The Non-structural Protein 4A of Dengue Virus Is an Integral Membrane Protein Inducing Membrane Alterations in a 2K-regulated Manner*

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Dengue virus (DV) is a positive sense RNA virus replicating in the cytoplasm in membranous compartments that are induced by viral infection. The non-structural protein (NS) 4A is one of the least characterized DV proteins. It is highly hydrophobic with its C-terminal region (designated 2K fragment) serving as a signal sequence for the translocation of the adjacent NS4B into the endoplasmic reticulum (ER) lumen. In this report, we demonstrate that NS4A associates with membranes via 4 internal hydrophobic regions, which are all able to mediate membrane targeting of a cytosolic reporter protein. We also developed a model for the membrane topology of NS4A in which the N-terminal third of NS4A localizes to the cytoplasm, while the remaining part contains three transmembrane segments, with the C-terminal end localized in the ER lumen. Subcellular localization experiments in DV-infected cells revealed that NS4A resides primarily in ER-derived cytoplasmic dot-like structures that also contain dsRNA and other DV proteins, suggesting that NS4A is a component of the membrane-bound viral replication complex (RC). Interestingly, the individual expression of DV NS4A lacking the 2K fragment resulted in the induction of cytoplasmic membrane alterations resembling virus-induced structures, whereas expression of full-length NS4A does not induce comparable membrane alterations. Thus, proteolytic removal of the 2K peptide appears to be important for induction of membrane alterations that may harbor the viral RC. These results shed new light on the role of NS4A in the DV replication cycle and provide a model of how this protein induces membrane rearrangements and how this property may be regulated.

Dengue virus (DV), a member of the family Flaviviridae, is the causative agent of dengue fever, the most prevalent arthropod borne viral illness in humans. More than 50–100 million cases of dengue fever are reported annually in the tropical and subtropical regions of the world, where the mosquito vectors Aedes aegypti and Aedes albopictus are endemic. Of these patients, ~1% develop more severe and often lethal symptoms called dengue hemorrhagic fever and dengue shock syndrome (1).

Flaviviruses are enveloped viruses that contain a single-stranded RNA genome of positive polarity ~11 kb in length. This RNA contains a type I cap at its 5'-terminus (2) and is translated by the host cell translation machinery in close association with cellular membranes, giving rise to the 3391-amino acid long polyprotein. The structural components of the virus (proteins C, prM, and E; see Fig. 1A) are encoded in the 5’ one-fourth of the genome, whereas the non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5; see Fig. 1A) are encoded in the remainder of the genome (3). Processing of the polyprotein into the individual components is carried out by a combination of different host cell enzymes and the cytoplasmic viral NS2B-3 protease complex (4–6). In addition to its protease activity NS3 also contains an RNA helicase and nucleotide triphosphatase activity important for the replication of the viral RNA (7,8), which seems to occur in close association with virus-induced cellular membrane structures designated viral replication complexes (RCs) (9, 10). Most of the other NS proteins seem to be directly or indirectly involved in viral RNA replication as well. NS5 is a multifunctional protein containing an RNA-dependent RNA polymerase (RdRp) activity and a methyltransferase activity involved in capping of progeny viral RNA genomes (11, 12). The role of NS1 in viral replication is not understood as yet, but it has been reported that mutations in NS1 affected the initiation of minus-strand RNA synthesis (13, 14).

Not much is known about the functions of the hydrophobic flavivirus proteins NS2A, NS4A, and NS4B. It has been suggested that they contribute to the inhibition of the interferon-α/β (IFN-α/β) response (15–18). Furthermore, NS2A may target the viral RC to membrane organelles (9), and NS4B may modulate viral replication via an interaction with NS3 (19).

Thus far, the small hydrophobic protein NS4A (~16 kDa) is only poorly characterized, and its proper function for the viral infection–mediated membrane rearrangements remains unresolved. The 2K fragment of NS4A is a component of the membrane-bound viral replication complex (RC); RdRp, RNA-dependent RNA polymerase; IFN-α/β, interferon-α/β; KUNV, Kunjin virus; YFV, Yellow Fever virus; ER, endoplasmic reticulum; DMEM, Dulbecco’s modified Eagle’s medium; eGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; h p.i., hours postinfection; dsRNA, double-stranded RNA; EM, electron microscopy; LD, low density; HD, high density; TMS, transmembrane segment; pTMS, predicted transmembrane segment; SFV, Semliki Forest Virus; DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride; Pipes, 1,4-piperazineethanesulfonic acid; IFM, immunofluorescence microscopy.
Characterization of DV NS4A

replication cycle is still unknown. The observations that Kunjin virus (KUNV) NS4A localizes to the presumed sites of RNA replication and polyprotein processing (9) and that an interaction between NS4A and NS1 is required for RNA replication (20) suggest that flavivirus NS4A is involved in some steps of viral RNA amplification, perhaps by anchoring replicase components to intracellular membranes.

The N terminus of NS4A is generated in the cytoplasm by the viral two-component protease NS2B-3, whereas the C-terminal 23 amino acid residues of NS4A seem to act as a signal sequence for the translocation of NS4B into the lumen of the ER (see Fig. 1A). This signal sequence (designated the 2K fragment) is removed from the N terminus of NS4B by the host signalase in the ER lumen. Signalase cleavage at the 2K-4B site requires a prior NS2B-3 protease-mediated cleavage at the so called 4A/2K site just N-terminal of the 2K fragment (21). Recently, it was reported that the KUNV NS4A-2K precursor can induce intracellular membrane rearrangements, which may form the scaffold for the viral RC (22). However, it is not yet clear how NS4A associates with cellular membranes, or how it induces membrane rearrangements. Answering these questions requires characterization of the NS4A membrane topology and intracellular targeting.

