 shows the amino acid alignments of VAP-A and VAP-B and their predicted functional domains. VAP-A and VAP-B are composed of 242 and 243 amino acids, respectively. VAP-B shows 63% amino acid identity to VAP-A. VAP has three structural domains. The first 124 amino acids share high sequence similarity with the nematode major sperm protein and are conserved among all VAP family members (50). The central region on the protein contains an amphipathic helical structure and is predicted to form a coiled-coil protein-protein interaction motif (159 to 196 aa) and a hydrophobic carboxy-terminal transmembrane domain (TMD) (223 to 243 aa). The homology between their N-terminal regions is higher than that between their C-terminal regions (32, 48).

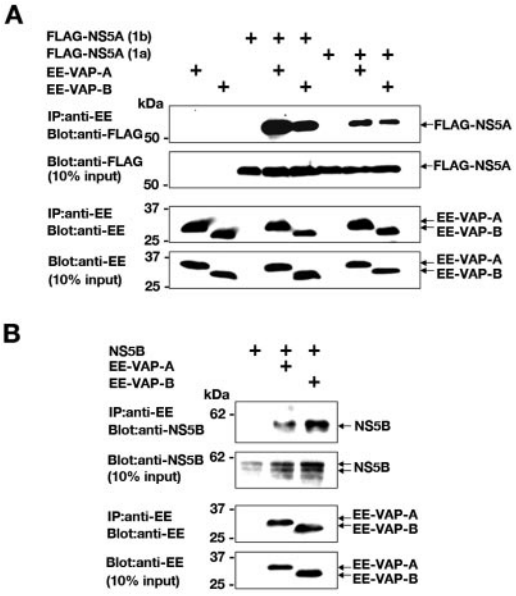

FIG. 2. VAP-A and VAP-B bind to both NS5A and NS5B in mammalian cells. N-terminally FLAG-tagged NS5A of genotype 1b, FLAG-NS5A (1b) of genotype 1a, FLAG-NS5A (1a), and N-terminally EE-tagged VAP (EE–VAP-A or EE–VAP-B) were coexpressed in HEK293T cells and immunoprecipitated with anti-EE antibody. The resulting precipitates were examined by immunoblotting using anti-FLAG antibody (A). NS5B was coexpressed with EE-tagged VAP-A or VAP-B and immunoprecipitated with anti-EE antibody, and NS5B in the precipitates was detected by anti-NS5B antibody (B). One-tenth of the lysates used in immunoprecipitation are shown as the 10% input. The data in each panel are representative of three independent experiments.

VAP-B interacts with NS5A and NS5B in mammalian cells. To confirm the specific interaction, FLAG-tagged NS5A was coexpressed with EE-tagged VAP-A or VAP-B in 293T cells, and cell lysates were immunoprecipitated by specific antibodies. NS5A was coprecipitated with VAP-A and VAP-B to similar extents (Fig. 2A). We also obtained the same results in the reverse experiments (data not shown). Recently, it was shown that hyperphosphorylation of NS5A disrupts interaction with VAP-A and negatively regulates HCV RNA replication, sug-

gesting that adaptive mutations detected in the HCV replicon prevent phosphorylation-dependent dissociation of the RNA replication complex (9). Amino acid residues at Tyr2185 and Lys2187 of NS5A genotype 1b were defined as key determinants for VAP-A binding, and the replacement of these residues with those of genotype 1a (Ala and Gly, respectively) reduced binding to VAP-A in yeast and enhanced hyperphosphorylation of NS5A (9). However, as shown in Fig. 2A, the NS5As of both the 1a and 1b genotypes were coimmunoprecipitated with VAP-A and -B in mammalian cells. Since a previous report indicated that VAP-A interacts with not only NS5A but also NS5B (12), we next examined the interaction of VAP-B with NS5B. EE-tagged VAP-A or VAP-B was coexpressed with NS5B in 293T cells and immunoprecipitated with anti-EE-tag antibody. NS5B was coprecipitated with VAP-B, as well as VAP-A (Fig. 2B). These results indicate that VAP-B participates in the complex of HCV NS proteins in a manner similar to that of VAP-A.

