Conserved GXXXG- and S/T-Like Motifs in the Transmembrane Domains of NS4B Protein Are Required for Hepatitis C Virus Replication[▽]

Qingxia Han,¹ Jason Aligo,¹ David Manna,¹ Kerry Belton,¹ Sree V. Chintapalli,^{2,3} Yoojin Hong,^{2,4} Randen L. Patterson,^{2,3} Damian B. van Rossum,^{2,3} and Kouacou V. Konan^{1*}

Department of Biochemistry and Molecular Biology,¹ Center for Computational Proteomics in the Huck Institutes of the Life Sciences,² Department of Biology,³ and Department of Computer Science and Engineering,⁴ The Pennsylvania State University, University Park, Pennsylvania

Received 3 November 2010/Accepted 4 April 2011

Hepatitis C virus (HCV) nonstructural protein 4B (NS4B) is an integral membrane protein, which plays an important role in the organization and function of the HCV replication complex (RC). Although much is understood about its amphipathic N-terminal and C-terminal domains, we know very little about the role of the transmembrane domains (TMDs) in NS4B function. We hypothesized that in addition to anchoring NS4B into host membranes, the TMDs are engaged in intra- and intermolecular interactions required for NS4B structure/function. To test this hypothesis, we have engineered a chimeric JFH1 genome containing the Con1 NS4B TMD region. The resulting virus titers were greatly reduced from those of JFH1, and further analysis indicated a defect in genome replication. We have mapped this incompatibility to NS4B TMD1 and TMD2 sequences, and we have defined putative TMD dimerization motifs (GXXXG in TMD2 and TMD3; the S/T cluster in TMD1) as key structural/functional determinants. Mutations in each of the putative motifs led to significant decreases in JFH1 replication. Like most of the NS4B chimeras, mutant proteins had no negative impact on NS4B membrane association. However, some mutations led to disruption of NS4B foci, implying that the TMDs play a role in HCV RC formation. Further examination indicated that the loss of NS4B foci correlates with the destabilization of NS4B protein. Finally, we have identified an adaptive mutation in the NS4B TMD2 sequence that has compensatory effects on JFH1 chimera replication. Taken together, these data underscore the functional importance of NS4B TMDs in the HCV life cycle.

Hepatitis C virus (HCV) is an enveloped, positive-sense RNA virus responsible for 170 million cases of chronic infections worldwide. HCV is the only member of the genus *Hepacivirus* in the family *Flaviviridae* (51, 63), which includes other human pathogens, such as West Nile virus and dengue virus. Translation of the virus genome yields at least three structural proteins (core, E1, and E2), the highly hydrophobic p7 peptide, and six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The NS proteins, including NS3 to NS5B, are sufficient to promote HCV replication *in vitro* (10, 43). However, with the advent of the HCV cell culture system, many of the NS proteins (NS2, NS3, and NS5A) have been reported to play an active role in HCV assembly (5, 46, 50, 69, 73), further supporting the idea that the NS proteins in general have multiple functions in the HCV life cycle.

NS3 is illustrative of multifunctionality. Its N-terminal serine protease activity is responsible for processing the NS proteins into their mature forms, whereas the C-terminal helicase activity may be required for the unwinding of HCV RNA (35, 66). Similarly, NS4A is a cofactor of NS3 serine protease; it also assists NS3 in binding to host membranes (72) and facilitates the association of NS3 with the HCV replication complex

(RC). NS5A may have multiple functions, including inhibition of the interferon response to virus infection and HCV RNA binding (5, 27, 29). NS5B is the HCV RNA-dependent RNA polymerase (RdRp) (53) responsible for HCV RNA synthesis and works in conjunction with both viral and host factors for efficient HCV genome replication (7, 53, 65).

NS4B is a 27-kDa hydrophobic protein (30) whose known biochemical functions include GTPase and ATPase activities (17, 70). NS4B also binds to the 3' end of the negative-sense viral RNA (18), suggesting that NS4B might facilitate the binding of HCV RNA to the HCV RC. Disruption of the GTPase or RNA-binding activity leads to impairment of HCV replication, suggesting that these activities are important during the early steps of the viral life cycle.

Like most positive-sense RNA viruses, HCV replicates its genome in association with remodeled cytosolic membranes termed the membranous web (16). In HCV, expression of NS4B alone induces membranous web formation (16, 37). The membranous web appears as dots or foci in fluorescence microscopy, and disruption of these foci has been associated with decreased HCV replication efficiency (3, 20, 44, 65, 67). Not surprisingly, in cells replicating the virus genome, NS4B foci contain all the components of the HCV RC, including the replicase proteins (NS3, NS4A, NS4B, NS5A, and NS5B), host factors (3, 48), and viral RNA. Coexpression studies have indicated that NS4B interacts with other NS proteins (NS2, NS3, NS4A, NS5A, and NS5B), most of which are involved in HCV RNA synthesis (2–4, 16, 21, 24, 31). These findings suggest that

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, The Pennsylvania State University, 308 Althouse Laboratory, University Park, PA 16802. Phone: (814) 863-8254. Fax: (814) 863-7024. E-mail: kvk10@psu.edu.

[▽] Published ahead of print on 20 April 2011.

NS4B might provide the scaffold for the recruitment of the replicase proteins to the HCV RC. In addition, NS4B has been reported to oligomerize (74); however, to date, there is no evidence showing that oligomerization promotes membranous-web formation and/or NS4B interaction with the RC.

The precise membrane topology of NS4B protein is still unknown. However, topology prediction programs, coupled with several reports (3, 20, 25, 26, 44), suggest that NS4B has an N-terminal domain (NTD) (amino acids [aa] ~1 to 79), a transmembrane domain (TMD) region composed of at least four TMDs (TMD1 [aa ~80 to 101], TMD2 [aa ~113 to 134], TMD3 [aa ~140 to 159], and TMD4 [aa ~169 to 191]), and a C-terminal domain (CTD) made up of 70 amino acids (aa ~192 to 261). The CTD is believed to be on the cytosolic side of the endoplasmic reticulum membrane, whereas the location of the NTD remains controversial.

How these domains contribute to NS4B structure and function is still the subject of intense study. It is now believed that both the NTD and the CTD contribute to (i) the formation of NS4B foci (3, 20, 25, 26), (ii) NS4B oligomerization (74), and (iii) NS4B association with membranes through the amphipathic alpha-helices (25, 26). However, the identity of the NS4B domains interacting with replicase proteins remains unknown. Moreover, we know little about the four TMDs, which constitute approximately one-third of the NS4B protein. Given the cumulative size of these TMDs, the following questions arise: why would NS4B need four TMDs merely to insert the protein into host membranes? If these TMDs are doing more than anchoring NS4B into membranes, how else are they contributing to NS4B function? In short, what is the role(s) of the TMDs in NS4B structure and function?

To elucidate the role of the TMDs in NS4B function, we have engineered JFH1 chimeras containing various Con1 NS4B TMD sequences. If the TMDs merely anchor NS4B into the membrane, we hypothesized that exchanging them between different HCV genotypes would have no effect on replication or virus production. However, if the TMDs play a role in genotype-specific NS4B function, exchanging such domains would negatively impact replication. In this report, we show that the TMDs are required for HCV genome replication. Further, we have identified putative motifs that have been associated with TMD-TMD interaction in host and virus proteins. The significance of these unexpected findings is discussed.

MATERIALS AND METHODS

Cells. Huh7.5 cells, a human hepatoma cell line highly permissive for HCV replication (11), were kindly provided by Apath, LLC (St. Louis, MO). The cells were grown as monolayers in Advanced Dulbecco's modified Eagle's medium (Advanced DMEM; Invitrogen, Carlsbad, CA) supplemented with 1.5% fetal bovine serum (FBS), L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5% CO₂ incubator.

Construction of plasmids. All nucleotide and amino acid numbers refer to the JFH1 genome (71). pJFH1 (71) and pJFH1/GND (71) vectors, encoding wild-type (WT) and replication-defective JFH1 genomes, respectively, were kindly provided by Takaji Wakita (Tokyo Metropolitan Institute for Neuroscience) (71). These vectors were used to generate the various JFH1 chimeras, including the full-length genome and the subgenomic luciferase (Luc)-expressing replicons. Plasmid pLuc-JFH1, which contains the T7 promoter sequence fused to nucleotides (nt) 1 to 389 of the JFH1 consensus sequence, followed by the firefly luciferase gene, the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), and the nucleotides spanning the area from the beginning of the

NS3 gene to the 3' nontranslated region (3' NTR) of JFH1, was constructed according to the methods described previously by Kato et al. (34) and Targett-Adams and McLauchlan (68).

To construct the plasmids containing the full-length JFH1-derived chimeras (Fig. 1B), the N-terminal, transmembrane, or C-terminal domain of the JFH1 NS4B gene was replaced by the corresponding fragment from Con1 by using the overlap extension PCR protocol. Since most of the chimeric genomes were engineered by the same approach, we will use pJ/C1-NS4B to illustrate how the constructs were made. First, the NsiI (5' end)-flanked F1 fragment (nt 5257 to 5485) was amplified from pJFH1 by using the JFH1 forward primer NsiI-F (5'-CACCTCACACACCCTGGGACGAA-3') and reverse primer P12 (5'-GT AAGGGAGGTGTGAGGCGCATTCTCCATCTCATC-3' [nucleotides in boldface correspond to the 5' end of Con1 NS4B]). Next, the F2 fragment, composed mainly of the Con1 NS4B gene, was amplified from pEGFP-N2-NS4B (3) by using forward primer P11 (5'-GATGAGATGAGGAATGCGCTCACACT CCCTT AC-3' [underlined nucleotides correspond to the 3' end of JFH1 NS4A; nucleotides in boldface correspond to the 5' end of Con1 NS4B]) and reverse primer P4 (5'-CGGAGCCAGGATCCGGAGCATGGCGTGGAGCAGT-3' [underlined nucleotides correspond to the 5' end of JFH1 NS5A; nucleotides in boldface are in the 3' end of Con1 NS4B]). The F1 and F2 fragments were purified, mixed, and used as templates to amplify the recombinant F3 fragment by using forward primer NsiI-F and reverse primer P4. The F4 fragment, corresponding to the N-terminal two-thirds of JFH1 NS5A (nt 6269 to 7470), was amplified by using forward primer P3 (5'-ACTGCTCACGCCATGCTCCGGA TCCTGGCTCCG-3' [nucleotides in boldface are at the 3' end of Con1 NS4B; underlined nucleotides are at the 5' end of JFH1 NS5A]) and reverse primer RsrII-R (5'-CCAGGGGACGTCGGACCGCCGAT-3' [RsrII site in boldface]). To amplify the final chimeric F5 fragment, the NsiI-F forward and RsrII-R reverse primers were used, and the purified PCR products F3 and F4 served as templates. The chimeric F5 fragment was purified, digested with NsiI and RsrII, and inserted into the NsiI- and RsrII-cut pJFH1 vector. The other chimeric constructs, shown in Fig. 1B, were constructed similarly.

