Characterization of Dengue Virus NS4A and NS4B Protein Interaction

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ABSTRACT
Flavivirus replication is mediated by a membrane-associated replication complex where viral membrane proteins NS2A, NS2B, NS4A, and NS4B serve as the scaffold for the replication complex formation. Here, we used dengue virus serotype 2 (DENV-2) as a model to characterize viral NS4A-NS4B interaction. NS4A interacts with NS4B in virus-infected cells and in cells transiently expressing NS4A and NS4B in the absence of other viral proteins. Recombinant NS4A and NS4B proteins directly bind to each other with an estimated $K_d$ (dissociation constant) of 50 nM. Amino acids 40 to 76 (spanning the first transmembrane domain, consisting of amino acids 50 to 73) of NS4A and amino acids 84 to 146 (also spanning the first transmembrane domain, consisting of amino acids 101 to 129) of NS4B are the determinants for NS4A-NS4B interaction. Nuclear magnetic resonance (NMR) analysis suggests that NS4A residues 17 to 80 form two amphipathic helices (helix $\alpha_1$, comprised of residues 17 to 32, and helix $\alpha_2$, comprised of residues 40 to 47) that associate with the cytosolic side of endoplasmic reticulum (ER) membrane and helix $\alpha_3$ (residues 52 to 75) that transverses the ER membrane. In addition, NMR analysis identified NS4A residues that may participate in the NS4A-NS4B interaction. Amino acid substitution of these NS4A residues exhibited distinct effects on viral replication. Three of the four NS4A mutations (L48A, T54A, and L60A) that affected the NS4A-NS4B interaction abolished or severely reduced viral replication; in contrast, two NS4A mutations (F71A and G75A) that did not affect NS4A-NS4B interaction had marginal effects on viral replication, demonstrating the biological relevance of the NS4A-NS4B interaction to DENV-2 replication. Taken together, the study has provided experimental evidence to argue that blocking the NS4A-NS4B interaction could be a potential antiviral approach.

IMPORTANCE
Flavivirus NS4A and NS4B proteins are essential components of the ER membrane-associated replication complex. The current study systematically characterizes the interaction between flavivirus NS4A and NS4B. Using DENV-2 as a model, we show that NS4A interacts with NS4B in virus-infected cells, in cells transiently expressing NS4A and NS4B proteins, or in vitro with recombinant NS4A and NS4B proteins. We mapped the minimal regions required for the NS4A-NS4B interaction to be amino acids 40 to 76 of NS4A and amino acids 84 to 146 of NS4B. NMR analysis revealed the secondary structure of amino acids 17 to 80 of NS4A and amino acids 84 to 146 of NS4B. The study has advanced our knowledge of the molecular function of flavivirus NS4A and NS4B proteins. The results also suggest that inhibitors of the NS4A-NS4B interaction could be pursued for flavivirus antiviral development.

The four serotypes of dengue virus (DENV-1 to DENV-4) are the causative pathogens of dengue disease, which has become a major public health threat. DENV infection causes flu-like illness known as dengue fever (DF). Some DENV-infected patients can develop life-threatening disease, known as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (1). DENV causes about 390 million human infections annually, leading to 96 million cases with manifest symptoms (2). Neither an approved vaccine nor an antiviral is currently available for prevention and treatment of DENV infection. Better understanding of the molecular mechanisms of DENV replication will benefit vaccine and antiviral development.

DENV is a member of genus Flavivirus within family Flaviviridae. In addition to DENV, many flaviviruses are important human pathogens, including West Nile virus (WNV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), and tick-borne encephalitis virus (TBEV). The plus-sense, single-stranded genomic RNA of flavivirus contains a 5′ untranslated region (UTR) with a type I cap structure, a single open reading frame (ORF), and a nonpolyadenylated 3′ UTR. The genome encodes a polyprotein that is processed co- and posttranslationally by cellular and viral proteases into three structural proteins (capsid [C], premembrane [prM], and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). DENV infection induces endoplasmic reticulum (ER)-derived vesicles to accommodate viral replication complexes (3). Nonstructural proteins are
mainly involved in viral replication. NS3 serves as a protease (using NS2B as a cofactor) (4), RNA helicase, nucleotide triphosphatase, and RNA triphosphatase (5–7). NS5 functions as a methyltransferase (8–10), weak guanylyltransferase (11), and RNA-dependent RNA polymerase (12). The glycosylated NS1 protein is essential for viral replication (13, 14).

Four nonstructural proteins (NS2A, NS2B, NS4A, and NS4B) are integral membrane proteins. NS2A contains five transmembrane domains (TMDs) and is important for viral replication and assembly (15, 16). NS2B is a cofactor for NS3 protease (17). Membrane proteins NS4A and NS4B are linked by a conserved 23-amino-acid-long signal peptide with a molecular weight of 2,000 (2K). The cleavage between NS4A and 2K by NS2B/NS3 proteinase is a prerequisite for the cleavage between 2K and NS4B by a host signalase (18). DENV-2 NS4A contains 127 amino acids and contains two TMDs (19); the first 48 amino acids of DENV-2 NS4A were reported to form an amphipathic helix that mediates oligomerization (20). DENV-2 NS4B consists of 248 amino acids and contains three TMDs (21). NS4A and NS4B play multiple roles in viral replication and virus-host interactions: (i) WNV NS4A and its C-terminal 2K peptide induce ER membrane rearrangement. Removal of 2K results in a redistribution of NS4A to the Golgi apparatus (22); however, expression of DENV-2 NS4A without 2K causes ER membrane alterations resembling virus-induced structures, whereas expression of NS4A in tandem with 2K fails to induce membrane rearrangement (19). This evidence suggests that 2K regulates NS4A’s function in modulating the ER membrane through distinct mechanisms in different flaviviruses. (ii) Interaction between DENV NS4A and cellular vimentin regulates the formation of virus replication complexes (23). (iii) Flavivirus NS4A protein induces autophagy to prevent cell death and facilitate viral replication (24). (iv) WNV NS4A regulates the ATPase activity of NS3 helicase (25), while DENV NS4B interacts with the helicase domain of NS3 and dissociates it from single-strand RNA (26). As noted in the accompanying article by Zou et al. (27), the DENV NS3–NS4B interaction was mapped to the NS3 helicase (subdomains 2 and 3) and the NS4B cytoplasmic region (amino acids 129 to 165). (v) NS4A and NS4B genetically interact with NS1 to modulate viral replication. The replication defect of YFV containing NS1 mutations could be restored by an adaptive mutation in viral NS4A (28). The replication defect of WNV containing NS1 mutations could be compensated by a mutation in viral NS4B (29). (vi) An N-terminal cytoplasmic mutant of DENV-1 NS4A is defective in viral replication. The replication defect can be rescued by mutations in the TMD3 of NS4B (30). (vii) Both NS4A and NS4B can induce the unfolded protein response in the host and inhibit interferon (IFN) signaling (31–33). These results suggest that NS4A and NS4B may function cooperatively in viral replication and host response. However, the direct interaction between NS4A and NS4B remains to be established and characterized.