NS5A colocalizes with VAP-B in ER and Golgi compartments. To determine the subcellular localization of NS5A and VAP-B in mammalian cells, HeLa cells were cotransfected with plasmids encoding enhanced green fluorescent protein (EGFP)-tagged NS5A and FLAG-tagged VAP-B or FLAG-tagged VAP-A and examined by immunofluorescence analysis. EGFP-NS5A was colocalized exclusively with FLAG-VAP-B in the cytoplasm, as seen in FLAG-VAP-A (Fig. 3A). To further determine the precise subcellular localization of NS5A and VAP-B, the ER and Golgi apparatus were stained with specific antibodies against PDI and giantin, respectively. NS5A and VAP-B were colocalized with PDI and giantin in HeLa cells transfected with the plasmids (Fig. 3B), indicating that NS5A and VAP-B are colocalized in the membranes of the ER or ER-derived compartment. VAP-B was localized in a diffuse ER-like network, in small vesicles clustered around the nucleus, and predominantly in a perinuclear/Golgi region. Similar to the case with VAP-A, the colocalization of NS5A with VAP-B in the ER and Golgi apparatus suggests that NS5A specifically interacts with VAP-B under intracellular conditions.

Dimerization of VAP-A and VAP-B and interaction with NS5A. Immunoprecipitation analyses revealed that NS5A and NS5B interact with VAP-A and VAP-B. Therefore, it might be reasonable to speculate that VAP-A and VAP-B interact with each other and are involved in RNA replication through the formation of a replication complex. It has been demonstrated that VAP-A interacts with VAP-A or VAP-B through their TMDs and forms a homodimer and a heterodimer in vitro (32). We constructed expression plasmids encoding mutant VAP-A and VAP-B lacking their TMDs and examined their dimer formation with authentic VAPs in vivo. Although coprecipitation of authentic VAP (FLAG–VAP-B or FLAG–VAP-A) with VAP-B–HA was clearly detected, no interaction between TMD deletion mutants (FLAG–VAP-AΔTMD or FLAG–VAP-BΔTMD) and VAP-B–HA was observed (Fig. 4A and B). Furthermore, a TMD deletion mutant, HA–VAP-BΔTMD, which lost the ability to form a dimer with VAP-B and VAP-A, retained the ability to bind to FLAG-NS5A (Fig. 4C), although the efficiency of interaction with NS5A was reduced. These results indicate that TMDs of VAP-A and VAP-B are required for hetero- and homodimerization, but not for binding to NS5A. A region other than the TMD should be involved in the specific interaction between VAP-B and HCV NS5A. The coiled-coil domain of VAP-A was reported to be critical for binding to NS5A (48). Therefore, we examined whether the coiled-coil domain of VAP-B is also involved in interaction with NS5A. FLAG-NS5A was coimmunoprecipitated with EE–VAP-B but not with EE–VAP-BΔcoiled-coil, which lost the coiled-coil domain but retained the TMD (Fig. 4D), suggesting that the coiled-coil domain is also essential for interaction between NS5A and VAP-B.