Plasmids pLuc-J/C1-NS4B, pLuc-J/C1-A, pLuc-J/C1-B, pLuc-J/C1-C, and pLuc-JGND were constructed by directly replacing the fragment between the EcoRI site (nt -24) and the SpeI site (nt 4106) of pJFH1 or its derived chimeric vectors (Fig. 1B) with an EcoRI- and SpeI-cut fragment (from pLuc-JFH1) that has the HCV 5' NTR followed by the luciferase gene, the EMCV IRES sequence, and part (nt 3431 to 4106) of the NS3 gene. For the plasmids containing the smaller swaps between JFH1 and Con1 NS4B transmembrane domain sequences (Fig. 1C), blunt-end ligation was performed. To engineer the pLuc-J/C1-B(1-2) vector, for example, forward primer Nsi-F and a phosphorylated reverse primer, Con14B-TMD2-R (5'-CACAAGCACCTTCCCAAGGCCTAT-3'), were combined with the template, pJ/C1-NS4B, to amplify the F-A fragment (nt 5257 to 5899) with an NsiI site at the 5' end. Next, the F-B fragment (nt 5900 to 7476) was amplified from the pJFH1 template by using the phosphorylated forward primer JFH1-NS4B-TMD3-F (5'-GACATCTGGCAGGATATGGT GCGG 3') and reverse primer RsrII-R. The purified F-A and F-B fragments were cut by NsiI and RsrII, respectively, and were ligated with the NsiI- and RsrII-digested pLuc-JFH1 vector.

The blunt-end ligation approach was also used to introduce the single or double mutations into pLuc-JFH1 (including those in the GXXXG- and S/T-like motif residues) or the chimeric replicon constructs. For example, to introduce the NS4B S113N mutation into pLuc-J/C1-B, fragment F1 was amplified from pLuc-J/C1-B by using forward primer NsiI-F and the phosphorylated reverse primer S113N-R (5'-TGGGAGGAGCAAGTTGGGCGGCCAC-3'). Similarly, fragment F2 was amplified from pLuc-J/C1-B with the phosphorylated forward primer S113N-F (5'-ACGCTGCTCTGCTTTCGTAGGC-3' [mutated nucleotide in boldface]) and reverse primer RsrII-R. The F1 and F2 fragments were purified, cleaved with NsiI and RsrII, respectively, and ligated into the NsiI- and RsrII-cleaved pLuc-JFH1 vector. The NS5B E72K mutation was introduced into pLuc-J/C1-B in a similar way by using the RsrII (in NS5A) and EcoRV (in NS5B) restriction enzymes. Because there is an HpaI site in Con1 NS4B, the NS5A T462I mutation was introduced into pLuc-J/C1-B in two steps. First, an F1 fragment (with RsrII at the 5' end) and an F2 fragment with the NS5A T462I mutation and the HpaI site at the 3' end were cleaved with the respective enzymes. The resulting fragments were ligated into the RsrII- and HpaI-cleaved pLuc-JFH1 vector. The recombinant vector pLuc-JFH1-NS5A(T462I) was digested with the RsrII and XbaI enzymes; the fragment (nt 7461 to 9678) containing the NS5A T462I mutation was purified and ligated into RsrII- and XbaI-digested pLuc-J/C1-B to generate the recombinant vector pLuc-J/C1-B-NS5A(T462I). To engineer plasmid pLuc-J/C1-B(S113N+T462I+K72E), the RsrII-HpaI fragment (nt 7461 to 8015) was first amplified from JConTMD-AD1 virus cDNA by using forward primer RsrII-S (5'-GGCGGGCGCCGCCGAAT

FIG. 1. (A) Alignment of HCV NS4B protein sequences from genotypes 2a (strain JFH1) and 1b (strain Con1). The sequences were aligned using the SIM alignment tool with the graphical viewer program LALNVIEW (<http://expasy.org/tools/sim-prot.html>). Stars mark conserved residues. Note that NS4B proteins from JFH1 and Con1 share 72.4% amino acid sequence identity. The TMDs were selected based on topology prediction programs TopPred (<http://mobile.pasteur.fr/cgi-bin/portal.py?form=toppred>), TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), and SOSUI (<http://bp.nuap.nagoya-u.ac.jp/sosui/>). Light shading indicates nonconservative amino acid substitutions; a couple of the conservative changes are highlighted by dark shading. (B and C) Schematics of the WT and chimeric JFH1 genomes used in this study to examine the effects of the NS4B TMD region on virus production (B) and genome replication (C). NS4B was divided into three domains: A, B, and C represent the NTD, the TMDs, and the CTD, respectively. TMD1, TMD2, TMD1-2, TMD2-3, TMD2-4, and TMD3-4 are represented by the numbers only in construct designations. Note that constructs with ranges 1-2, 2-3, 2-4, and 3-4 include the indicated TMDs and the IL(s); for example, 1-2 includes TMD1, TMD2, and the IL. The Con1 IL, between JFH1 TMD1 and TMD2, is shown as a small gray rectangle in the Luc-J/C1-B(1-2)-Loop construct. Transient HCV replication was measured via the luciferase reporter gene product under the control of the HCV IRES. (D) Huh7.5 cells were electroporated with 10 μ g of WT or chimeric JFH1 RNA. At 24, 48, or 72 hpt, the cell supernatant was collected, and virus infectivity was measured using the limiting dilution assay (75). The results are expressed as FFU/ml. (E) Naïve Huh7.5 cells were infected at an MOI of 0.01 with the same viruses used for panel D. At 3, 6, 9, or 12 dpi, virus infectivity was measured. Titers are expressed as FFU/ml. The results are representative of two independent experiments, each containing triplicate samples. (F) Replication efficiencies of intergenotypic J/C1-NS4B, J/C1-A, J/C1-B, and J/C1-C chimeras. Huh7.5 cells were electroporated with 10 μ g of luciferase-expressing replicon RNA. At 4, 24, 48, or 72 hpt, cell extracts were prepared. HCV replication was quantified via luciferase reporter activity. RLU, relative light units. The values represent the fold increase in luciferase activity over that at 4 h, normalized to that of the defective RNA-dependent RNA polymerase replicon (Luc-GND). The results are representative of at least two independent experiments, each containing triplicate samples.

FIG. 1—Continued.

CCGCGGGT-3') and reverse primer HpaI-R (5'-TTCCAGGAGGTCTTCC ACACGGAC-3'). The purified fragment was digested by RsrII and HpaI and was subcloned into the RsrII- and HpaI-cleaved pLuc-JFH1 vector to generate the construct pLuc-JFH1-(T462I+K72E). The recombinant vector, pLuc-JFH1-(T462I+K72E), was digested with RsrII and XbaI, and the isolated fragment (containing the two mutations T462I and K72E) was ligated into RsrII- and XbaI-cut pLuc-J/C1-B(S113N).

To express NS4B protein singly in Huh7.5 cells, the NS4B sequence was amplified from WT and mutant pLuc-JFH1 replicons containing single or double mutations in NS4B TMDs by using forward primer JFH1-NS4B-F(XhoI) (5'-C GCGCTCGAGATGGCCTCTAGGGCGGCTCTCATC-3' [the XhoI site is in boldface]) and reverse primer JFH1-NS4B-R(NotI) (5'-ATACCGCGGCGCT TATCAGCATGGGATGGGGCAGTCCTC-3' [the NotI site is in boldface]). The PCR products were purified, digested by XhoI and NotI, and ligated into the XhoI- and NotI-cut pEGFP-N2 vector (Clontech). Note that the enhanced green fluorescent protein (EGFP) reporter gene was deleted from the vector after digestion with XhoI and NotI.

Antibodies. A rabbit polyclonal antibody to HCV NS4B was obtained from Covance (Denver, PA). A mouse monoclonal antibody to HCV NS4B was obtained from Abcam (Cambridge, MA). An anti-HCV NS5A antibody was kindly provided by Craig Cameron (Penn State, University Park, PA). Alexa Fluor-conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, CA).

DNA transfection. For each experiment, Huh7.5 cells were trypsinized and grown overnight in 100-mm-diameter dishes or 6-well plates to obtain 70 to 80% confluent monolayer cells. Prior to transfection, the cells were washed with phosphate-buffered saline (PBS) and were fed with 10 ml of fresh complete medium (for 10-cm-diameter dishes) or 2 ml of complete medium per well for 6-well plates. Cells were transfected according to the TransIT-LT1 protocol from Mirus (Madison, WI). The DNA mixture was added to each dish and was

incubated at 37°C for 24 h. With this procedure, DNA transfection efficiency was usually 70 to 90%.