In this study, we demonstrated a direct interaction between DENV-2 NS4A and NS4B. We mapped amino acids 40 to 76 of NS4A and amino acids 84 to 146 of NS4B to be the determinants for the NS4A-NS4B interaction. Nuclear magnetic resonance (NMR) studies revealed the secondary structure of NS4A residues 17 to 80 and identified the residues within this region that may participate in the NS4A-NS4B interaction. Functional analysis using a DENV-2 genome-length RNA showed that the identified NS4A residues were important for viral replication. Furthermore, the replication defects of NS4A mutant viruses correlated with the mutational effect on the NS4A-NS4B interaction, demonstrating the biological relevance of the NS4A-NS4B interaction.

MATERIALS AND METHODS

Cells, viruses, and antibodies. Baby hamster kidney (BHK-21) cells were purchased from the American Type Culture Collection (ATCC) and maintained in high-glucose Dulbecco’s modified Eagle medium (DMEM) (Life technologies) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and 1% penicillin-streptomycin (Life Technologies). HEK 293T cells were grown in low-glucose DMEM (Life Technologies) containing 10% FBS and 1% penicillin-streptomycin. DENV-2 (strain New Guinea C [NGC]; GenBank accession number AF938403) was generated from its corresponding infectious cDNA clone (pACYC-NGC FL) (15). The following antibodies were used in this study: negative-control mouse IgG2b (Millipore), mouse monoclonal antibody (MAB) against DENV-2 NS4B protein (clone 44-4-7) (34), mouse MAB against DENV-2 NS4A protein (generated in-house [unpublished data]), mouse MAB D2DC1 against DENV-2 capsid (35), mouse MAB 4G2 (ATCC) against DENV E protein, mouse anti-enhanced green fluorescent protein (EGFP) MAB (Roche), rabbit anti-EGFP polyclonal antibody (pAb) (Abcam), rabbit anti-hemagglutinin (HA) and anti-Flag (Sigma) pAbs, mouse anti-Flag (Sigma) MAB, mouse anti-DENV-2 NS4 MAB, rabbit anti-DENV 2 NS3 and NS4A (GeneTex) pAbs, Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG (Life technologies), goat anti-mouse or goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) (Sigma), and protein A conjugated to HRP (Sigma).

Plasmid construction. Standard molecular biology procedures were performed for all plasmid constructions. The fragments encoding full-length (FL) or peptides of NS4A and NS4B were amplified from plasmid pACYC-NGC FL (15) and fused in tandem with an HA or Flag tag by PCR using corresponding primer pairs. The tag positions of individual constructs are indicated in each experiment (see below). The PCR products were digested with EcoRI and XhoI restriction enzymes and cloned into mammalian expression vector pXJ (15) to generate the following constructs: the full-length NS4A protein with a C-terminal Flag tag (pXJ-NS4A-Flag), the NS4A peptide consisting of residues 50 to 76 with an N-terminal Flag tag [pXJ-Flag-NS4A (50–76)], the NS4A peptide consisting of residues 40 to 76 with an N-terminal Flag tag [pXJ-Flag-NS4A (40–76)], the full-length NS4B protein with an N-terminal 2K signal peptide and a C-terminal HA tag [pXJ-2K-NS4B-HA], and the NS4B peptide consisting of residues 84 to 146 with a C-terminal HA tag [pXJ-NS4B (84–146)-HA]. For C-terminally EGFP-tagged NS4A or NS4B constructs, the NS4A or NS4B fragment was amplified by PCR, digested with EcoRI and BamHI restriction enzymes, and inserted into the plasmid pXJ-EGFP (15) to generate constructs pXJ-NS4A-Flag-EGFP and pXJ-NS4B peptide-EGFP (where the peptide is indicated as above). For site-directed mutagenesis, all NS4A mutants were first introduced into a subclone TA-E (containing cDNA from nucleotide 5,426 to the 3’ end of the genome of DENV-2, strain NGC) individually by using a QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Inc.). The DNA fragment containing each NS4A mutation was cloned into the plasmid pACYC-NGC FL (15). To construct pXJ-NS4A-EGFP encoding mutant NS4A-EGFP proteins, the corresponding NS4A fragments were amplified from the plasmid pACYC-NGC FL containing the designated NS4A mutations, digested with EcoRI and BamHI restriction enzymes, and inserted into the plasmid pXJ-EGFP, resulting in constructs of the form pXJ-NS4A-mutant-EGFP. All constructs were validated by restriction enzyme digestion and cDNA sequencing. Primer sequences are available upon request.

Immunofluorescence assay (IFA). Cells grown on an eight-well Lab-Tek chamber slide (Thermo Fisher Scientific) were washed once by phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS at room temperature for 30 min, and then permeabilized with 0.1% (vol/vol)
vol) Triton X-100 in PBS for 10 min. After cells were blocked with PBS containing 1% bovine serum albumin (BSA) for 1 h, they were incubated with primary antibody for 1 h, followed by three washes with PBS containing 0.05% Tween 20 (PBST buffer). Afterwards, the cells were incubated with the secondary antibody Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 594 goat anti-rabbit IgG (1:10,000) for 1 h in PBST buffer containing 1% BSA. After three washes with PBST, the cells were mounted in mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc.). Fluorescence images were acquired with a Leica DM4000 B system.

For confocal study, BHK-21 cells were infected with DENV-2 NGC (multiplicity of infection [MOI] of 10) in wells of an eight-well chamber slide at 37°C for 24 h. After that, the cells were fixed, permeabilized, and incubated with the antibodies described above. Confocal images were acquired by using a Zeiss LSM 510 META laser scanning confocal microscope equipped with an oil immersion 100× objective lens (Biopolis Shared Facilities, Singapore). Images were merged using Fiji image software.

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**Co-IP.** HEK 293T cells in a 10-cm dish were transfected with the indicated plasmid DNA (as detailed for each experiment in the text) using an X-tremeGENE 9 DNA transfection reagent (Roche). At 48 h posttransfection (p.t.), the cells were lysed in 1 ml of immunoprecipitation (IP) buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5% n-dodecyl-β-D-maltoside (DDM), and 1× EDTA-free protease cocktail [Roche]) by rotation at 4°C for 1 h. Cell lysates were clarified by centrifugation at 20,000 × g at 4°C for 30 min, and the supernatants were subjected to coimmunoprecipitation (co-IP) using protein G-conjugated magnetic beads, according to the manufacturer’s instructions (Millipore). Briefly, 200 μl to 400 μl of the cell lysates was mixed with 2 μg of antibodies in a 500-μl volume containing 250 to 400 mM sodium chloride to form immune complexes at 4°C overnight. Subsequently, the immune complexes were precipitated by protein G-conjugated magnetic beads at 4°C for 1 h with rotation. After five washes with PBS containing 0.1% Tween 20, the bound proteins were eluted in 4× lysis buffer of sodium dodecyl sulfate (LDS) sample buffer (Life Technologies) containing 100 mM dithiothreitol (DTT) by heating at 70°C for 15 min on an Thermomixer (Eppendorf) with shaking at 1,200 rpm. Eluates were analyzed by SDS-PAGE and Western blotting.