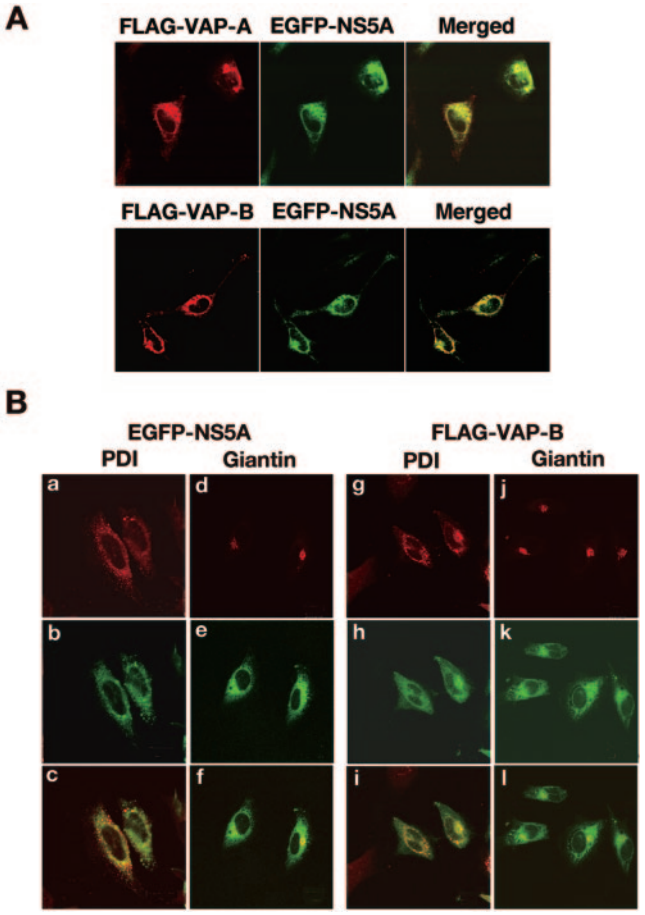

FIG. 3. Intracellular localization of VAPs and NS5A in mammalian cells. (A) N-terminally FLAG-tagged VAP (FLAG–VAP-A or FLAG–VAP-B) was coexpressed with N-terminally EGFP-fused NS5A of genotype 1b (EGFP-NS5A) in HeLa cells, fixed with 4% paraformaldehyde-PBS, permeabilized with 0.5% Triton X-100, and stained with anti-FLAG antibody and AlexaFluor 594-conjugated anti-mouse IgG antibody. (B) EGFP-NS5A of genotype 1b (b and e) or FLAG–VAP-B (h and k) was expressed and then stained with anti-PDI (a and g) or anti-giantin (d and j) antibodies and AlexaFluor 594-conjugated anti-mouse IgG antibody. FLAG–VAP-B was stained with biotinylated anti-FLAG antibody and fluorescein isothiocyanate-conjugated streptavidin. Overlapped images are shown in panels c, f, i, and l.

Two separate domains in NS5A are critical for binding to VAP-B. Since NS5A specifically interacts with VAP-B, we tried to determine the region of NS5A responsible for interaction with VAP-B. Various deletion mutants of FLAG-tagged NS5A were prepared as shown in Fig. 5A. The mutants covering regions from amino acids 1 to 75, but not 1 to 50, and those

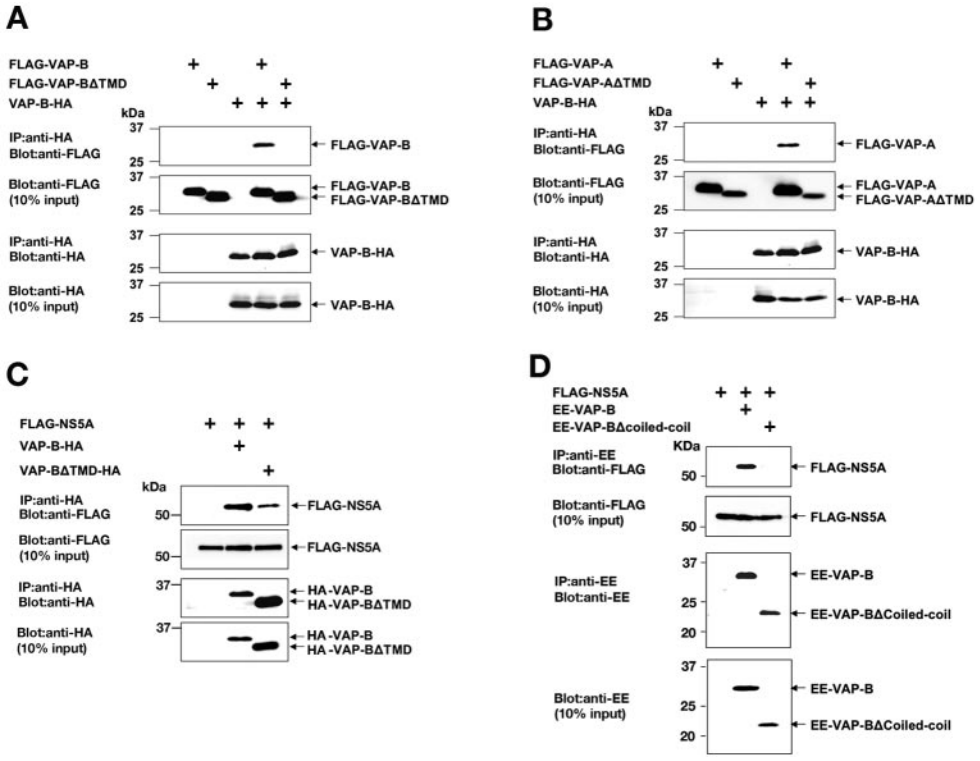

FIG. 4. VAP-B dimerizes with VAP-B and VAP-A through the TMD and interacts with NS5A via the coiled-coil domain. C-terminally HA-tagged VAP-B (VAP-B–HA) was coexpressed with FLAG–VAP-B or FLAG–VAP-B with TMD deleted (FLAG–VAP-BΔTMD). VAP-B–HA was immunoprecipitated with anti-HA antibody, and the immunoprecipitates were immunoblotted with anti-FLAG antibody (A). Interaction of VAP-B–HA with FLAG–VAP-A or FLAG–VAP-A with TMD deleted (FLAG–VAP-AΔTMD) was examined in a similar way (B). FLAG-NS5A was coexpressed with HA–VAP-B or HA–VAP-BΔTMD, and immunoprecipitates with anti-HA antibody and immunoprecipitates were immunoblotted with anti-FLAG antibody (C). FLAG-NS5A was coexpressed with EE–VAP-B or with EE–VAP-B in which the coiled-coil domain was deleted (EE–VAP-BΔcoiled-coil). EE-tagged VAP-B proteins were immunoprecipitated with anti-EE antibody, and immunoprecipitates were immunoblotted with anti-FLAG antibody (D). One-tenth of the lysates used in immunoprecipitation are shown as the 10% input. The data in each panel are representative of three independent experiments.