Metabolic labeling and immunoprecipitation (IP). Huh7.5 cells were seeded at 1×10^6 /10-cm-diameter dish approximately 24 h prior to transfection. Before transfection, the cells were washed once in PBS and were fed with fresh Advanced DMEM-1.5% FBS. For polyprotein processing analysis, the cells were cotransfected with 10 μ g of various DNA constructs. At 24 h posttransfection, the cells were trypsinized and washed twice with PBS. Each sample was then resuspended in 1 ml of DMEM without cysteine and methionine (Invitrogen) and was incubated at 37°C with gentle rotation for 1 h. After starvation, the cells were labeled with 200 μ l of 500- μ Ci/ml Express 35 S protein labeling mix (Perkin-Elmer) for 1 h for polyprotein processing or for 30 min for the examination of NS4B stability. The cells were either lysed immediately in 1 ml of ice-cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride [PMSF], and Complete protease inhibitor cocktail [Roche]) or lysed after a 2-h chase in complete medium containing 10 mM cysteine-methionine (pH 7.4). Cell lysates were precleared by incubation for 1 h at 4°C with Protein A/G Plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). The supernatants were then incubated with an HCV NS4B-specific polyclonal antibody at a dilution of 1:250 for 3 to 12 h at 4°C, mixed with protein A/G Plus agarose, and incubated for 2 h at 4°C. Protein A/G agarose Plus-bound complexes were collected by centrifugation at $5,000 \times g$ for 5 min and were washed once with RIPA buffer, once with RIPA buffer-500 mM NaCl, and once more with RIPA buffer. To examine the immunoprecipitates by SDS-polyacrylamide gel electrophoresis (PAGE), the samples were resuspended in 30 μ l of loading buffer (38), heated at 95°C for 5 to 10 min, and centrifuged at $14,000 \times g$ for 2 min. The supernatants were separated by 10% SDS-PAGE, fixed in 20% methanol-7% acetic acid for 10 min at room temperature, and dried for 1 h at

80°C. Labeled proteins were visualized on a PhosphorImager (Typhoon 8600; Amersham Pharmacia Biotechnology Inc./Molecular Dynamics, Piscataway, NJ).

In vitro transcription and electroporation of viral RNA into Huh7.5 cells. Plasmid DNA constructs containing WT or chimeric genomes or subgenomic replicons were linearized with XbaI and were purified using the Cycle Pure kit (Omega Bio-Tek, Norcross, GA). RNA was synthesized using the T7 RiboMAX Express large-scale RNA production system kit (Promega, Madison, WI) according to the manufacturer's instructions. The RNA was then isolated using Trizol (Invitrogen). Prior to electroporation, subconfluent Huh7.5 cells were trypsinized and resuspended in complete DMEM. The cells were then washed three times and were resuspended at a concentration of 1.25×10^7 /ml in PBS. Briefly, 10 μ g of JFH1 RNA was mixed with 2.5×10^6 Huh7.5 cells in 0.2 ml ice-cold PBS and was electroporated with an ElectroSquarePorator (BTX) in a 0.2-mm-gap cuvette. The electroporator was set at 820 V, and 3 pulses of 99 μ s at 1.1-s intervals were used. The actual voltage was around 690 V for each sample. For the luciferase assay, the cells were left to recover for 10 min at room temperature before being diluted into 10 ml of DMEM–10% FBS. The cells (0.5 ml of suspension) were then seeded into a 24-well plate and were subsequently harvested at 4 h, 24 h, 48 h, and 72 h for the Luc assay.

Titration of HCV infection. Virus supernatant titers were determined by end-point dilution assays as described previously (75, 76). Briefly, Huh7.5 cells were seeded into 96-well plates at a density of 6×10^3 /well. Samples were serially diluted 10-fold in complete growth medium and were used to infect the seeded cells. Following 3 days of incubation, the cells were immunostained with an NSSA-specific antibody. Positive foci were counted, and the infectivity titer was calculated from the average of the number of foci counted in the last and second-to-last wells of the dilution series that still contained positive foci. The viral titer was expressed as focus-forming units (FFU) per milliliter of supernatant.

Analysis of the adapted-virus nucleotide sequence. Viral RNA was extracted from 0.25 ml of filtered infectious virus supernatant by using the Trizol LS reagent (Invitrogen). The RNA was converted into cDNA by using SuperScript III (Invitrogen) and the specific primer 9470R binding at the 3' NTR (59). By using a series of overlapping primers, six PCR fragments, which covered the whole HCV genome, were then amplified and sequenced (59).

Luciferase assay. At the time of the Luc assay, the medium was removed, and the cells were washed once in PBS. One hundred microliters of ice-cold $1 \times$ Cell Culture Lysis Reagent (CCLR) buffer (made from a $5 \times$ stock; Promega) was added to each well of the 24-well plate, and the plates were gently rocked at room temperature for 15 min to lyse the cells. The lysate was removed from the plate and was transferred to a 1.5-ml tube on ice. The supernatant was transferred to a fresh tube after a brief spin at $12,000 \times g$ in a microcentrifuge. Twenty microliters of the lysate was then added to 20 μ l of the Luc assay substrate (Promega) and was quickly mixed by vortexing prior to the enzyme assay in a luminometer.

Indirect immunofluorescence. Huh7.5 cells were seeded onto coverslips in 10-cm-diameter dishes or 6-well plates as described above. At 24 h posttransfection, the cells on coverslips were washed with PBS and were fixed for 10 min in 4% formaldehyde–PBS. The cells were then permeabilized for 5 min at room temperature in 0.05% Triton X-100–PBS, followed by staining with an anti-NSSA rabbit polyclonal antibody (or with a mouse monoclonal antibody against NS4B) and an Alexa Fluor 488- or 594-conjugated secondary antibody. After three washes in PBS, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI)–PBS for 10 min at room temperature, followed by three more washes in PBS. The cells were mounted on glass slides in Vectashield (Vector Laboratories, Inc., Burlingame, CA), and the coverslips were sealed with nail polish. Immunostained samples were analyzed by fluorescence microscopy (Zeiss Axiovert 200M) with a $63 \times$ lens. Digital images were taken with an AxioCam MRm charge-coupled device (CCD) camera. Optical sections were deconvolved using AxioVision software to exclude out-of-focus information. All images were saved as tagged-image format files (TIFF), which were imported to and processed in Adobe Photoshop. Colocalization of green (fluorescein isothiocyanate [FITC]) and red (Cy3) signals produces yellow.

Membrane floatation assay. For the membrane floatation assay, 4 100-mm-diameter dishes (7×10^5 cells/dish) of Huh7.5 cells were grown overnight and were transfected as described above. Transfected cells were resuspended in homogenization buffer (150 mM NaCl, 50 mM Tris [pH 7.4], 2 mM EDTA) containing protease inhibitors (1 mM PMSF and 1 tablet of Complete Mini [Roche, Nutley, NJ]). The cells were then lysed with 8 passages in a ball-bearing homogenizer. Cell lysates were spun at $2,500 \times g$ for 10 min at 4°C to pellet cellular debris and nuclei. A discontinuous iodixanol gradient (5%, 25%, and 30%) (3) was layered on the top of the lysate, and the samples were spun at $120,000 \times g$ for 4 h 25 min at 4°C in an 80 Ti rotor. A total of 8 fractions (867

μ l each) were collected from top to bottom. Each fraction was precipitated with 10% trichloroacetic acid (TCA), separated by 10% SDS-PAGE, and processed for Western blotting as described above. Typically, membrane-bound proteins were associated with fractions 1 to 4, whereas soluble proteins were prominent in fractions 5 to 8.

RESULTS

The Con1 NS4B transmembrane domain region is incompatible with efficient JFH1 replication. As an integral membrane protein, the NS4B transmembrane domains (TMDs) may function simply to anchor NS4B to host lipid bilayers. If this is true, we postulated that exchanging NS4B TMDs between different HCV genotypes should have no effect on replication or virus production. However, if the TMDs actively contribute to NS4B function, we predicted that exchanging them would have a negative impact on HCV production. To test this hypothesis, we examined the effect of exchanging NS4B sequences between HCV genotype 2a strain JFH1 (which displays robust virus production in cell culture) (41, 71, 75) and genotype 1b strain Con1 (which replicates poorly in cell culture) (57) (Fig. 1A). We engineered JFH1 virus chimeras in which full-length NS4B, the N-terminal domain (NTD), the TMD region, or the C-terminal domain (CTD) was replaced with the Con1 counterpart to give the J/C1-NS4B, J/C1-A, J/C1-B, and J/C1-C chimeras, respectively (Fig. 1B). Luciferase-expressing subgenomic replicons were also engineered in order to examine the impact of these chimeric RNAs on JFH1 replication efficiency (Fig. 1C).

The chimeric JFH1 genomes were electroporated into Huh7.5 cells; then, at 24, 48, and 72 h posttransfection (hpt), the supernatants were collected, and virus titers were determined as focus-forming units (FFU) per milliliter. While the JFH1 and J/C1-A viruses had similar titers (ca. 1×10^4 FFU/ml), the other chimeras (J/C1-NS4B, J/C1-B, and J/C1-C) displayed 5- to 15-fold lower virus production (Fig. 1D) by 72 hpt. To examine the kinetics of virus growth following infection, naïve Huh7.5 cells were infected at a multiplicity of infection (MOI) of 0.01, and at various times postinfection, the supernatant was collected, and virus titers were measured. As shown in Fig. 1E, the exchange of the entire JFH1 NS4B protein with that of Con1 (J/C1-NS4B) resulted in at least a 100-fold decrease in JFH1 production by 12 days postinfection (12 dpi). The defect in virus production was further mapped to NS4B sequences in the TMD region (J/C1-B) and the CTD (J/C1-C) (Fig. 1E). Note that exchanging the N-terminal domain (J/C1-A) had no effect on virus production. These results suggest that Con1 NS4B sequences within the TMD region and the CTD are incompatible with their JFH1 counterparts. The discrepancy between the electroporation (10 μ g RNA) results and the low-MOI (0.01) infection results may be explained in part by the kinetics of virus production in the two systems. At 48 hpt, 70 to 80% of the cells (transfected with JFH1 or chimeric genomes) stained positive for NS5A, suggesting that the high virus titers may represent a single step of virus growth because of high intracellular virus RNA levels. With infection at a low MOI, the difference in virus titers at 3 dpi may be the result of multiple cycles of virus infection. We propose that JFH1 releases more infectious virus at 1 dpi than the chimeric viruses and that this difference increases exponentially by 3 dpi as more naïve cells become infected.