Expression and purification of recombinant NS4A and NS4B proteins. DENV-2 NGC NS4B protein was expressed and purified by following a previously described protocol (36). A similar protocol was used to express and purify DENV-2 NS4A protein with some modifications. Briefly, the cDNA encoding the full-length NS4A was amplified from 

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**Expression and purification of recombinant NS4A and NS4B proteins.** DENV-2 NGC NS4B protein was expressed and purified by following a previously described protocol (36). A similar protocol was used to express and purify DENV-2 NS4A protein with some modifications. Briefly, the cDNA encoding the full-length NS4A was amplified from Escherichia coli Rosetta (Stratagene) cells. The expression of NS4A protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM when the cells reached an optical density at 600 nm (OD600) of 6.0 to 8.0. After growth for 16 h at 18°C, the cells were harvested, resuspended in a lysis buffer (20 mM Tris–HCl, 300 mM NaCl, 2 mM tris(2-carboxyethyl)phosphine (TCEP), 0.5× EDTA-free protease inhibitor cocktail, pH 8.5) and disrupted by sonication using a Digital Sonifier 450 (Branson). The cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatants were collected and applied to ultracentrifugation at 45,000 rpm for 1 h at 4°C using a Beckman type 70 Ti rotor. The pellets containing the membrane fractions were collected and solubilized with 1% (wt/vol) 6-cyclohexyl-1-hexyl-β-D-maltoside (Cymal-6; Anatrace) in lysis buffer overnight at 4°C. Following another ultracentrifugation step at 45,000 rpm for 1 h, the supernatants were collected and loaded onto a HisTrap Fast Flow column (GE Healthcare) which was precolumn buffer with buffer A (20 mM Tris, 300 mM NaCl, 2 mM TCEP, and 0.1% Cymal-6, pH 8.5). The protein was eluted using a linear gradient of imidazole at concentrations from 40 to 300 mM. The fractions containing (His)6-NS4A proteins were pooled, concentrated, and subjected to gel filtration using a HiLoad Superdex 200 16/60 column (GE Healthcare) in buffer A. The peak fractions containing (His)6-NS4A proteins were pooled and concentrated to approximately 5 to 10 mg/ml before storage at −80°C.

Expression and purification of NS4A (17–80). The cDNA encoding residues M17 to K80 of NS4A [NS4A (17–80)] of DENV-2 (NGC strain) was synthesized and cloned into the NdeI and Xhol sites of pET29b. The resulting plasmid pET29-NS4A encodes NS4A (17–80) with a C-terminal (His6) tag to facilitate protein purification. The plasmid was transformed in E. coli competent cells, and cells were plated onto an LB plate containing kanamycin. NS4A (17–80) was induced and purified using a protocol similar to that used for other membrane proteins (37). Briefly, two to three colonies were inoculated in 20 ml of M9 medium and incubated at 37°C overnight with shaking. The culture was then transferred into M9 medium supplemented with kanamycin. When the OD600 reached 0.6 to 0.8, the protein was induced overnight at 37°C by the addition of IPTG to a final concentration of 1 mM. The E. coli cells were collected and resuspended in a lysis buffer that contained 20 mM Tris–HCl, pH 7.8, 300 mM NaCl, and 2 mM β-mercaptoethanol. Cells were then broken by sonication in an ice bath, and the cell lysate was cleared by centrifugation at 40,000 × g for 20 min. The pellet was solubilized in a urea buffer containing 8 M urea, 300 mM NaCl, 10 mM SDS, and 20 mM Tris–HCl, pH 7.8. The solution was cleared by centrifugation at 40,000 × g for 20 min at room temperature. The resulting supernatant was then mixed with a nitritotriacetic acid-saturated nickel (Ni2+–NTA) resin, and the resin was then loaded on a gravity column. The resin was washed with 10 column volumes of urea buffer containing 20 mM imidazole. The resin was further washed with 10 column volumes of lysis buffer with 10 mM SDS and another 10 column volumes of lysis buffer with 15 mM dodecylphosphocholine (DPC). Protein was eluted in an elution buffer that contained 20 mM sodium phosphate, pH 6.5, 300 mM imidazole, and 15 mM DPC. Purified protein from Ni2+–NTA resin was concentrated from 8 ml to 1 ml using a 3-kDa-molecular-mass-cutoff concentrator and loaded on a Superdex 200 10/300 GL column that was equilibrated with a gel filtration buffer containing 20 mM sodium phosphate, pH 6.5, 15 mM DPC, and 1 mM DTT. The sample was concentrated to 200 to 400 μl before it was transferred into an NMR tube for data acquisition.

ELISA. An enzyme-linked immunosorbent assay (ELISA) was performed according to a previous report (38) with minor modifications. In brief, purified DENV-2 NS4A protein was diluted in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6) and incubated in 96-well ELISA plates (Nunc) (250 ng/well) at 4°C overnight. Next day, the plates were incubated at room temperature for 2 h in a blocking buffer (10 mM Tris–HCl, pH 8.0, and 150 mM NaCl [TBST buffer], supplemented with 2% FBS). After three washes with TBST buffer plus 0.05% Triton X-100, blocked wells were incubated with serial dilutions of DENV-2 NS4B protein in blocking buffer at room temperature for 2 h. After another three washes with TBST buffer plus 0.05% Triton X-100, the plates were incubated with a mouse anti-NS4B MAb (clone 44-4-7), followed by incubation with a secondary antibody (goat anti-mouse antibody conjugated to HRP). After extensive washing, the plates were incubated with the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Thermo Scientific) at room temperature for less than 3 min. The reactions were stopped by addition of equal volumes of 1 M sulfuric acid. The absorbance at 450 nm was measured using a Safire II plate reader (Tecan). Two sets of controls were used for background subtraction: (i) wells coated with NS4A were not sequentially incubated with NS4B; (ii) NS4A-free wells were sequentially incubated with NS4B. The concentration of NS4B at which half of NS4A proteins were bound by NS4B was defined as the Kd (dissociation constant), which was calculated using the one-site specificity model in Prism, version 5, software.

In situ PLA. A proximity of ligation assay (PLA) was performed using a Duolink in situ kit (Olink Bioscience) according to the manufacturer’s...
instructions. Briefly, 2.5 × 10^5 BHK-21 cells were seeded into each well of an eight-well chamber. Next day, cells were cotransfected with pXJ-Flag-NS4A (50–76) and pXJ-NS4B (84–146)-HA or with pXJ-Flag-NS4A (40–76) and pXJ-NS4B (84–146)-HA. Cells transfected with pXJ-Flag-NS4A (84–146)-HA alone were set as a negative control. At 48 h p.t., the cells were fixed by 4% paraformaldehyde in PBS for 30 min at room temperature and then permeabilized by 0.1% Triton X-100 in PBS for 10 min. After cells were blocked in PBS containing 1% FBS and 0.05% Tween 20 for 1 h, they were incubated simultaneously with two different primary antibodies (mouse anti-Flag MAb and rabbit anti-HA pAb) for 1 h. The following steps, including incubation with PLA probes, ligation, amplification, and preparation for imaging, were performed according to the manufacturer’s instructions. Images were acquired using a Leica DM4000 B microscope system equipped with 20× and 63× objectives. For each experiment, 15 images per well were acquired under the identical settings of the camera parameters. About 30 PLA-positive cells were randomly chosen, and the mean fluorescence intensity in each cell was quantified and calculated using Fiji image software. An unpaired two-tailed Student’s t test was performed in Prism, version 5, software.