from amino acids 325 to 447, but not 350 to 447, exhibited binding to VAP-B, suggesting that two separate regions of NS5A (amino acids 51 to 75 and 325 to 349) are involved in physical association with VAP-B. Further mutational analyses of NS5A revealed that regions from amino acids 1 to 70, but not 1 to 65, and those from amino acids 340 to 447, but not 345 to 447, interact with VAP-B (Fig. 5B and C), suggesting that amino acids 66 to 70 and 340 to 344 are required for interaction with VAP-B. According to Tellinghuisen et al., NS5A consists of three domains, domain I (amino acids 1 to 213), domain II (amino acids 250 to 342), and domain III (amino acids 356 to 477) (46, 47). In our results, the region from amino acids 340 to 344, which is essential for the physical interaction with VAP-B, belongs to the connecting segment between domains II and III of NS5A. Ala substitution analyses revealed that an NS5A construct covering amino acids 260 to 447 that replaced the five amino acid residues between 340 and 344 with Ala abrogated interaction with VAP-B (Fig. 5D), whereas that covering 75 N-terminal amino acids carrying an Ala substitution of between 66 and 70 residues retained binding activity to VAP-B (data not shown). Therefore, we focused on the region between 340 and 344 to determine the amino acid residues in NS5A responsible for specific binding to VAP-B. A FLAG-tagged full-length NS5A carrying an Ala substitution between amino acid residues 340 and 344 (FLAG-NS5A/340-344A) exhibited a clear reduction of binding to EE–VAP-B compared with the authentic NS5A (Fig. 5E). To further determine the critical amino acids of NS5A responsible for specific binding to VAP-B, each amino acid between 340 and 344 of the NS5A construct covering amino acids from 260 to 447 was replaced with Ala, and the effect of each substitution on the interaction with VAP-B was examined by immunoprecipitation. As summarized in Fig. 5F, the four amino acid residues 341 to 344 in the polyproline cluster region of NS5A, which are highly conserved among HCV genotypes, are suggested to be involved in the interaction with VAP-B.

VAP-B plays an important role in HCV RNA synthesis. To determine whether VAP-B is involved in HCV replication, cell lysates isolated from Huh-7 cells harboring a subgenomic HCV replicon were used for an in vitro RNA synthesis assay. Chicken anti-human VAP-B antibody raised against synthesized peptides specifically detected endogenous and overexpressed VAP-B (Fig. 6A). Cytoplasmic fraction from the HCV replicon was added to an assay mixture containing [α-^{32}P]CTP and incubated at 30°C for 4 h in the presence or absence of antibodies. Labeled RNA was analyzed by 1% formaldehyde agarose gel electrophoresis as described previously (2). Replication of the subgenomic HCV RNA was inhibited by the