We sought to determine whether the defect in the J/C1-B or J/C1-C chimera could be explained by a decrease in HCV replication. This is important given that NS4B (i) remodels host membranes to form the HCV replication complex (16, 37) and (ii) has nucleoside triphosphatase (NTPase) and RNA-binding activities required for HCV replication (17, 18). To examine the impact of the chimeras on JFH1 replication, we engineered various luciferase-expressing subgenomic replicons. As seen in Fig. 1F, expression of JFH1 replicons containing full-length Con1 NS4B (Luc-J/C1-NS4B) or the Con1 NS4B TMD region (Luc-J/C1-B) resulted in at least a 10-fold decrease in JFH1 replication. No such drop was observed in replicons expressing the Con1 NS4B NTD (Luc-J/C1-A) or CTD (Luc-J/C1-C). These data imply that the J/C1-B virus is defective in genome replication, whereas the J/C1-C virus is defective in virus production. A detailed analysis of the role of the NS4B CTD in JFH1 production will be the subject of future experimentation.

The incompatibility in the TMD region does not affect NS4B subcellular distribution or processing. In Huh7.5 cells, NS4B protein displays foci when expressed alone or in the context of the viral genome (3, 44, 48, 65). To determine whether exchanging the TMD region could alter NS4B subcellular distribution or processing, the cells were cotransfected either with a vector alone or with various replicon DNA constructs and a T7 polymerase-expressing vector. As expected, cells expressing the vector alone (Fig. 2Ai and iii) displayed background fluorescence for NS4B or for NS5A, another replicase protein. In contrast, cells expressing the JFH1 polyprotein exhibited the typical foci characteristic of NS4B subcellular distribution (Fig. 2Aiv); these foci overlap with those from NS5A (Fig. 2Av and vi). Similarly, NS4B and NS5A foci merged in cells expressing the J/C1-NS4B (Fig. 2Avii to ix) or J/C1-B (Fig. 2Ax to xii) polyprotein. These results suggest that JFH1 chimeras have no observable effect on the subcellular distribution of NS4B protein or its colocalization with JFH1 NS5A. Consistent with these data, we found that NS4B was processed in J/C1-NS4B and J/C1-B polyproteins (Fig. 2B). However, the molecular weight of NS4B in J/C1-NS4B and J/C1-B is slightly higher than that of the JFH1 counterpart. These data can be explained in part by the fact that NS4B proteins from JFH1 and Con1 share only 72.4% amino acid sequence identity (Fig. 1A).

The incompatibility in the JFH1 and Con1 NS4B TMD regions can be mapped to the TMD1 and TMD2 sequences. Since the NS4B TMD region includes at least four putative TMD sequences and three intervening loops (ILs), we sought to ascertain whether it was the putative TMD helices, their ILs, or both that could not be exchanged between the JFH1 and Con1 genomes. Thus, we engineered JFH1 chimeras containing various TMDs from the Con1 NS4B TMD region (Fig. 1C). As shown in Fig. 3A, exchanging the NS4B TMD3-4 sequence [Luc-J/C1-B(3-4)] resulted in a <2-fold drop in JFH1 replication. In contrast, the presence of the Con1 NS4B TMD1-2 sequence [Luc-J/C1-B(1-2)] led to at least a 60-fold decrease in JFH1 replication. These findings suggest that the JFH1 NS4B sequence that includes TMD1, TMD2, and their IL is incompatible with its Con1 counterpart. We confirmed these findings by showing that JFH1 replicates poorly when it contains the Con1 NS4B NTD fused to the TMD1-2 sequence [Luc-J/C1-A-B(1-2)] (Fig. 3B) but replicates fairly well when it

has Con1 NS4B TMD3-4 fused to the CTD sequence [Luc-J/C1-B(3-4)-C] (Fig. 3B).

To further map the incompatible region, we engineered JFH1 chimeras containing Con1 NS4B TMD1, TMD2, or their IL sequence (Fig. 1C). As shown in Fig. 3C, replacement of the JFH1 NS4B TMD2 sequence [Luc-J/C1-B(2)] led to a 10-fold decrease in replication, whereas the Con1 TMD1 sequence [Luc-J/C1-B(1)] had a more severe impact on this activity (a drop of ca. 100-fold). Interestingly, exchanging the IL between the TMD1 and TMD2 sequences [Luc-J/C1-B(1-2)-Loop] had no effect on JFH1 replication. These data imply that the putative Con1 NS4B TMD1 and TMD2 sequences are incompatible with their JFH1 counterparts. In an attempt to restore Con1 TMD2 compatibility, we engineered JFH1 constructs containing the Con1 TMD2-3 [Luc-J/C1-B(2-3)] or TMD2-4 [Luc-J/C1-B(2-4)] sequence. Whereas Luc-J/C1-B(2-4) replicated as well as Luc-J/C1-B(1-2) RNA (Fig. 3C), Luc-J/C1-B(2-3) was severely defective relative to any other replicon containing the Con1 TMD2 sequence (Fig. 3C). These findings indicate that the severity of the incompatibility depends on the combination of the Con1 TMD helices present in the JFH1 genome.

The defect in J/C1-B replication can be complemented by a mutation in the NS4B TMD2 sequence. To gain insight into the mechanism underlying the deficiency in the JFH1 chimeras containing Con1 NS4B TMD sequences, we took advantage of the chimeric virus containing the Con1 NS4B TMD region (J/C1-B). Thus, Huh7.5 cells were infected with the J/C1-B virus at an MOI of 0.01 and were passaged every 3 days to generate revertant virus. After 56 days of passaging, the supernatant virus titer was found to be as high as 10^5 FFU/ml. As shown in Fig. 4A, infection of Huh7.5 cells with the resulting virus, called J/C1-B-Ad1, at an MOI of 0.01 generated virus titers above 10^5 FFU/ml by 6 dpi, an increase of ca. 100-fold over the titers for JFH1-infected cells at this time point. As expected, infection with the parental J/C1-B virus led to very low virus titers relative to those with JFH1 or J/C1-B-Ad1.

To confirm that the J/C1-B-Ad1 virus was adapted, the genome from a cDNA pool obtained from the infected cell culture supernatant was sequenced, revealing three mutations. One mutation was in Con1 NS4B TMD2 (S113N [S1828N in the polyprotein]), the second in JFH1 NS5A (T462I [T2438I in the polyprotein]), and the third in JFH1 NS5B (K72E [K2514E in the polyprotein]). To test whether any of these mutations can rescue J/C1-B replication, the NS4B S113N, NS5A T462I, and NS5B K72E mutations were engineered, singly or together, into the Luc-J/C1-B replicon, followed by transfection of Huh7.5 cells and a luciferase assay as described above. As shown in Fig. 4B, the introduction of NS5A T462I into the Luc-J/C1-B replicon had no impact on JFH1 replication, whereas the NS5B K72E mutation resulted in a decrease of ca. 2-fold at 72 hpt. Interestingly, the NS4B S113N mutation led to a >10-fold increase in Luc-J/C1-B replication efficiency at 72 hpt. No such increase was observed when the combined NS4B S113N, NS5A T462I, and NS5B K72E mutations were introduced into the Luc-J/C1-B sequence. These results suggest that NS4B mutation, but not NS5A or NS5B mutation, can rescue the defective Luc-J/C1-B replicon. Since S113 is in the Con1 NS4B TMD2 sequence, these data also argue for a putative genetic interaction involving NS4B TMD2. The NS5A T462I

FIG. 2. (A) Subcellular distribution of NS4B and NS5A proteins in cells expressing JFH1 chimeras. Ten micrograms of JFH1 and various chimeric replicon DNA constructs were cotransfected into Huh7.5 cells with 5 μ g of a T7 polymerase-expressing vector. At 24 hpt, the cells were processed for fluorescence microscopy with a mouse NS4B (dilution, 1:250)- or rabbit NS5A (dilution, 1:500)-specific antibody and an Alexa Fluor 488- or 594-conjugated secondary antibody (1:1,000). Bars, 10 μ m. (B) Huh7.5 cells were cotransfected as described for panel A. At 24 hpt, the cells were labeled with 100 μ Ci [35 S]methionine for 1 h, and the lysates were immunoprecipitated with a rabbit polyclonal antibody specific for HCV NS4B (1:250). Immunoprecipitated proteins were separated by 10% SDS-PAGE and were visualized by autoradiography using a PhosphorImager.

mutation has been reported to enhance the virus production, but not the genome replication, of both JFH1 and a JFH1-based reporter virus (28). Therefore, the high titer of the J/C1-B-Ad1 virus may be due to increased efficiencies at more than one step in the virus life cycle.

Since the adaptive mutation S113N was in NS4B TMD2, we predicted that this mutation would have a compensatory effect on the JFH1 TMD2 chimera [J/C1-B(2)]. To test this hypothesis, we engineered the S113N mutation into the J/C1-B(2) replicon. We also introduced an A113N mutation into JFH1 to test its impact on JFH1 replication. As shown in Fig. 4C,

introduction of the S113N mutation into Luc-J/C1-B(2) led to an increase of ca. 10-fold in replication, with levels similar to that of WT JFH1. However, the A113N mutation did not significantly alter the replication efficiency of JFH1 (Fig. 4C). These findings indicate that the S113N mutation has a compensatory effect on the JFH1 TMD2 chimera.

The NS4B TMD1 and TMD2 sequences have putative protein-protein interaction motifs. The findings that the replication efficiencies of J/C1-B and J/C1-B(2) can be rescued by a mutation in a Con1 NS4B TMD2 residue suggest that differences in the amino acids between the Con1 and JFH1 NS4B

FIG. 3. (A) Huh7.5 cells were transfected with 10 μ g of J/C1-B(1-2) or J/C1-B(3-4) chimeric RNA. At 4, 24, 48, or 72 hpt, the cell extracts were tested for luciferase activity as for Fig. 1F. (B) Huh7.5 cells were transfected with 10 μ g of J/C1-A-B(1-2) or J/C1-B(3-4)-C replicon RNA, and at 4, 24, 48, or 72 hpt, JFH1 replication efficiency was determined as for panel A. (C) The cells were transfected with 10 μ g of J/C1-B(1-2)-Loop, J/C1-B(1), J/C1-B(2), J/C1-B(1-2), J/C1-B(2-3), or J/C1-B(2-4) chimeric RNA, and at 4, 24, 48, or 72 hpt, JFH1 replication was assessed as for panel A. The results in panels A, B, and C are representative of two independent experiments, each containing triplicate samples.