**SDS-PAGE and Western blotting.** Proteins were separated on 12% Mini-Protein precast gels (Bio-Rad) or Bolt 4 to 12% Bis-Tris gels (Life Technologies) and transferred onto a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot Turbo Transfer System (Bio-Rad). The blots were blocked in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) supplemented with 5% skim milk for 1 h at room temperature, followed by 1 h of incubation with primary antibodies. After three washes with TBST buffer, the blots were incubated with secondary antibodies (goat anti-mouse or goat anti-rabbit antibodies conjugated to HRP or protein A conjugated to HRP in TBST buffer plus 5% milk for 1 h. After another three washes with TBST buffer, the blots were incubated with Amersham ECL Prime Western blotting detection reagent (GE Healthcare), Chemiluminescence signals were detected by exposing the blots to Kodak X-ray films or a C-DiGit blot scanner (Li-Cor).

**Resonance assignment.** Uniformly 13C/15N-labeled NS4A (17–80) was prepared using the aforementioned method and concentrated to 0.5 mM in a buffer containing 20 mM sodium phosphate, pH 6.5, 1 mM DTT, 200 mM DPC, and 10% D2O. All the NMR spectra were recorded at 313 K (3458 ppm) in a buffer containing 20 mM sodium phosphate, pH 6.5, 1 mM DTT, 200 mM DPC, and 10% D2O. The pulse programs were from the Topspin, version 2.1, program library. Data acquired were processed with NMRPipe (39) and analyzed using NMRView (40). Backbone resonance assignment was obtained based on the experiments including two-dimensional (2D)-1H-15N-heteronuclear single-quantum coherence (HSQC) and three-dimensional (3D) HNCA, HN(CO)CA, HN(CO)CACB, HNCO, and HBHAACONH experiments. Secondary structure was analyzed by analysis of 13C chemical shift (41) and by TALOS+ (42).

**Structural model of NS4A (17–80).** The structural model of NS4A (17–80) was generated by XPLOR-NIH (43) using mainly backbone dihedral angle restraints derived from TALOS+ (42). Structure determination was carried out using a randomized template. Simulated annealing was performed, and energy minimization was carried out as previously described (44). Simulated annealing was carried out with a starting temperature of 3,500 K and 15,000 K cooling steps. The structure was energy minimized with Powell energy minimization. One model out of 50 calculated ones was selected for presentation.

**RNA transcription, electroporation, and virus production.** Wild-type (WT) and mutant DENV-2 genome-length RNAs were transcribed *in vitro* using a T7 mMessage mMachine kit (Ambion) from cDNA plasmids prelinearized by XbaI. The RNA transcripts (10 µg) were electroporated into BHK-21 cells according to a protocol described previously (45). After electroporation, cells were immediately seeded on an eight-well chamber slide. At the indicated time points (see the details in Fig. 8), the cells were subjected to an immunofluorescence assay as described above. For virus production, the transfected cells were seeded in one T-75 flask. After incubation at 37°C for 24 h, the medium was replaced with fresh DMEM plus 2% FBS to remove extracellular input RNAs. Afterwards, the cells were maintained at 30°C for another 4 days. On every day from day 1 to day 5 p.t., 500 µl of culture fluids was collected, clarified by centrifugation at 415 × g for 5 min, and stored at −80°C. Virus titers were quantified by a standard cytopathic effect (CPE)-based plaque assay (46).

**Surrogate plaque assay by immunostaining.** A surrogate plaque assay was used to quantify the viruses that did not generate obvious plaques by the above standard plaque assay (46). In brief, 1.5 × 10^5 BHK-21 cells per well were seeded into a 24-well plate. On the next day, 100 µl of undiluted virus sample or of a series of 10-fold diluted virus samples was added to individual wells. After 1 h of incubation at 30°C with 5% CO2, the inoculum in each well was replaced with about 0.6 ml of overlay medium (RPMI 1640 medium plus 0.8% methylcellulose [Aquacide H; Merck, CA] and 2% FBS). After incubation at 37°C with 5% CO2 for 5.5 days, the cells were washed twice with PBS to completely remove the overlay medium, followed by fixation with 3.7% formaldehyde in PBS for 30 min. After that, cells were washed once with PBS and permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 10 min. After being blocked with PBS containing 1% FBS for 1 h at room temperature, cells were incubated with a mouse MAb against E protein (4G2) for 1 h, followed by three washes with PBST buffer. Afterwards, cells were incubated with a goat anti-mouse IgG conjugated to HRP (1:1,000 dilution) for 1 h. After three washes with PBST, cells were stained by DAB (3,3′-diaminobenzidine) Plus substrate kits (Life Technologies) according to the manufacturer’s instructions.

**RESULTS**

**Intracellular NS4A-NS4B interaction.** Three experiments were performed to demonstrate the NS4A-NS4B interaction in cells. First, we examined colocalization of NS4A and NS4B in DENV-2-infected BHK-21 cells using confocal microscopy. Both NS4A and NS4B showed similar ER staining patterns that extended throughout the cytoplasm, which resembled the distribution of NS3 in infected cells (Fig. 1A). Statistical analysis showed that the IFA signals of NS4A and NS4B significantly overlapped each other (Pearson coefficient of >0.5). This result confirms the previous reports that NS4A and NS4B colocalize with other viral proteins (e.g., NS3) and double-stranded RNA (dsRNA) at the viral replication site (19, 21).

Second, we performed a co-IP experiment to examine the NS4A-NS4B interaction in the context of viral infection. BHK-21 cells infected with DENV-2 were subjected to co-IP analysis in which mouse anti-NS4A MAB, mouse anti-NS4B MAB, or mouse IgG2b (negative control) was used for pulldown. As shown in Fig. 1B, anti-NS4A MAB pulled down NS4A protein together with NS4B and NS3 proteins but not capsid protein; in a reciprocal experiment, anti-NS4B MAB pulled down NS4B protein together with NS4A and NS3 proteins but not the capsid protein. In contrast, the negative-control mouse IgG2b could not pull down any viral proteins (capsid, NS3, NS4A, or NS4B). The results suggest that NS4A interacts with NS4B in virus-infected cells; such an interaction could be mediated through direct NS4A/NS4B binding or through an intermediate viral/host protein.