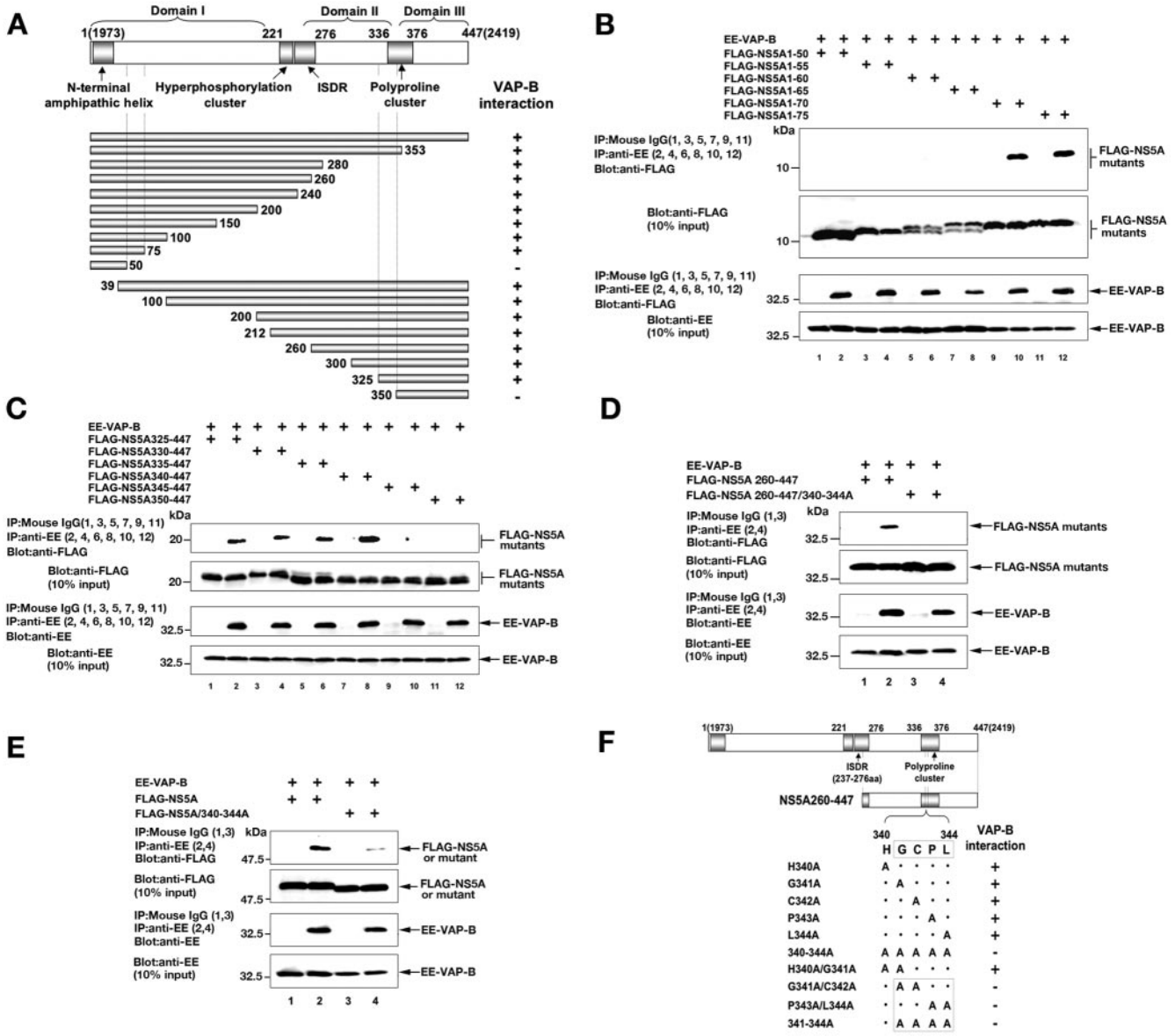

FIG. 5. Two regions of NS5A are required for VAP-B binding. N-terminal or C-terminal deletion mutants of NS5A were introduced into pEF-FLAG pGBK puro vector and coexpressed with EE–VAP-B. EE–VAP-B was immunoprecipitated with anti-EE antibody, and immunoprecipitates were immunoblotted by anti-FLAG antibody. The reverse combination of immunoprecipitation was also examined. The results are summarized in panel A. Four functional domains in the NS5A protein and three domains based on the locations of the blocks of low-complexity sequence (46) are indicated. The numbers in parentheses indicate amino acid residues in the HCV polyprotein. To further determine the critical amino acids of NS5A for specific binding to VAP-B, deletion mutants of the N-terminal region from residues 1 to 75 (B) or those of the C-terminal region from residues 325 to 447 (C) were immunoprecipitated with EE–VAP-B. Replacement of the five residues 340 to 344 with Ala was introduced into a truncated NS5A possessing residues 260 to 447, FLAG-NS5A 260-447/340-344A (D), or full-length NS5A, FLAG-NS5A/340-(E), to examine the interaction with VAP-B. Further precise mutations were introduced into NS5A possessing residues 260 to 447. The resulting mutants were coexpressed with EE–VAP-B and immunoprecipitated as described above. The results are summarized in panel F. Four amino acids (Gly, Cys, Pro, and Leu) responsible for interaction with VAP-B are indicated by dotted squares. Plus and minus indicate binding and nonbinding, respectively (A and F). One-tenth of the lysates used in immunoprecipitation are shown as the 10% input. The data in each panel are representative of three independent experiments.

antibody to VAP-B but not by a control chicken immunoglobulin G (IgG) (Fig. 6B), suggesting that VAP-B plays a critical role in HCV replication. Aizaki et al. suggested that VAP-A sequesters NS5A at an appropriate site, such as the raft-like domain on the intracellular compartment, and that the TMD of VAP-A plays an important role in subcellular localization and dimerization (2). We demonstrated that the TMD of VAP is required for hetero- and homodimerization of VAP-A and VAP-B but not for interaction with NS5A (Fig. 4). Gao et al. indicated that a truncated VAP-A mutant lacking the TMD inhibited the association of HCV NS proteins with insoluble membrane fractions and reduced both the expression level of