TMD sequences could disrupt putative protein-protein interactions required for the role of NS4B in JFH1 replication. Thus, we sought to map the residues responsible for the incompatibility in the NS4B TMD1 and TMD2 sequences by replacing the amino acids in the JFH1 sequences for which nonconservative substitutions are shown in Fig. 1A with their Con1 counterparts (A113S and S121A; A85S was not tested because we failed to engineer the construct). Surprisingly, replacement of each of these amino acids had no negative impact on JFH1 replication (data not shown), suggesting that either (i) these residues may act synergistically or (ii) the 12 conservative amino acid changes (Fig. 1A, S93Q, S83T, L86I, S91T, I96L, L98F, G114A, T116S, G117A, V120G, L123I, and V124A) could play a major role in the incompatibility of TMD1 and TMD2 sequences. Indeed, NS4B TMD2 substitutions that included the JFH1 Val₁₂₀ residue [in VS(120/121)GA] led to an approximately 5-fold decrease in JFH1 replication (data not shown). Since a mutant with the S121A substitution alone replicated as well as JFH1, these data are consistent with the interpretation that conservative amino acid changes play a role in the incompatibility between the Con1 and JFH1 NS4B TMD sequences. Thus, we searched for reported TMD helix-helix interaction motifs and tested the possibility that their residues play a role in NS4B function in HCV replication.

A common feature of the TMD helices involved in protein interactions is the presence of the dimerization motif GlyXXXGly (GXXXG). This motif is found in host factors (e.g., amyloid precursor protein) and in virus proteins (e.g., HIV-1 gp41 and the HCV E1 protein) (Fig. 5A) (13, 15, 54, 55). Indeed, a GXXXG-like motif is also found in the HCV NS4B TMD2 and TMD3 sequences (Fig. 5A and B). A helical-wheel representation of the JFH1 NS4B TMD2 sequence was generated, and the interface containing the putative TMD dimerization motif is indicated in Fig. 5C. Since Gly has a small side chain (—H), GXXXG motifs tend to bring TMD helices closer together to allow interaction with the surrounding residues. Replacement with Ala may not have a negative impact on helix interaction, because the methyl (—CH₃) group in the side chain of Ala is only slightly larger than the —H group in Gly. However, the bulkier side chain in Leu is likely to push helices far apart, thus inhibiting helix-helix interaction and protein function.

To test whether the TMD2 G₁₂₅XXXG₁₂₉-like motif is required for NS4B function, G125A/L or G129A/L mutations were engineered, and their impacts on JFH1 replication were determined. As shown in Fig. 5D, the G129A mutant replicon behaved like JFH1, whereas the G129L mutation led to a total loss of JFH1 replication. Interestingly, the G125A and G125L mutations resulted in severely defective replicons. These data suggest that the G₁₂₅XXXG₁₂₉-like motif is required for NS4B function in HCV replication. To examine the contribution of the surrounding residues to NS4B function, we mutated Val128 or Phe118 to Ala (V128A or F118A), since these residues are often found in TMD helices and are reported to be engaged in hydrogen (H) bond interactions (1, 39). As shown in Fig. 5D, whereas the V128A mutation had a negligible impact on JFH1 replication, the F118A mutation resulted in a completely defective replicon. These data indicate that TMD2 residue F118 is required for the NS4B role in HCV replication.

FIG. 4. (A) Comparison of virus yields in cells infected with JFH1, J/C1-B, or the adapted virus J/C1-B-Ad1. Huh7.5 cells were infected with JFH1 chimeras at an MOI of 0.01. At 3, 6, 9, and 12 dpi, the culture supernatant was collected, and released virus titers were determined as for Fig. 1D. (B) Impact of the second-site mutations on HCV replication. Huh7.5 cells were transfected with the Luc-J/C1-B replicon containing the NS4B S113N, NS5A T462I, and NS5B K72E mutations, singly or together. At 4, 24, 48, and 72 hpt, the cell extracts were prepared, followed by a luciferase assay as described for Fig. 1F. (C) Effect of the NS4B S113N mutation on the replication efficiency of the JFH1 TMD2 chimera. Huh7.5 cells were transfected with Luc-JFH1 and Luc-J/C1-B (2) replicons containing the NS4B A113N and NS4B S113N mutations, respectively. At 4, 24, 48, and 72 hpt, the cell lysates were tested for luciferase activity as for panel B. The results in panels B and C are representative of two independent experiments with triplicate wells.

The Ser/Thr (S/T) cluster-like dimerization motif is a less common TMD-TMD interaction motif. This motif has been shown in model membranes to mediate helix interaction (15, 39) via H bonding of the hydroxyl ($-\text{OH}$) group in the side chain of Ser and/or Thr. As shown in Fig. 6A, a highly conserved S/T-like motif is found in the NS4B TMD1 sequence. A helical-wheel representation of the JFH1 NS4B TMD1 sequence was also made, and the interface containing the putative S/T dimerization motif is indicated in Fig. 6B. To investigate the role of the S/T cluster-like motif in NS4B function, Ser and Thr residues in the putative helix-helix dimerization interface were mutated to Ala. Since Ala has a $-\text{CH}_3$ group in its side chain, such a substitution is predicted to disrupt any putative H bonding between Ser (or Thr) in TMD1 and an interacting residue in another TMD. As seen in Fig. 6C, the S91A mutation had a negligible impact on the replication efficiency of JFH1. Interestingly, the S83A mutation led to a 10-fold decrease in replication, whereas the TS(87/88)AA or TT(94/95)AA mutations resulted in completely defective replicons (Fig. 6C). These data argue that some of the S/T residues in the TMD1 sequence are required for NS4B function in HCV replication.

Some TMD mutations result in disruption of NS4B foci.

One important role of NS4B in HCV replication is its ability to induce a distinct ultrastructural membrane alteration called the membranous web (16, 37), which appears as dots or foci in fluorescence microscopy (3, 24, 44). Since most of the chimeric or mutant replicons were severely defective in replication, we examined the impact of these changes on NS4B subcellular distribution. First, Huh7.5 cells were transfected with JFH1-derived replicon RNAs, and the subcellular distribution of NS4B was examined at 24 or 48 hpt. The results indicated that detection of NS4B fluorescence in the cells correlates with the replication efficiency of each replicon RNA, since NS4B fluorescence could not be detected in cells transfected with replication-defective genomes (data not shown). To circumvent this limitation, WT, chimeric, and mutant JFH1 NS4B proteins were expressed alone and were examined under fluorescence microscopy. As expected, WT NS4B and mutant NS4B S91A (which replicates like a WT replicon) proteins displayed the typical foci (Fig. 7ii and v), whereas no such foci were found in the vector alone (Fig. 7i). The mutations in NS4B TMD1 [S83A, TS(87/88)AA, TT(94/95)AA] or TMD2 (G125A, G129L), which negatively affected replication, showed no observable effect on the subcellular distribution of NS4B (Fig. 7iii, iv, vi, vii, and viii). Interestingly, two TMD2 mutations (F118A and G125L) that led to a complete defect in HCV replication showed severe disruption of NS4B foci (Fig. 7ix and x). These results suggest that the TMD2 sequence contributes to NS4B focus formation. Note that all the chimeric NS4B proteins displayed foci similar to those of the WT JFH1 NS4B protein (data not shown). These findings suggest that the defect in replication efficiency in the chimeric RNAs cannot be attributed to disruption of the membrane-associated HCV replication complex.

The presence of WT-like NS4B foci in the NS4B chimeras, and in most of the mutant proteins, led us to predict that these proteins are not defective in membrane association. To test this hypothesis, Huh7.5 cells were transfected with various NS4B DNA constructs, followed by a membrane floatation

FIG. 5. (A) Amino acid sequence motif commonly found in transmembrane helices known to dimerize. The GXXXG motif is found in glycoprotein A (GpA), amyloid precursor protein (APP) (54), adrenoceptor (Adr.) (12), integrin (45, 77), HIV-1 gp41 (52), vesicular stomatitis virus glycoprotein (VSV-G) (14), and HCV E1 (13, 55) helices. GXXXG-like motifs can be seen in NS4B TMD2 and TMD3 sequences. (B) Comparison of the NS4B TMD2 amino acid sequences from several HCV isolates and hepatitis B virus B (GBV-B), another member of the *Flaviviridae*. (C) Helical-wheel representation of the JFH1 NS4B TMD2 sequence. Gly residues 125 and 129, in the square boxes in TMD2, are part of the putative GXXXG motif. The TMD2 dimerization interface, which contains the $G_{125}XXXG_{129}$ -like motif, is also shown. Stars mark nonconservative amino acid changes between JFH1 and Con1. (D) Huh7.5 cells were transfected with 10 μ g of a JFH1 replicon containing various mutations targeting the Gly residues [J-NS4B(G125A), -(G125L), -(G129A), and -(G129L)] or the Phe and Val residues in the dimerization interface [J-NS4B(F118A) and -(V128A)]. At 4, 24, 48, or 72 hpt, cell extracts were prepared, and the replication efficiency of JFH1 was examined as for Fig. 1F. The results are representative of two independent experiments, each containing triplicate samples.

assay as reported by Aligo et al. (3). Indeed, when the chimeras [J/C1-B(1) and J/C1-B(2)] and the G125A mutant protein were tested, they were all found to be membrane associated, since their subcellular distribution was similar to that of WT JFH1 NS4B and of calnexin (Fig. 8A), an integral membrane protein. The profile for these proteins was different from that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a soluble protein (Fig. 8A). Note that the NS4B profile of J/C1-B(2) was slightly different from that of WT JFH1, but these profiles were

found to be similar in Fig. 8B. We attempted to investigate the NS4B profiles from the F118A and G125L proteins, since these proteins displayed a reticular distribution in Fig. 7. Surprisingly, we were unable to obtain enough protein from F118A mutant- and G125L mutant-transfected cells for detection by Western blotting after floatation. These findings suggest that the F118A and G125L mutant NS4B proteins might be unstable. However, we cannot completely rule out the possibility that the apparent instability of the NS4B F118A and G125L

FIG. 6. (A) Comparison of the NS4B TMD1 amino acid sequences from HCV isolates and GBV-B. Note that the HCV NS4B TMD1 sequences are enriched in Ser and Thr residues. (B) Helical-wheel representation of the JFH1 NS4B TMD1 sequence. Ser and Thr appear to be enriched on one face of the helix; they are reminiscent of the Ser/Thr (S/T) cluster motif (15). The TMD dimerization interface, which contains the S/T cluster-like motif, is shown. Stars mark nonconservative amino acid changes between JFH1 and Con1. (C) Huh7.5 cells were transfected with 10 μ g of JFH1 replicons containing various mutations targeting Ser and Thr residues in the putative TMD1 S/T cluster [J-NS4B(S83A), -(TS87/88AA), -(S91A), and -(TT94/95AA)]. At 4, 24, 48, or 72 hpt, cell extracts were prepared, and the replication efficiency of JFH1 was analyzed as for Fig. 1F. The results are representative of two independent experiments, each containing triplicate samples.

mutants could be due to poor recognition by the NS4B-specific antibody.