Third, we used a co-IP experiment to demonstrate NS4A-NS4B interaction in the absence of other viral proteins. HEK 293T cells were cotransfected with two plasmids: one plasmid expressing full-length NS4B with an N-terminal 2K signal peptide and a C-terminal EGFP tag (construct 2K-NS4B-EGFP) and another plasmid expressing full-length NS4A with a C-terminal Flag tag (construct NS4A-Flag). The 2K peptide was included in construct 2K-NS4B-EGFP to ensure the correct topology of NS4B; when 2K-NS4B-EGFP is expressed inside cell, a host protease cleaves 2K-NS4B-EGFP to generate 2K and
As shown in Fig. 1C, the EGFP antibody was able to pull down NS4B-EGFP together with NS4A-Flag (Fig. 1C, lane 3). In addition to NS4B-EGFP, a band corresponding to the molecular mass of EGFP was also detected; this band varied from experiment to experiment (see below), possibly representing a degradation product of NS4B-EGFP. In contrast, when EGFP was coexpressed with NS4A-Flag, the EGFP antibody pulled down EGFP but not together with NS4A-Flag (Fig. 1C, lane 2). As a control, NS4A-Flag alone did not bind to beads or EGFP antibody under the same co-IP conditions (Fig. 1C, lane 1). Next, we performed a reciprocal co-IP experiment using full-length NS4A with a C-terminal EGFP tag (construct NS4A-EGFP) and full-length NS4B with an N-terminal 2K signal peptide and an N-terminal HA tag (construct 2K-NS4B-HA). The EGFP antibody was able to pull down NS4A-EGFP together with 2K-NS4B-HA (Fig. 1C, lane 5). In contrast, the EGFP antibody could pull down EGFP but not NS4B-HA (Fig. 1C, lane 4). Taken together, the results suggest that NS4A interacts with NS4B in the context of viral replication or when it is expressed in the absence of other viral proteins.

**Interaction of recombinant NS4A and NS4B proteins.** An ELISA was used to examine the in vitro NS4A-NS4B interaction. Recombinant DENV-2 NS4A and NS4B proteins were expressed and purified from *E. coli*. SDS-PAGE analysis showed that both proteins reached purities of >90% (Fig. 2A). For NS4A proteins, besides a monomer (17.2 kDa), several SDS-resistant oligomer species (dimer or higher-order oligomers) were detected, suggesting that recombinant NS4A protein can oligomerize in vitro. This result is in agreement with a recent report that the DENV NS4A protein could oligomerize (20). For NS4B protein, two bands, representing a monomer (28 kDa) and dimer (56 kDa), were observed. The result is consistent with our previous result that NS4B could dimerize (36). Western blotting showed that antibodies against DENV-2 NS4A or NS4B could specifically detect various oligomeric forms of recombinant proteins (Fig. 2B). Next, the recombinant NS4A and NS4B proteins were used for an ELISA.
The ELISA plate was coated with NS4A proteins, blocked with 2% FBS, and incubated with serial dilutions of NS4B proteins. The captured NS4B was detected using an NS4B mouse mAb and a goat anti-mouse pAb conjugated to HRP (secondary Ab). The absorbance at a wavelength of 450 nm was measured. The averages and standard deviations of quadruplicates from one of three independent experiments are shown. The $K_d$ was estimated using the one-site specificity model in Prism, version 5.

**Amino acids 40 to 76 of NS4A are responsible for interaction with NS4B.** To define the regions that are responsible for the NS4A-NS4B interaction, we performed a co-IP assay using a series of truncated peptides of NS4A and full-length NS4B. Based on the topology of NS4A (Fig. 3A), we prepared a panel of C-terminally truncated NS4A peptides and tested their interaction with full-length NS4B. HEK 293T cells were cotransfected with two plasmids: one plasmid expressing a full-length 2K-NS4B-HA (construct NS4A-peptide-EGFP) and another plasmid expressing an NS4A peptide with a C-terminal HA tag (construct 2K-NS4B-HA) and another plasmid expressing an NS4A peptide with a C-terminal EGFP tag (construct NS4A-peptide-EGFP). As shown in Fig. 3B, an EGFP antibody pulled down various NS4A-peptide-EGFP constructs together with different amounts of 2K/NS4B-HA (representing both cleaved NS4B-HA and uncleaved 2K-NS4B-HA; see below). Similar levels of 2K/NS4B-HA were pulled-down by an NS4A-EGFP peptide consisting of residues 1 to 76 [NS4A (1–76)-EGFP], NS4A (1–101)-EGFP, NS4A (1–122)-EGFP, and NS4A (1–127)-EGFP (full-length NS4A). In contrast, only trace amounts of 2K/NS4B-HA were pulled down by NS4A (1–50)-EGFP and NS4A (1–60)-EGFP. Notably, when 2K-NS4B-HA was expressed in HEK 293T cells, two bands could be detected in the cell lysate and IP eluates (Fig. 3B to D; see Fig. 9A). This may be caused by an inefficient cleavage between 2K and NS4B that is mediated by a host signalase. For simplicity, these two bands were collectively labeled 2K/NS4B-HA. The cleavage efficiency at the 2K and NS4B junction varied between experiments (compare Fig. 1C with 3A to D), possibly due to the HEK 293T cells with different passage numbers which could affect the amount/efficiency of the host signalase. In addition, the variation in EGFP cleavage was observed for different NS4A peptides through an unknown mechanism. Nevertheless, the results demonstrate that deletion of residues 77 to 127 of the NS4A C terminus does not significantly affect the NS4A-NS4B interaction.

Next, we performed N-terminal deletion analysis of NS4A (Fig. 3C). The NS4A (40–127)-EGFP peptide and full-length NS4A-EGFP pulled down similar amounts of 2K/NS4B-HA, demonstrating that the N-terminal 39 residues of NS4A are not required for the NS4A-NS4B interaction. In contrast, the NS4A (50–127)-EGFP peptide almost lost its ability to pull down NS4B protein, suggesting that residues 40 to 50 of NS4A contribute to the NS4A-NS4B interaction. The results presented in Fig. 3B and C suggest that amino acids 40 to 76 of NS4A represent the minimal region required for NS4B interaction. To confirm this conclusion, we prepared two more NS4A-EGFP peptides (consisting of residues 40 to 76 and 50 to 76) and compared their abilities to bind NS4B (Fig. 3D). As expected, the NS4A (40–76)-EGFP peptide bound NS4B as efficiently as the full-length NS4A (1–127)-EGFP. In contrast, the NS4A (50–76)-EGFP peptide almost lost its binding to NS4B. The results clearly indicate that amino acids 40 to 76 of NS4A are responsible for the NS4A-NS4B interaction.

**Amino acids 84 to 146 of NS4B are required for interaction with NS4A.** We used an approach similar to that described above to define the region of NS4B required for NS4A interaction. In this case, HEK 293T cells were cotransfected with two plasmids: one plasmid expressing a full-length NS4A with a C-terminal Flag tag (construct NS4A-Flag) and another plasmid expressing truncated NS4B with a C-terminal EGFP tag (construct NS4B-peptide-EGFP). Based on the topology of NS4B (Fig. 4A), we first tested the effect of a C-terminal truncation of NS4B on the NS4A-NS4B interaction (Fig. 4B). For the C-terminally truncated NS4B-peptide-EGFP, the 2K sequence was retained at the N terminus of each construct to ensure a correct topology on ER membrane. As shown in Fig. 4B, full-length NS4B-EGFP consisting of residues 1 to 248 and the peptide consisting of residues 1 to 146 exhibited similar binding activities to NS4A-Flag; in contrast, the NS4B (1–130)-EGFP peptide almost lost NS4B-binding activity. These re-
Results suggest that C-terminal residues 147 to 248 of NS4B are not required for the NS4A-NS4B interaction.