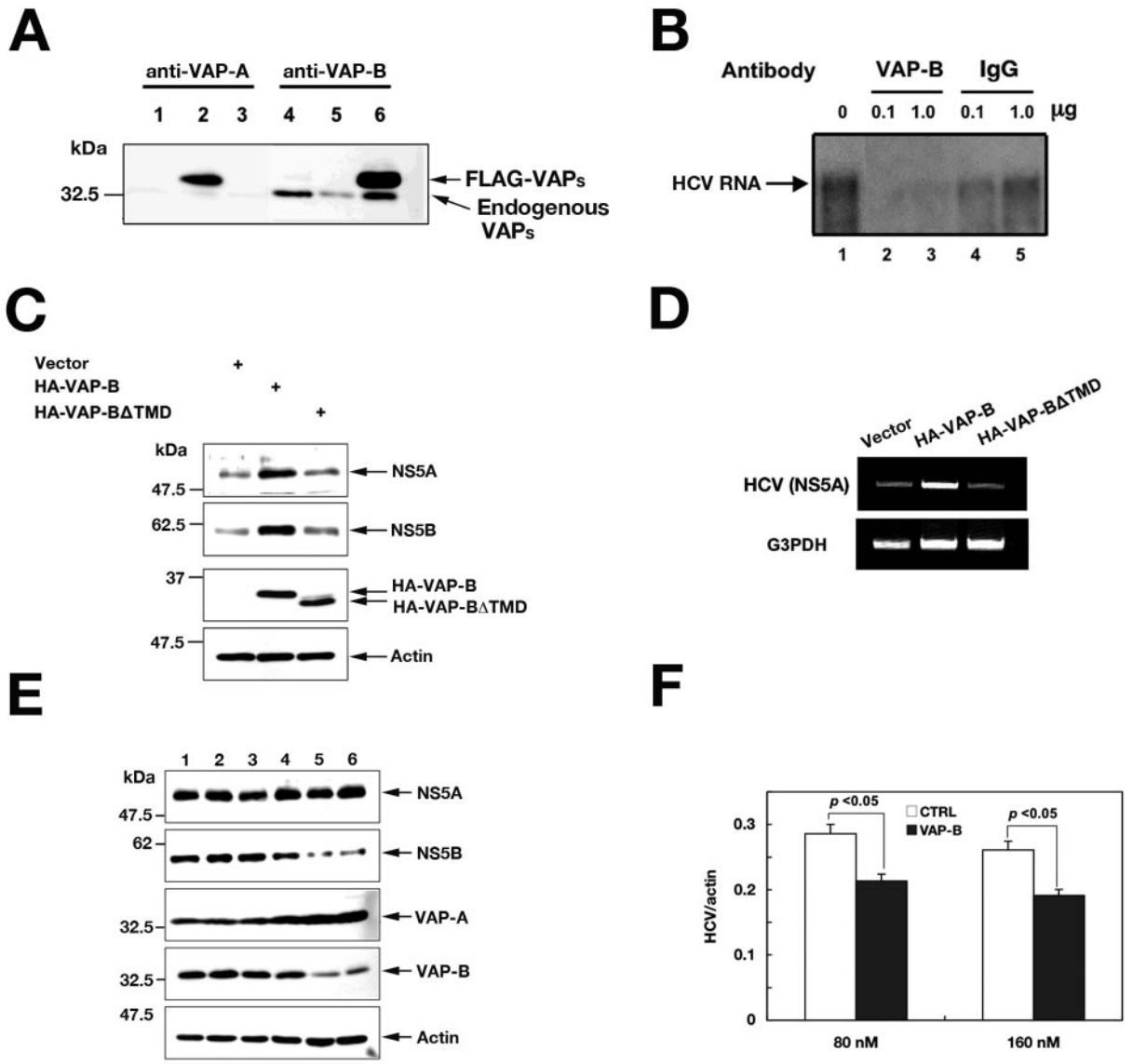

FIG. 6. VAP-B is involved in HCV replication. (A) FLAG–VAP-A (lanes 2 and 5) or FLAG–VAP-B (lanes 3 and 6) was expressed in HEK293T cells and examined by immunoblotting using anti-human VAP-A mouse monoclonal and anti-human VAP-B chicken polyclonal antibodies. (B) In vitro RNA synthesis was carried out in the presence of various concentrations of anti-human VAP-B chicken polyclonal antibody or control chicken IgG. RNA extracted from each fraction was analyzed by agarose gel electrophoresis and autoradiographed. (C) Empty plasmid, expression plasmid of N-terminally HA-tagged VAP-B (HA–VAP-B), or N-terminally HA-tagged VAP-BΔTMD (HA–VAP-BΔTMD) was transfected into HCV replicon cells. Expression of NS5A and NS5B was examined by immunoblotting. (D) HCV RNA was detected by reverse transcription-PCR using primer pairs against NS5A, and expression of G3PDH was used as a control. (E) siRNA against VAP-B or control was transfected into the HCV replicon cells. Lane 1, untreated; lane 2, treated with siFECTOR; lanes 3 and 4, control siRNA was transfected; lanes 5 and 6, VAP-B siRNA was transfected. Expression of NS5A, NS5B, VAP-A, VAP-B, and beta-actin was determined by immunoblotting at 96 h posttransfection. (F) siRNA against VAP-B or control was transfected into the HCV replicon cells. The results are expressed as standard deviations. The significance of the difference in means was determined by the Student *t* test. The data in each panel are representative of three independent experiments.