Some TMD mutations negatively impact NS4B stability. The NS4B TMD swaps in the chimeric replicons, or the mutations in the GXXXG- and S/T-like motifs, may lead to misfolding of NS4B protein and thus may negatively impact its stability. However, the fluorescence and membrane floatation results suggest that most of the NS4B proteins are stable except for two mutant proteins, the F118A and G125L proteins, which could not be detected in the membrane floatation assay. To investigate the stability of these proteins, we employed two approaches. First, Huh7.5 cells expressing NS4B protein alone were metabolically labeled with [35 S]methionine, followed by immunoprecipitation (IP) with an NS4B-specific antibody, separation of the protein by SDS-PAGE, and visualization using a PhosphorImager. As shown in Fig. 9A, all the NS4B proteins tested, including the F118A and G125L proteins, were translated and appeared to be relatively stable after a 2-h chase. However, when the same constructs were expressed in cells for 48 h, followed by immunoblotting, the levels of the F118A and G125L proteins were significantly lower than that of JFH1

NS4B (Fig. 9B). A smaller decrease was observed with J/C1-B(1) protein (Fig. 9B). The differences in NS4B protein accumulation in Fig. 9B cannot be due to different transfection efficiencies, since the pulse-chase results in Fig. 9A indicate that all of the DNA constructs could be successfully transfected into cells and translated at similar rates. Taken together, these results suggest that only two mutations, F118A and G125L, destabilize NS4B protein.

DISCUSSION

Since NS4B is a multipass transmembrane protein, its putative TMDs contribute to at least one-third of the protein size. This implies that in addition to anchoring NS4B to host membranes, the TMDs could actively participate in the folding of the protein via intramolecular interactions. Further, as a scaffolding protein, NS4B may need intermolecular interactions in the context of host membranes (i) to recruit host and viral proteins to the RC and (ii) to oligomerize so as to form the typical NS4B foci. Thus, we have hypothesized that exchanging the NS4B TMD sequences between the HCV Con1 and JFH1

FIG. 7. Subcellular distribution of various NS4B proteins. Huh7.5 cells were transfected with 1.5 μ g of WT or mutant [S83A, TS(87/88)AA, S91A, TT(94/95)AA, G125A, G129L, F118A, or G125L] JFH1 NS4B plasmid DNA. At 48 hpt, the cells were processed for fluorescence microscopy with a rabbit polyclonal antibody specific for NS4B (dilution, 1:200) and an Alexa Fluor 594-conjugated secondary antibody (1:1,000). Bars, 10 μ m.

strains could lead to a defect in JFH1 virus production and virus genome replication. In light of the findings that the TMD sequences are not identical for different HCV genotypes, the small differences in amino acids could give rise to incompatibility between the NS4B proteins. Further, the importance of the TMDs in NS4B function is illustrated by the findings that several drug resistance mutations leading to NS4B-induced apoptosis, or HCV replication, can be mapped to the NS4B TMD1 and TMD2 sequences (58).

To test the prediction described above, we engineered JFH1

chimeric RNAs containing all or parts of the Con1 NS4B protein. In this report, we have identified the TMDs as major determinants of NS4B function in HCV replication. The TMDs of Con1 and JFH1 are generally incompatible, implying that the NS4B TMDs from different genotypes may not bind efficiently to each other or do not allow the chimeric NS4B protein to interact effectively with other replicase proteins. Our finding that a mutation in the Con1 TMD2 sequence restored the replication efficiency of the defective TMD2 chimera further supports this interpretation. More importantly, we have

FIG. 8. Huh7.5 cells were transfected with 10 μ g of various JFH1 DNA constructs. At 24 hpt, 1.25 mg of cell extract was subjected to membrane floatation, followed by immunoblotting with an NS4B-, calnexin-, or GAPDH-specific antibody. Six hundred micrograms of crude lysate was used for controls. Lanes 1 to 4, membrane fractions; lanes 5 to 8, soluble fractions.

identified two putative helix dimerization motifs in the TMDs: the S/T-like motif in TMD1 and the GXXXG-like motif in both TMD2 and TMD3. Mutagenesis of residues in the putative dimerization motifs in TMD1 and TMD2 generally resulted in a loss of JFH1 replication, suggesting that these motifs might play a role in NS4B interactions in the context of the host membranes. These interactions may contribute to NS4B stability, as evidenced by the fact that two mutations in the TMD2 sequence led to the destabilization of the mutant proteins. Finally, we have shown that the JFH1 chimera with the Con1 NS4B CTD is defective in virus production, not replication. This finding confirms the previous report by Jones et al. (33) indicating that NS4B plays a role in virion production.

In contrast to the roles of the NTD (9, 20, 25) and CTD (3, 26, 33, 40, 56), we know very little about the role of the TMDs in NS4B function. Few studies concerning the NS4B TMD region have been reported previously. For example, Lindström et al. (42) have used alanine scanning mutagenesis to demonstrate that certain residues in the loop regions of TMD1-2, TMD2-3, and TMD3-4 are required for HCV replication, but the underlying mechanism is still unknown. Einav et al. (17) reported that NS4B GTPase activity is required for HCV genome replication but is not associated with NS4B foci, the platform for HCV RC assembly (3, 44, 48, 65). The exact role of the TMD helices has been overlooked in part because of the assumption that these domains merely insert NS4B into host membranes. However, TMDs are now recognized as major

FIG. 9. Huh7.5 cells were transfected with 10 μ g of various JFH1 DNA constructs. At 24 hpt, the cells were labeled with 100 μ Ci [35 S]methionine for 30 min; then they were chased for 2 h, and the lysates were immunoprecipitated with an HCV NS4B-specific antibody (dilution, 1:250). (A) Immunoprecipitated proteins were separated by 10% SDS-PAGE and were visualized by autoradiography using a PhosphorImager. (B) Alternatively, 100 μ g of protein was separated by SDS-PAGE, followed by immunoblotting with an NS4B-specific antibody (1:1,500).

instigators of protein-protein interactions, thanks to biochemical, biophysical, and genetic analyses of these domains (8, 12, 13, 15, 39, 54, 64, 77). Some specific motifs have been found responsible for TMD helix homo- or heterodimerization. GXXXG, the most widespread motif, was initially found in the human erythrocyte sialoglycoprotein glycophorin A (GpA) TMD (47). It was later identified in several host and viral integral membrane proteins whose TMDs have been reported to homo- or heterodimerize (8, 12, 13, 15, 52, 54, 55). The GXXXG motif tends to induce membrane helix interactions because its short side chain (—H) promotes helix-helix association via van der Waals forces and H bond interactions (39). Interestingly, two recent studies have shown that the GXXXG motif in the HCV E1 TMD sequence is required for E1-E2 heterodimerization (13, 55), an event that may be linked to HCV entry and budding from the cells.

In NS4B protein, a GXXXG-like motif was found in TMD2 (G₁₂₅XXXG₁₂₉) and TMD3 (G₁₄₃XXXG₁₄₇). Since exchanging TMD2 between Con1 and JFH1 resulted in a significant decrease in JFH1 replication, we chose to investigate the role of the putative GXXXG motif in NS4B function in light of the findings that the nonconservative changes in amino acid sequences in TMD2 play little to no role in the incompatibility between the Con1 and JFH1 sequences. Whereas the G125A mutation led to a severe defect in replication efficiency, the G129A mutation did not. If Gly₁₂₅ and Gly₁₂₉ are part of a GXXXG-like motif, then the WT phenotype of the G129A mutation could be explained in part by the facts that (i) both Ala and Gly residues have smaller side chains than Leu, allowing close packing of the helices to occur and (ii) two HCV genotypes have Ser129 or Ala129 in NS4B TMD2 (Fig. 5B), suggesting that this position can tolerate changes with residues that have small side chains. In contrast, the replication-defective G125A phenotype may indicate that this position cannot

tolerate small residues such as Ala in TMD2 (47). Indeed, variants of the GXXXG motif, including GXXXA, AXXXG, SXXXG, and AXXXA, have also been reported to mediate membrane-spanning domain interactions (6, 32, 36). While mutation of the Gly to residues with a bulkier side chain, such as Leu, will definitely destroy the GXXXG-mediated TMD interactions, the impact of a G-to-A or G-to-S mutation may depend on the context of the motif.

The GXXXG motif predicts that replacement with bulky residues, such as Leu (with large side chains), will reduce van der Waals and H bond interactions between interacting TMDs. Thus, the findings that the G125L and G129L mutations lead to severe decreases in JFH1 replication efficiency suggest that Gly₁₂₅ and Gly₁₂₉ may be part of a GXXXG-like motif. However, G₁₂₉ is also the first residue of a previously reported Walker A motif (G₁₂₉SIGLGK₁₃₅) (19). Therefore, the phenotype of G129L mutation may just reflect the loss of NS4B GTPase activity. The finding that G129A mutant RNA replicates as well as JFH1 RNA may suggest that Gly₁₂₉ has a dual role in NS4B function: in addition to its reported role in nucleotide binding, we propose that Gly₁₂₉ is part of a GXXXG-like motif. Future experiments will seek to (i) identify NS4B TMD2-interacting partners, (ii) test whether the conserved Gly residues in both TMD2 and TMD3 are functionally important, mimicking the GXXXG motif, and thus may play a role in protein-protein interaction, and (iii) determine how mutations in this motif affect NS4B properties, including GTPase activity and RNA-binding ability.