Next, we prepared two NS4B-EGFP peptides (consisting of residues 84 to 146 and 130 to 165) to further map the region of NS4B required for the NS4A-NS4B interaction. We did not engineer the 2K sequence to the N termini of NS4B (84–146)-EGFP and NS4B (130–165)-EGFP. Based on the topology model of NS4B, NS4B (84–146)-EGFP should form the right topology on the ER membrane, and NS4B (130–165)-EGFP should locate in the cytosol. As shown in Fig. 4C, full-length NS4B-EGFP consisting of residues 1 to 248, and peptides consisting of residues 1 to 146 and 84 to 146 yielded equivalent levels of NS4A-binding activity; however, the NS4B (130–165)-EGFP peptide lost NS4A-binding activity (Fig. 4C). Collectively, the results indicate that residues 84 to 146 of NS4B are required for the NS4A-NS4B interaction.

NS4A (40–76) and NS4B (84–146) peptides are in close proximity when coexpressed inside cells. The above results prompted us to directly examine the interaction between the NS4A (40–76) and NS4B (84–146) peptides. Due to the small size of these peptides (<7 kDa), it is technically difficult to detect their interaction through fusion with small tags (such as HA and Flag) for co-IP and Western blot analysis. Initially, we attempted to overcome this difficulty by fusing the NS4A and NS4B peptides to bigger tags (EGFP and glutathione S-transferase [GST]). Unfortunately, we found that GST showed a strong nonspecific binding to EGFP in the absence of any NS4A and NS4B peptides when they were coexpressed in cells (data not shown), thus excluding the feasibility of this approach. As an alternative method, we used an in situ proximity of ligation assay (PLA) to examine the spatial proximity of NS4A/NS4B peptides intracellularly. PLA allows for detection of two molecules in proximity of ~40 nm in cells (47, 48). The PLA was performed by transfecting BHK-21 cells with two plasmids: one plasmid expressing the Flag-NS4A (40–76) peptide or Flag-NS4A (50–76) peptide (the Flag tag was at the N terminus of NS4A peptides) and another plasmid expressing the NS4B (84–146)-HA peptide (the HA tag was at the C terminus of the NS4B peptide). Figure 5A shows equivalent numbers of transfected cells expressing Flag-tagged NS4A and/or HA-tagged NS4B peptides, indicating that similar transfection efficiencies were achieved for different plasmids. The PLA showed very weak fluorescence signals (red) in cells transfected with Flag-NS4A (50–76) and NS4B (84–146)-HA (Fig. 5B, left panel). In contrast, cells transfected with Flag-NS4A (40–76) and NS4B (84–146)-HA produced about 4-fold stronger fluorescence signals (Fig. 5B and C). No fluorescence signal was detected in cells without plasmid transfection or in cells transfected with NS4B (84–146)-HA plasmid alone (data not shown). Overall, the PLA results suggest that the NS4A (40–76) and NS4B (84–146) peptides are in close proximity when coexpressed intracellularly.

Secondary structure of NS4A amino acid residues 17 to 80. We used NMR to analyze the structure of an NS4A fragment rep...
resenting amino acids 17 to 80 that included the mapped NS4B-binding site (residues 40 to 76), referred to as NS4A (17–80). NS4A (17–80) was selected from a series of N-terminal deletion peptides of NS4A (including the peptide consisting of residues 1 to 80); only the NS4A (17–80) peptide could be successfully expressed and purified and gave good NMR spectra for further study. Recombinant NS4A (17–80) was expressed in *E. coli* and purified in DPC micelles. The C-terminally (His)_6-tagged peptide was purified through a Ni^2+/-HCl column followed by gel filtration. SDS-PAGE analysis of the purified NS4A (17–80) showed a single band of 98% purity (Fig. 6A). The 1H-15N-HSQC spectrum of NS4A (17–80) was obtained. The dispersion of cross-peaks in the spectrum suggested the feasibility of analyzing the structure of NS4A (17–80) using NMR spectroscopy. The backbone resonances of NS4A (17–80) were assigned using 3D triple-resonance and nuclear Overhauser effect spectroscopy (NOESY) experiments. Almost complete backbone assignments for NS4A (17–80) in DPC micelles were obtained (Fig. 6B).

Secondary structure analysis was conducted using both the Cα chemical shift difference from random coil values and TALOS+ (Fig. 6C and D). NS4A (17–80) contains three helical fragments: helix α1 consists of residues 17 to 32, helix α2 contains residues 40 to 47, and helix α3 includes residues 52 to 75 and transverses membrane (Fig. 6C and D). Interproton NOE connectivity was plotted against residue number based on a 15N-HSQC-NOESY experiment (Fig. 6E). Although we did not observe a large number of NOE restraints, which might be due to the dynamic nature or the size of the protein/micelle complex, the observation of NOEs between the Hα of residue i and HN of residue i+3/4 suggests the presence of helical structures under the current experimental conditions. One structural model of NS4A (17–80) was generated using the XPLOR-NIH package with the dihedral angles restraints derived from TALOS+ (Fig. 6F). Helix α3 should be buried in the membrane, while helices α1 and α2 may interact with the ER membrane. Although the tertiary structure of NS4A (17–80) could not be determined due to the lack of long-range distance restraints, the secondary structural information is still useful for understanding the function of NS4A. A charge surface representation of the model of NS4A (17–80) was obtained and is shown in Fig. 6G. As expected, the transmembrane domain helix α3 is hydrophobic. The surface charges of helices α1 and α2 are very similar because both helices are amphipathic. Helix wheel representations of the three helices were generated to further understand their structures (Fig. 6H): helices α1 and α2 exhibit an amphipathic feature with one surface formed by hydrophobic residues and another surface formed by hydrophilic residues. The above
results are in general agreement with a previous report that the first 48 amino acids of DENV-2 NS4A form an amphipathic helix structure (20).

NMR analysis of the interaction between NS4A (17–80) and NS4B (84–147). With the resonance assignment of NS4A (17–80), we tested its interaction with a peptide corresponding to residues 84 to 147 of DENV-2 NS4B (strain NGC) (purity of 90%; synthesized by AAT Bioquest, Inc.). Figure 7A shows the \(^{1}H-^{15}N\)-HSQC spectra of NS4A (17–80) in the absence and presence of NS4B (84–147). In the presence of NS4B (84–147), line broaden-
ing of cross-peaks in the $^1$H-$^15$N-HSQC of NS4A (17–80) was observed, suggesting that the two peptides interact with each other in DPC micelles. Affected residues of NS4A (17–80) in the presence of NS4B peptide were mapped onto the structural model (Fig. 7B). In the HSQC spectrum, cross-peaks of residues (Fig. 7B, purple spheres), including Q19, A21, V30, A34, L48, L52, E53, T54, L56, L57, L61, A62, T63, T65, G66, G67, F69, L72, and G75, exhibited line broadening when NS4B peptide was added, suggesting that the interacting NS4A and NS4B peptides were undergoing intermediate exchange. Such intermediate exchange