NS5A and HCV RNA replication in replicon cells (12). To determine the possible implication of VAP-B in HCV replication, VAP-B or VAP-BΔTMD was expressed in Huh-7 RNA replicon cells. In contrast with the previous data, overexpression of VAP-B increased NS5A and NS5B expression and enhanced the replication of HCV replicon cells, but no effect was observed in cells expressing VAP-BΔTMD (Fig. 6C and D). To confirm the role of VAP-B in HCV replication, we examined the effect of the knockdown of endogenous VAP-B from the HCV replicon cells by siRNA. At 96 h posttransfection, the expression of VAP-B in cells transfected with the siRNA targeted to VAP-B was reduced to half the levels of cells transfected with a control siRNA, whereas the expression of VAP-A was slightly increased. Although NS5B expression was reduced by the VAP-B knockdown, NS5A was not affected (Fig. 6E). HCV RNA

replication exhibited 25% and 27% reductions by the transfection of 80 and 160 nM siRNA, respectively, to VAP-B (Fig. 6F). Collectively, these results suggest that VAP-B plays an important role in the sequestration of NS5A and NS5B in the HCV RNA replication complex.

DISCUSSION

Although there are conflicting data in the literature, it is accepted that NS5A is a multifunctional protein with critical roles in HCV replication, as well as in the establishment and maintenance of persistent infection (26). Tu et al. were the first to successfully isolate VAP-33 (VAP-A) as a binding partner of NS5A by a yeast two-hybrid screening of the human liver library; they also indicated an association between VAP-A and not only NS5A, but also NS5B, in mammalian cells (48). Gao et al. (12) further demonstrated that NS5A interacts with NS4B, the only HCV NS protein possessing an intrinsic ability to associate with lipid rafts; and the interaction of NS5A, NS5B, NS4B, and other NS proteins with VAP-A on lipid rafts plays a crucial role in the formation of the HCV RNA replication complex (26). Evans et al. indicated that NS5A from the Con1 strain (genotype 1b) is strongly associated with VAP-A, whereas NS5A from the H77 strain (genotype 1a) was unable to bind VAP-A in yeast (9). The determinants of subtype-specific binding to VAP-A were mapped to amino acids 2185 and 2187, and the substitution of these amino acids of the Con1 strain into those of the H77 strain abrogated both the binding to VAP-A and the replication of the subgenomic replicon. However, these defects in binding to VAP-A and in the replication of the subgenomic replicon were suppressed in the highly adaptive S2204I mutation in NS5A. The S2204I adaptive mutation was shown to disrupt NS5A hyperphosphorylation (5), and the loss of NS5A hyperphosphorylation was shown to correlate with the adaptive mutation's ability to suppress the replication defect caused by the VAP-A-noninteracting mutations (9).