Our study has also identified an S/T cluster-like motif in the NS4B TMD1 sequence. This motif is less common among integral membrane proteins, but it has been found in TMD libraries of model membranes (15, 39). Indeed, owing to their uncharged but polar properties, Ser and Thr residues in the hydrophobic environment of membranes tend to be engaged in helix-helix interactions via H bonds (15, 39). Replacing these Ser and Thr residues with Ala should have no negative impact on NS4B function unless these changes disrupt H-bonding interactions in the side chains (15, 39). Indeed, except for TMD1 residue Ser₉₁, mutagenesis of residues in the putative S/T cluster [S83A, TS(87/88)AA, TT(94/95)AA] led to a significant decrease in HCV replication. These data imply that some residues in the S/T cluster-like motif might be engaged in interaction, probably via their —OH group in the side chain. Thus, future studies will seek to (i) reveal the putative NS4B TMD1-interacting partner(s), (ii) define the role of the S/T cluster-like motif in this activity, and (iii) investigate how mutations in this putative motif affect NS4B activities.

As mentioned above, the TMD sequences may participate in (i) the NS4B interactions with host and viral proteins, (ii) the folding of NS4B protein, and/or (iii) NS4B oligomerization (74). The replication defect in the JFH1 chimeras containing the entire Con1 NS4B or its TMD region may be due in large part to the disruption of the interaction between NS4B and host/viral proteins. As with the smaller TMD helix swaps, since both TMD2 and TMD3 have GXXXG-like motifs in their sequences, we postulated that Con1 TMD2 and TMD3 would be more compatible via interaction of their helices, stimulating the replication of the JFH1 chimera. We predicted that, if this is correct, a JFH1 chimera containing the Con1 TMD2-3 sequence would replicate better than the chimera with the Con1

TMD2 sequence alone. Surprisingly, Luc-J/C1-B(2-3) replication was severely impaired relative to that of Luc-J/C1-B(2) (Fig. 3C). Further, Luc-J/C1-B(2-4) replicated better than Luc-J/C1-B(2-3) (Fig. 3C). These findings suggest that the NS4B TMDs are perhaps engaged in complex intra- and intermolecular interactions, which remain to be uncovered. For visualization of the TMDs, we performed homology modeling to predict the conformation of the JFH1 NS4B TMD region that has an amino acid sequence homologous to that of known protein structures (see the supplemental figures at <http://bmb.psu.edu/directory/kvk10>) (22, 23, 49, 60–62).

This model shows Gly₁₂₉ at the interface between the endoplasmic reticulum membrane and the cytosol. However, this is a static model, which does not take into account any potential movement that could occur in NS4B TMDs. Considering that (i) the NS4B molecule has two proposed amphipathic helices and that (ii) membranes are dynamic, the proposed model may represent an “off” state of the TMDs. Thus, if our model is true, conformational changes must occur for NS4B enzymatic activity (GTPase) to be achieved. Although this model needs to be confirmed using structural approaches, it nevertheless predicts putative intramolecular NS4B interactions involving the TMDs. Further, the findings that the defect in J/C1-B and J/C1-B(2) chimera replication can be rescued by a second-site mutation in the NS4B TMD2 sequence suggest that at least the TMD2 region is engaged in genetic interactions. Whether such interactions are intra- or intermolecular will be the subject of future studies.

NS4B expression results in the formation of foci that serve as a platform for the recruitment of the HCV RC (24). To understand the mechanism underlying the defect in the chimeric or mutant JFH1 replicon RNAs, we examined the subcellular distribution of various JFH1 NS4B proteins. As expected, all the chimeric NS4B proteins displayed WT-like foci (data are shown only for J/C1-NS4B and J/C1-B), suggesting that these chimeric proteins might be defective in their ability to (i) recruit host/virus factors to the HCV RC, (ii) bind to nucleoside triphosphates (GTP/ATP), or (iii) bind to the HCV RNA.

Further, whereas most of the mutant proteins displayed a WT-like NS4B subcellular distribution, the NS4B F118A and G125L mutations resulted in significant disruption of NS4B foci (Fig. 7). Additionally, several attempts to investigate the membrane association of NS4B F118A and G125L proteins were unsuccessful, whereas the binding of mutant G125A, J/C1-B(1), and J/C1-B(2) NS4B proteins (Fig. 8) to host membranes was not significantly altered from that of WT NS4B. These findings suggested that either the mutant NS4B F118A and G125L proteins are unstable or the constructs resulted in low transfection efficiency of these plasmids. However, the findings from pulse-chase and immunoblot experiments (Fig. 8) are consistent with the interpretation that the F118A and G125L mutations destabilize NS4B protein. The F118A phenotype may be explained by a disruption of the TMD2 interaction(s) required for NS4B folding. The phenotype of the G125L mutant was surprising, since the G125A mutation had no significant effect on NS4B stability. Since the G125A and G125L mutations negatively impact replication, we interpret these results to mean that the G125A mutation disrupts a single intra- or intermolecular TMD2 interaction, whereas the G125L mutation may have a more global effect on such inter-

actions. However, we do not rule out the possibility that the F118A and G125L mutations can destabilize the TMD2 helix, thus rendering NS4B unstable. Studies are under way to test whether such mutations destabilize NS4B in the context of the replicase complex.

In conclusion, this study has revealed, for the first time, the importance of the TMDs in the NS4B role in HCV replication. Future efforts will focus on using genetic, biochemical, and biophysical approaches to further investigate the contribution of the TMDs to NS4B function in the HCV life cycle.

ACKNOWLEDGMENTS

We are grateful to Takaji Wakita, Charles Rice, Craig Cameron, and Biao He for reagents, Suresh Sharma for technical help, and Craig Cameron for suggestions and critical reading of the manuscript.

This work was supported by grant K22 CA129241 from the National Institutes of Health. It was also supported by the Searle Young Investigators Award (to R.L.P.), National Institutes of Health grant R01 GM087410-01 (to R.L.P. and D.B.V.R.), and a grant from the Pennsylvania Department of Health using Tobacco Settlement Funds (to D.B.V.R.).

The Pennsylvania Department of Health specifically disclaims responsibility for any analyses, interpretations, or conclusions.