FIG 6 Structural model of NS4A (17–80). (A) SDS-PAGE analysis of the purified DENV-2 NS4A (17–80) peptide. The peptide was separated by Bolt 4 to 12% Bis-Tris gels (Life Technologies) with 2-(N-morpholino)ethanesulfonic acid (MES) running buffer. (B, C, and D) $^1$H-$^15$N-HSQC spectra of NS4A (17–80) in DPC micelles. The assignment of $^1$H-$^15$N-HSQC spectra of NS4A (17–80) is shown in panel B. The cross-peaks are labeled with the residue names and numbers. Deviations of the observed $\chi_1$ chemical shift values from corresponding random coil chemical shift values are indicated in panel C. An amino acid with a positive value is helical in solution. The secondary structure analysis of NS4A (17–80) in DPC micelles was analyzed by TALOS$^+$ in panel D. Positive value is the possibility (0, 1) of the corresponding amino acid to be a helix. (E) NOE connectivity of NS4A (17–80) in DPC micelles. Residues in a helix exhibited H$\alpha$-$^1$H NOEs. (F) Ribbon representation of structural model of NS4A (17–80). The three helices are shown in different colors. The membrane is shown as a gold box. (G) Color-coded electrostatic surface potential for NS4A (17–80). Positive and negative potentials are shown in blue and red, respectively. (H) Three helix wheel representations of the three helices of NS4A (17–80). Hydrophobic residues such as Leu, Val, and Ile are highlighted in black. Positively charged residues, including His, Arg, and Lys, are highlighted in cyan. Residues Gln and Asn are highlighted in pink. Amino acids Gly and Ala with short side chains are highlighted in green. Ser and Thr are highlighted in gold. Asp and Glu are highlighted in red.

FIG 7 NMR study of the interaction between NS4A (17–80) and NS4B (84–147). (A) Superimposed $^1$H-$^15$N-HSQC spectra of NS4A (17–80) in the absence and presence of NS4B peptide. To test the binding ability between NS4B (84–147) and NS4A (17–80), the NS4B (84–147) peptide was directly added into the NS4A (17–80) solution. The $^1$H-$^15$N-HSQC spectra of 0.4 mM $^15$N-labeled NS4A (17–80) in the absence and presence of 1 mM NS4B peptide were recorded and compared. (B) Residues of NS4A that are affected by NS4B binding. Residues that showed line broadening upon addition of NS4B peptide are shown in purple spheres, and residues that showed chemical shift perturbation are highlighted in cyan spheres. This figure and similar ones were made using PyMOL (www.pymol.org). (C) Amino acid sequence alignment of region 40 to 76 in NS4A among four serotypes of DENV. The amino acid positions of NS4A are numbered according to the DENV-2 NGC strain (GenBank accession number AF038403). Identical (*), conserved (:), and semiconserved (·) residues are indicated. The residues for mutagenesis in this study are shaded, and their corresponding positions are labeled.
suggests that the binding affinity between NS4A and NS4B peptides might be in the range of micromolar to nanomolar, which is consistent with the ELISA binding $K_d$ value (Fig. 2). Cross-peaks of NS4A residues (Fig. 7B, cyan spheres), including K20, R22, A40, A44, and L60, exhibited chemical shift perturbation when NS4B peptide was added, suggesting that these residues may participate in the NS4A-NS4B interaction or that they are affected by the NS4A-NS4B interaction. Interestingly, most affected residues were located in the transmembrane domain, suggesting that the transmembrane helix $\alpha_3$ of NS4A is critical for interaction with NS4B. Only a few residues from the two amphipathic helices, $\alpha_1$ and $\alpha_2$, were affected by the addition of the NS4B peptide, again suggesting that the transmembrane helix $\alpha_3$ of NS4A is the main binding site with the NS4B (84–147) peptide.

**Functional analysis of NS4A mutations.** To characterize the biological relevance of the NMR-identified NS4A residues that may participate in the NS4A-NS4B interaction, we analyzed the conservation of these residues within NS4A (40–76) among the four serotypes of DENV. The NS4A (40–76) region was selected for such analysis because this region was defined to be essential for NS4B interaction (Fig. 3). As shown in Fig. 7C, 11 residues within NS4A (40–76) are identical among DENV-1 to DENV-4, including two alanine residues (A40 and A44) and four nonalanine residues (L48, E53, T54, L57, L60, T65, G67, F71, and G75).

To analyze the function of these amino acids, we mutated each of the 11 residues (Ala to Gly; non-Ala residues to Ala) in a DENV-2 genome-length RNA. The mutational effect on viral replication was examined. Equal amounts of mutant and WT RNAs were electroporated into BHK-21 cells. The transfected cells were examined for viral RNA expression by IFA (Fig. 8A), the plaque morphology of resulting virus (Fig. 8B), and virus production by plaque assay (Fig. 8C). The 11 mutants showed distinct levels of viral replication and could be divided into four groups. (i) Compared with WT, mutants F71A and G75A exhibited similar numbers of IFA-positive cells, plaque morphology, and viral yields. Sequencing the genomic RNAs of resulting viruses confirmed that the engineered mutations were retained without any extra mutations throughout their genomes. (ii) Mutants E53A, L57A, and G67A had reduced numbers of IFA-positive cells and decreased viral yields (especially on day 1 p.t.). The plaques formed by mutants L57A and G67A were smaller than those of the WT virus, as determined by a standard plaque assay. Interestingly, mutant E53A did not form plaques, as determined by a standard cytopathic effect (CPE)-based plaque assay, but could be detected using an immune-staining assay (Fig. 8B, lower panel). Sequencing analysis showed that mutant E53A, L57A, and G67A viruses kept the engineered mutations without extra mutations throughout the viral genomes. (iii) Mutants A40G and A44G generated few IFA-positive cells on days 3 to 4 p.t. and $<1,000$ PFU/ml virus. Mutant A40G formed smaller plaques than WT virus, whereas mutant A44G displayed plaques similar to those of the WT virus. Sequencing analysis showed that mutants A40G and A44G kept the engineered mutations without extra mutations throughout the genomic RNAs. (iv) Mutants L48A, T54A, L60A, and T65A generated no IFA-positive cells. No plaques or infectious viruses could be detected for the T54A or L60A mutants, $<40$ PFU/ml virus was produced for mutant L48A, and $<1,000$ PFU/ml virus was produced for mutant T65A. Sequencing the genomic RNAs of recovered viruses (collected on day 5 p.t.) showed that mutant L48A had changed to L48V and that mutant T65A reverted to the WT T65. Collectively, the results indicate that group iv mutations are lethal or severely defective in viral replication, that group i mutations have no effect on replication, and that group ii and iii mutations attenuate replication to different levels (but are not lethal).