To gain more insight into interaction between NS5A and host proteins involved in HCV replication, we screened human libraries by the yeast two-hybrid system using NS5A as bait and identified VAP-B as a binding protein to NS5A. VAP-B is ubiquitously expressed as VAP-A in human tissues, including liver (32). NS5A can bind to both VAP-A and VAP-B and is colocalized in intracellular compartments, such as the ER and Golgi apparatus. The coiled-coil domain of VAP-B is responsible for their interaction with NS5A, as previously reported in VAP-A (48). In the present study, two regions in NS5A are suggested to be important for VAP-B binding. One region is the N-terminal 70 residues, especially from 66 to 70 (2037 to 2042 aa in the HCV polyprotein), although replacement of these 5 residues with Ala could not abrogate binding to VAP-B. The other is identified at the C-terminal polyproline cluster, and replacement of these four residues from 341 to 344 (2313 to 2316 aa in the HCV polyprotein) with Ala in a full-length NS5A reduced VAP-B binding. Two class II polyproline motifs (consensus PXXPXR) are identified in the polyproline cluster and can bind the SH3 domains of a number of cellular signaling proteins, including Grb2 (45), amphiphysin II (56), and Src family tyrosine kinases (25). Pro343 and Leu344 in the C-terminal VAP-B binding region are part of the first class II polyproline motif. The overlapping of VAP-B's binding region with other cellular signaling proteins may suggest interplay between cellular signaling and replication of HCV. A previous observation indicated that the interaction between NS5A and VAP-A was genotype specific, and amino acid residues critical for the interaction were mapped to amino acids 2185 and 2187 in yeast (9). However, the same authors indicated that NS5A derived from either the1a or 1b genotype expressed in Huh-7 cells interacted equally well with a glutathione *S*-transferase fusion VAP-A expressed in bacteria in vitro, and an attempt at selective interaction of hypophosphorylated NS5A from replicon cells with VAP-A was not successful (9). Furthermore, in our study, no clear difference was detected between native NS5A and the S2204I mutant in binding to VAP-A or VAP-B by immunoprecipitation analyses in mammalian cells (data not shown). In addition, the data in Fig. 2 clearly indicate that NS5A genotype 1a binds to both VAP-A and VAP-B, even though this genotype carries the VAP-A-noninteracting mutations (A2185 and G2187). This discrepancy might be explained by the differences between the experimental systems, including the condition of cell lines, the intracellular ratio of VAP-A and VAP-B, and the phosphorylation status of NS5A. Evans et al. proposed that hyperphosphorylated p58 NS5A represents a closed conformation that cannot interact with VAP-A, whereas hypophosphorylated p56 NS5A represents an open conformation capable of strong interaction with VAP-A (9). The phosphorylation of NS5A is a critical modification that controls not only its interaction with VAP-A, but also RNA replication in Huh7 replicon cells (9, 31). Further study will be needed to elucidate the relationship between the phosphorylation status of NS5A and the capability of binding to VAP-B.

The inhibition of HCV RNA replication by the specific antibody to VAP-B in vitro indicated that VAP-B is a component of the HCV RNA replication complex. Furthermore, the reduction of VAP-B expression by siRNA induced the suppression of NS5B expression but not of NS5A, as seen in the knockdown experiment with VAP-A (12). This suggested that VAP plays an important role in the participation of NS5B in the replication complex. VAP could form hetero- and homodimers through their TMDs and interact with NS5A through their coiled-coil domains (Fig. 4). VAP-C is a splicing variant of VAP-B missing 60% of the C terminus. Therefore, VAP-C cannot interact with VAP-A, VAP-B, or NS5A. Although it is difficult to determine precisely the participation of the monomer and dimer of VAP-A and VAP-B in the HCV replication complex, it might be plausible to speculate that VAP-A is expressed more abundantly than VAP-B and that the heterodimer of VAP-A and VAP-B is more active as an HCV replication complex than those of the monomeric or homodimeric forms. Therefore, overexpression of VAP-B, but not of VAP-A, enhanced HCV RNA replication by providing scaffolds in appropriate positions, like the raft-like domain in the ER/Golgi compartment, capable of changing the nonfunctional NS proteins into a replication-competent state, because only a small fraction of NS proteins are functional as replication complexes (20, 28). Furthermore, VAP-A might have a higher affinity to NS5B than VAP-B does, and overexpression of the TMD deletion mutant of VAP-A, but not that of VAP-B, exhibited a reduction of RNA replication (12). The

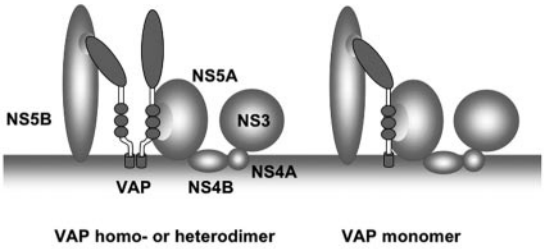

FIG. 7. Models of interaction between HCV NS proteins and VAP. Monomeric and hetero- or homodimeric forms of VAPs can interact with NS5A and NS5B through the coiled-coil domain and N-terminal region, respectively. NS4B can associate with lipid rafts and interact with NS5A (9). NS4A is a cofactor of NS3 and recruits NS3 to the HCV NS protein complex.

possible implication of monomeric and dimeric forms of VAPs in the replication complex of HCV is shown in Fig. 7.

In this study, we identified VAP-B as a novel binding protein to NS5A and NS5B and demonstrated its participation in HCV RNA replication. Elucidation of the precise roles of VAP-A and VAP-B in the phosphorylation of NS5A and in the formation of the replication complex through interaction with other HCV NS proteins and host proteins should provide clues to understanding the molecular mechanisms underlying the replication of HCV RNA and to developing novel therapeutics for chronic hepatitis C.

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