REFERENCES

- Adamian, L., and J. Liang. 2001. Helix-helix packing and interfacial pairwise interactions of residues in membrane proteins. *J. Mol. Biol.* **311**:891–907.
- Ali, N., K. D. Tardif, and A. Siddiqui. 2002. Cell-free replication of the hepatitis C virus subgenomic replicon. *J. Virol.* **76**:12001–12007.
- Aligo, J., S. Jia, D. Manna, and K. V. Konan. 2009. Formation and function of hepatitis C virus replication complexes require residues in the carboxy-terminal domain of NS4B protein. *Virology* **393**:68–83.
- Alter, M. J. 1997. Epidemiology of hepatitis C. *Hepatology* **26**:62S–65S.
- Appel, N., et al. 2008. Essential role of domain III of nonstructural protein 5A for hepatitis C virus infectious particle assembly. *PLoS Pathog.* **4**:e1000035.
- Asundi, V. K., and D. J. Carey. 1995. Self-association of *N*-syndecan (syndecan-3) core protein is mediated by a novel structural motif in the transmembrane domain and ectodomain flanking region. *J. Biol. Chem.* **270**:26404–26410.
- Bartenschlager, R., and S. Sparacio. 2007. Hepatitis C virus molecular clones and their replication capacity in vivo and in cell culture. *Virus Res.* **127**:195–207.
- Berger, B. W., et al. 2010. Consensus motif for integrin transmembrane helix association. *Proc. Natl. Acad. Sci. U. S. A.* **107**:703–708.
- Blight, K. J. 2007. Allelic variation in the hepatitis C virus NS4B protein dramatically influences RNA replication. *J. Virol.* **81**:5724–5736.
- Blight, K. J., A. A. Kolykhalov, and C. M. Rice. 2000. Efficient initiation of HCV RNA replication in cell culture. *Science* **290**:1972–1974.
- Blight, K. J., J. A. McKeating, and C. M. Rice. 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J. Virol.* **76**:13001–13014.
- Carrillo, J. J., J. F. Lopez-Gimenez, and G. Milligan. 2004. Multiple interactions between transmembrane helices generate the oligomeric $\alpha_1\beta$ -adrenoceptor. *Mol. Pharmacol.* **66**:1123–1137.
- Ciczora, Y., N. Callens, F. Penin, E. I. Pecheur, and J. Dubuisson. 2007. Transmembrane domains of hepatitis C virus envelope glycoproteins: residues involved in E1E2 heterodimerization and involvement of these domains in virus entry. *J. Virol.* **81**:2372–2381.
- Cleverley, D. Z., and J. Lenard. 1998. The transmembrane domain in viral fusion: essential role for a conserved glycine residue in vesicular stomatitis virus G protein. *Proc. Natl. Acad. Sci. U. S. A.* **95**:3425–3430.
- Dawson, J. P., J. S. Weinger, and D. M. Engelman. 2002. Motifs of serine and threonine can drive association of transmembrane helices. *J. Mol. Biol.* **316**:799–805.
- Egger, D., et al. 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* **76**:5974–5984.
- Einav, S., M. Elazar, T. Danieli, and J. S. Glenn. 2004. A nucleotide binding motif in hepatitis C virus (HCV) NS4B mediates HCV RNA replication. *J. Virol.* **78**:11288–11295.
- Einav, S., et al. 2008. Discovery of a hepatitis C target and its pharmacological inhibitors by microfluidic affinity analysis. *Nat. Biotechnol.* **26**:1019–1027.
- Einav, S., et al. 2008. The nucleotide binding motif of hepatitis C virus NS4B can mediate cellular transformation and tumor formation without Ha-ras co-transfection. *Hepatology* **47**:827–835.
- Elazar, M., P. Liu, C. M. Rice, and J. S. Glenn. 2004. An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. *J. Virol.* **78**:11393–11400.
- El-Hage, N., and G. Luo. 2003. Replication of hepatitis C virus RNA occurs in a membrane-bound replication complex containing nonstructural viral proteins and RNA. *J. Gen. Virol.* **84**:2761–2769.
- Eswar, N., et al. 2007. Comparative protein structure modeling using MODELLER. *Curr. Protoc. Protein Sci.* Chapter 2: Unit 2.9.
- Eswar, N., et al. 2006. Comparative protein structure modeling using Modeller. *Curr. Protoc. Bioinformatics* Chapter 5: Unit 5.6.
- Gosert, R., et al. 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. *J. Virol.* **77**:5487–5492.
- Gouttenoire, J., et al. 2009. Identification of a novel determinant for membrane association in hepatitis C virus nonstructural protein 4B. *J. Virol.* **83**:6257–6268.
- Gouttenoire, J., R. Montserret, A. Kennel, F. Penin, and D. Moradpour. 2009. An amphipathic α -helix at the C terminus of hepatitis C virus nonstructural protein 4B mediates membrane association. *J. Virol.* **83**:11378–11384.
- Guo, J. T., V. V. Bichko, and C. Seeger. 2001. Effect of alpha interferon on the hepatitis C virus replicon. *J. Virol.* **75**:8516–8523.
- Han, Q., et al. 2009. Compensatory mutations in NS3 and NS5A proteins enhance the virus production capability of hepatitis C reporter virus. *Virus Res.* **145**:63–73.
- Huang, L., et al. 2005. Hepatitis C virus nonstructural protein 5A (NS5A) is an RNA-binding protein. *J. Biol. Chem.* **280**:36417–36428.
- Hügler, T., et al. 2001. The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. *Virology* **284**:70–81.
- Ishido, S., T. Fujita, and H. Hotta. 1998. Complex formation of NS5B with NS3 and NS4A proteins of hepatitis C virus. *Biochem. Biophys. Res. Commun.* **244**:35–40.
- Itoh, R., and Y. Fujiki. 2006. Functional domains and dynamic assembly of the peroxin Pex14p, the entry site of matrix proteins. *J. Biol. Chem.* **281**:10196–10205.
- Jones, D. M., A. H. Patel, P. Targett-Adams, and J. McLauchlan. 2009. The hepatitis C virus NS4B protein can *trans*-complement viral RNA replication and modulates production of infectious virus. *J. Virol.* **83**:2163–2177.
- Kato, T., et al. 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* **125**:1808–1817.
- Kim, D. W., Y. Gwack, J. H. Han, and J. Choe. 1995. C-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase activity. *Biochem. Biophys. Res. Commun.* **215**:160–166.
- Kleiger, G., R. Grothe, P. Mallick, and D. Eisenberg. 2002. GXXXG and AXXXA: common alpha-helical interaction motifs in proteins, particularly in extremophiles. *Biochemistry* **41**:5990–5997.
- Konan, K. V., et al. 2003. Nonstructural protein precursor NS4A/B from hepatitis C virus alters function and ultrastructure of host secretory apparatus. *J. Virol.* **77**:7843–7855.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Langosch, D., and I. T. Arkin. 2009. Interaction and conformational dynamics of membrane-spanning protein helices. *Protein Sci.* **18**:1343–1358.
- Liefhebber, J. M., B. W. Brandt, R. Broer, W. J. Spaan, and H. C. van Leeuwen. 2009. Hepatitis C virus NS4B carboxy terminal domain is a membrane binding domain. *Virology* **496**:62.
- Lindenbach, B. D., et al. 2005. Complete replication of hepatitis C virus in cell culture. *Science* **309**:623–626.
- Lindström, H., M. Lundin, S. Haggstrom, and M. A. Persson. 2006. Mutations of the hepatitis C virus protein NS4B on either side of the ER membrane affect the efficiency of subgenomic replicons. *Virus Res.* **121**:169–178.
- Lohmann, V., et al. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**:110–113.
- Lundin, M., M. Monne, A. Widell, G. Von Heijne, and M. A. Persson. 2003. Topology of the membrane-associated hepatitis C virus protein NS4B. *J. Virol.* **77**:5428–5438.
- Luo, B. H., T. A. Springer, and J. Takagi. 2004. A specific interface between integrin transmembrane helices and affinity for ligand. *PLoS Biol.* **2**:e153.
- Ma, Y., J. Yates, Y. Liang, S. M. Lemon, and M. Yi. 2008. NS3 helicase domains involved in infectious intracellular hepatitis C virus particle assembly. *J. Virol.* **82**:7624–7639.
- MacKenzie, K. R., J. H. Prestegard, and D. M. Engelman. 1997. A transmembrane helix dimer: structure and implications. *Science* **276**:131–133.
- Manna, D., et al. 2010. Endocytic Rab proteins are required for hepatitis C virus replication complex formation. *Virology* **398**:21–37.
- Martí-Renom, M. A., et al. 2000. Comparative protein structure modeling of genes and genomes. *Annu. Rev. Biophys. Biomol. Struct.* **29**:291–325.
- Masaki, T., et al. 2008. Interaction of hepatitis C virus nonstructural protein

- 5A with core protein is critical for the production of infectious virus particles. *J. Virol.* **82**:7964–7976.
51. **Miller, R. H., and R. H. Purcell.** 1990. Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. *Proc. Natl. Acad. Sci. U. S. A.* **87**:2057–2061.
 52. **Miyauchi, K., et al.** 2006. Mutations of conserved glycine residues within the membrane-spanning domain of human immunodeficiency virus type 1 gp41 can inhibit membrane fusion and incorporation of Env onto virions. *Jpn. J. Infect. Dis.* **59**:77–84.
 53. **Moradpour, D., F. Penin, and C. M. Rice.** 2007. Replication of hepatitis C virus. *Nat. Rev. Microbiol.* **5**:453–463.
 54. **Munter, L. M., et al.** 2007. GxxxG motifs within the amyloid precursor protein transmembrane sequence are critical for the etiology of A β 42. *EMBO J.* **26**:1702–1712.
 55. **Op De Beeck, A., et al.** 2000. The transmembrane domains of hepatitis C virus envelope glycoproteins E1 and E2 play a major role in heterodimerization. *J. Biol. Chem.* **275**:31428–31437.
 56. **Paredes, A. M., and K. J. Blight.** 2008. A genetic interaction between hepatitis C virus NS4B and NS3 is important for RNA replication. *J. Virol.* **82**:10671–10683.
 57. **Pietschmann, T., et al.** 2009. Production of infectious genotype 1b virus particles in cell culture and impairment by replication enhancing mutations. *PLoS Pathog.* **5**:e1000475.
 58. **Rai, R., and J. Deval.** 1 February 2011. New opportunities in anti-hepatitis C virus drug discovery: targeting NS4B. *Antiviral Res.* doi:10.1016/j.antiviral.2011.01.009.
 59. **Russell, R. S., et al.** 2008. Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus. *Proc. Natl. Acad. Sci. U. S. A.* **105**:4370–4375.
 60. **Sali, A.** 1995. Comparative protein modeling by satisfaction of spatial restraints. *Mol. Med. Today* **1**:270–277.
 61. **Sali, A.** 1995. Modeling mutations and homologous proteins. *Curr. Opin. Biotechnol.* **6**:437–451.
 62. **Sali, A., L. Potterton, F. Yuan, H. van Vlijmen, and M. Karplus.** 1995. Evaluation of comparative protein modeling by MODELLER. *Proteins* **23**: 318–326.
 63. **Simmonds, P., et al.** 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* **42**:962–973.
 64. **Smith, S. O., et al.** 2001. Structure of the transmembrane dimer interface of glycoporphin A in membrane bilayers. *Biochemistry* **40**:6553–6558.
 65. **Stone, M., S. Jia, W. D. Heo, T. Meyer, and K. V. Konan.** 2007. Participation of Rab5, an early endosome protein, in hepatitis C virus RNA replication machinery. *J. Virol.* **81**:4551–4563.
 66. **Tackett, A. J., L. Wei, C. E. Cameron, and K. D. Raney.** 2001. Unwinding of nucleic acids by HCV NS3 helicase is sensitive to the structure of the duplex. *Nucleic Acids Res.* **29**:565–572.
 67. **Targett-Adams, P., S. Boulant, and J. McLauchlan.** 2008. Visualization of double-stranded RNA in cells supporting hepatitis C virus RNA replication. *J. Virol.* **82**:2182–2195.
 68. **Targett-Adams, P., and J. McLauchlan.** 2005. Development and characterization of a transient-replication assay for the genotype 2a hepatitis C virus subgenomic replicon. *J. Gen. Virol.* **86**:3075–3080.
 69. **Tellinghuisen, T. L., K. L. Foss, and J. Treadaway.** 2008. Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. *PLoS Pathog.* **4**:e1000032.
 70. **Thompson, A. A., et al.** 2009. Biochemical characterization of recombinant hepatitis C virus nonstructural protein 4B: evidence for ATP/GTP hydrolysis and adenylate kinase activity. *Biochemistry* **48**:906–916.
 71. **Wakita, T., et al.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* **11**:791–796.
 72. **Wölk, B., et al.** 2000. Subcellular localization, stability, and *trans*-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *J. Virol.* **74**:2293–2304.
 73. **Yi, M., Y. Ma, J. Yates, and S. M. Lemon.** 2007. Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. *J. Virol.* **81**:629–638.
 74. **Yu, G. Y., K. J. Lee, L. Gao, and M. M. Lai.** 2006. Palmitoylation and polymerization of hepatitis C virus NS4B protein. *J. Virol.* **80**:6013–6023.
 75. **Zhong, J., et al.** 2005. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **102**:9294–9299.
 76. **Zhong, J., et al.** 2006. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. *J. Virol.* **80**:11082–11093.
 77. **Zhu, J., et al.** 2009. The structure of a receptor with two associating transmembrane domains on the cell surface: integrin $\alpha_{IIb}\beta_3$. *Mol. Cell* **34**:234–249.