**NS4A mutations affected NS4A-NS4B interaction.** We performed co-IP experiments to examine whether the replication defect of NS4A mutants is linked to NS4A-NS4B interaction. Based on the genome-length RNA mutagenesis results (Fig. 8), we chose group i mutants (with a WT replication level) and group iv mutants (with abolished or severely defective viral replication). We also included mutant G67A in the NS4A-NS4B interaction analysis because this mutation was recently shown to inhibit NS4A oligomerization (our published results). To analyze the NS4A-NS4B interaction, we cotransfected HEK 293T cells with two plasmids: one plasmid encoding WT full-length NS4B with an N-terminal 2K peptide and C-terminal HA tag (2K-NS4B-HA) and another plasmid encoding WT or mutant full-length NS4A with a C-terminal EGFP tag (NS4A-EGFP). As shown in Fig. 9A, an anti-EGFP MAb pulled down the WT and mutant NS4A-EGFP proteins with similar efficiencies; however, the co-IP efficiency of 2K/NS4B-HA differed among the various NS4A-EGFP mutants. Figure 9B quantifies the co-IP efficiency, with the WT NS4A/NS4B pulldown efficiency set at 100%. For group i mutants, E71A and G75A did not significantly change the NS4A/NS4B pulldown efficiency. For group iv mutants, L48A and T54A reduced the pulldown efficiency by 31% and 22%, respectively, whereas L60A increased the pulldown efficiency by 32%; statistical analysis suggests that the above differences are significant. However, T65A did not significantly change the pulldown efficiency. For mutant G67A (that affected NS4A oligomerization), the mutation did not change the pulldown efficiency. Collectively, the results provide a general correlation between the mutational effect on viral replication and NS4A-NS4B interaction.

**DISCUSSION**

The goal of this study is to characterize DENV NS4A-NS4B protein interaction. Three lines of evidence support the NS4A-NS4B interaction. (i) Confocal microscopy showed colocalization of NS4A/NS4B in virus-infected cells (Fig. 1A). NS4A and NS4B proteins could pull down each other using infected cell lysates (Fig. 1B). (ii) NS4A and NS4B could pull down each other when the two proteins were transiently expressed in cells in the absence of other viral proteins (Fig. 1C). (iii) Recombinant NS4A and NS4B proteins interact with each other in an ELISA (Fig. 2C). The NS4A-NS4B interaction characterized in this study agrees with a previous report that flavivirus NS4A genetically interacts with NS4B (30). In that study, a chimeric virus of DENV-1 containing amino acids 27 to 34 of JEV NS4A was defective in replication; an adaptive mutation in TMD3 (T109I or L113F) of NS4B could restore the replication defect through enhancing viral RNA synthesis. Since the introduced mutations (residues 27 to 34) are located upstream of the NS4A region (residues 40 to 76) that is required for interaction with NS4B, mutations at residues 27 to 34 may affect the conformation of the NS4A (40–76) region, leading to a change in the NS4A-NS4B interaction; the adaptive mutation in NS4B TMD3 (T109I or L113F) may restore the NS4A-NS4B interaction and therefore compensate for viral replication. However, a recent study suggested that WNV NS4A did not interact with NS4B when the proteins were coexpressed in Vero cells (49).
The discrepancy could be due to the different detection methods (fluorescence resonance transfer and biologic fluorescence complementation) used in the WNV study; alternatively, the discrepancy could reflect a genuine difference in NS4A-NS4B interactions between WNV and DENV.

A monoclonal NS4B antibody (clone 44-4-7) was used to detect recombinant NS4B protein binding with NS4A in an ELISA (Fig. 2C). The epitope of antibody 44-4-7 was previously mapped to residues 141 to 147 of NS4B (34). In the current study, amino acids 84 to 146 of NS4B were found to be required for binding with...
NS4A. If residues 141 to 147 are already involved in binding with NS4A, how could the NS4B antibody detect the interaction in the ELISA? To reconcile the above observations, we speculate that residues 141 to 147 of NS4B might not be directly involved in the NS4A/NS4B binding; however, the presence of these residues is required for the correct conformation of the first transmembrane domain of NS4B (101–129) that directly participates in the NS4A-NS4B interaction. Therefore, when NS4B is bound to NS4A, residues 141 to 147 of NS4B remain available for binding to the NS4B antibody 44-4-7. Future experiments are needed to validate the above speculation.

In addition to the DENV-2 NS4A-NS4B interaction, recent studies showed that DENV-2 NS4A or NS4B alone could oligomerize (20, 36). The latter findings were confirmed by the current result that recombinant NS4A of DENV-2 formed high-order oligomers and that recombinant NS4B formed dimers on SDS-PAGE gels (Fig. 2A). These results raised the question of which oligomeric form of NS4A and NS4B participates in the NS4A-NS4B interaction. The current study has defined residues 40 to 76 of NS4A and residues 84 to 146 of NS4B to be the minimal regions required for the DENV-2 NS4A-NS4B interaction (Fig. 3 and 4). For NS4A, we recently found that residues 50 to 76 form the major determinant for DENV-2 NS4A oligomerization (our published results); this region is within residues 40 to 76 of NS4A that are required for the DENV-2 NS4A-NS4B interaction, suggesting that NS4A could not simultaneously form oligomers and interact with NS4B protein. Therefore, only the monomer of DENV-2 NS4A could bind to NS4B. For NS4B, the cytosolic loop (residues 129 to 165) and the C-terminal region (residues 166 to 248) are responsible for DENV-2 NS4B dimerization (36). NS4B cytosolic loop residues 129 to 146 are required for both DENV-2 NS4B dimerization and NS4A-NS4B interaction. Since C-terminal residues 166 to 248 alone of NS4B are sufficient for dimerization (36), NS4B could dimerize through residues 166 to 248 of its C terminus and simultaneously interact with NS4A through its residues 84 to 146. Thus, both the monomeric and dimeric forms of NS4B of DENV-2 could bind to NS4A.

Spatial and temporal regulation of various molecular complexes is required for a productive viral infection cycle. NS4A oligomerization, NS4B dimerization, and NS4A-NS4B interaction could occur at different steps of viral replication. The transition between homo-molecular oligomerization and hetero-molecular interactions could modulate the switch from one replication step to another. For instance, NS4A is known to be responsible for membrane modulation and is an essential component of the viral replication complex. During an early stage of DENV infection, NS4A may predominantly oligomerize; the oligomerization of NS4A induces membrane curvature to generate vesicle packets for replication complex formation. Once the vesicle packets are produced, the newly synthesized NS4A molecule no longer oligomer-
izes and predominantly exists in the monomeric form; alterna-
tively, NS4A molecules may dissociate from oligomers to
monomers. The abundance of NS4A and NS4B proteins at early
time points after infection could be a driving force for the
switch of NS4A oligomer to monomer; the monomeric NS4A
recruits NS4B (in the form of either monomer or dimer) to the
recruitment complex. The recruitment of NS4B to NS4A can be
efficient because of the higher affinity of NS4A/NS4B binding, with a Kd of 50 nM (Fig. 2C). Experiments are needed to validate this working model.

NMR analysis showed that NS4A (17–80) contains three heli-
cel fragments: helix α1 consists of residues 17 to 32, helix α2 con-
tains residues 40 to 47, and helix α3 includes residues 52 to 75 (Fig.
6C to F). The existence of helix α2 explains on a structural level
why deletion of residues 40 to 49 from NS4A (40–76) severely
reduced its ability to bind to NS4B (Fig. 3D). The amphipathic helix α2 interacts with the cytosolic side of the ER membrane and coordinates with the transmembrane helix α3 to interact with NS4B. Indeed, upon addition of the NS4B (84–147) peptide, dis-


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