In this report, we investigated the subcellular localization of DV NS4A and characterized both its membrane association and membrane topology in detail. Our experiments clearly show that DV NS4A is part of the viral RC and colocalizes with other viral proteins and RNA. We developed a model of the NS4A membrane topology, which could explain how this protein induces membrane alterations in a regulated manner.

EXPERIMENTAL PROCEDURES

Cells and Viruses—Cell monolayers of the human hepatoma cell line Huh-7 (23) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 2 mM l-glutamine, non-essential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (DMEM complete). Huh-7/T7 cells, constitutively expressing the T7-RNA-polymerase (24), were grown under the manufacturer’s protocol. Huh-7/T7 cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS. T7 RNA polymerase was added to the medium. After 16 h, the medium was changed to fresh medium and the cells were incubated for 4 h at 37 °C with occasional rocking. After 4 h, the inoculum was replaced by complete DMEM, and cells were incubated for various times.

Transfection—DNA constructs were transfected into Huh-7/T7 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s protocol.

Antibodies—The following primary antibodies were used: mouse monoclonal anti-dsRNA antibody (English & Scientific Probes, Eugene, OR), mouse monoclonal anti-DV2 E-antibody (ATCC), and mouse monoclonal anti-GFP antibody (Roche Applied Science, Mannheim, Germany). The secondary antibodies used in immunofluorescence microscopy were goat antibodies conjugated to AlexaFluor 546 and AlexaFluor 488 (Molecular Probes). Cellular DNA was stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes). The secondary antibodies used in Western blot analyses were mouse or rabbit secondary antibodies coupled to horseradish peroxidase (Sigma). For the generation of the anti-NS4A antiserum New Zealand White Rabbits were immunized with a mixture of synthetic DV NS4A-specific peptides over a period of 3 months. For immunofluorescence studies, the obtained monospecific polyclonal antisera were purified by affinity purification as described previously (25).

Plasmid Constructs—Standard molecular biology techniques were used for cloning (26). All plasmids were confirmed by restriction and sequence analysis using an ABI 310 sequencer and the Big Dye terminator cycle sequencing kit version 1.1 (Applied Biosystems, Foster City). The full-length DV2 NGC strain cDNA (a kind gift from Andrew Davidson, School of Medical Sciences, Bristol, UK) served as a template for polymerase chain reaction (PCR)-based amplification of the required DV genes. A previously described modified version of pTM-eGFP (25) served as the vector for the generation of pTM-NS4A-eGFP fusion constructs expressing different NS4A fragments fused to the enhanced green fluorescent protein (eGFP).

The restriction sites NcoI and SpeI were used for the insertion of PCR fragments encoding for the different NS4A regions. For the construction of pTM-eGFP-glyc encoding an N-glycosylation acceptor site (Asn-Ser-Thr-Ser-Ala) fused in-frame to the C terminus of eGFP, eGFP-glyc was amplified by PCR using the modified pTM-eGFP as a template, the sense primer 5′-CAC-GATAAATACATGGCACGGC-3′, and the antisense primer 5′-TGCTAGTACGTTAGCGAGGTCAATCTTGTACAGCTCGTCTACATGCCG-3′. The obtained PCR fragment was digested with Agel and SpeI and inserted into pTM-eGFP digested with the same restriction enzymes. PCR fragments encoding for the different NS4A regions were amplified using appropriate oligonucleotides and inserted into pTM-eGFP via the restriction sites NcoI and Agel. For the generation of pTM expression constructs encoding for NS4A either containing or lacking the 2K sequence the corresponding regions were amplified by PCR and inserted into pTM (27) using the restriction sites NcoI and SpeI.

Microscopy—Confocal fluorescence microscopy was performed as described previously (25). For cryoimmuno-EM transfected Huh-7/T7 cells were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in PHEM buffer (240 mM Pipes, 100 mM Hepes, 8 mM MgCl2, 40 mM EGTA; pH 6.9). Cells were scraped off the dish, pelleted, and incubated in 2.3 M sucrose overnight at 4 °C. Subsequently, cell pellets were mounted on silver pins, flash-frozen, and stored in liquid nitrogen. The specimens were sectioned with a Reichert Ultracut S ultramicrotome with a Reichert FCS cryoattachment using a Diatome diamond knife (Diatome, Biel, Switzerland). Immunocytochemical labeling of thawed cryosections was performed as
**Characterization of DV NS4A**

**Metabolic Labeling, Immunoprecipitation, and Deglycosylation**—Four hours after transfection of 5 × 10^5 Huh-7/T7 cells, they were washed, incubated for 30 min in DMEM without methionine and cysteine, and subsequently incubated for 3–6 h in the same medium containing 100 μCi of [35S]methionine/[35S]cysteine per ml (Expre35S35S protein labeling mix; PerkinElmer, Boston). Cells were then washed two times with PBS, and eGFP-fusion proteins were isolated by immunoprecipitation from cell lysates. In brief, cells were lysed with 1 ml of NPB (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride), centrifuged for 15 min at 20,000 × g, and supernatants were incubated overnight with a polyclonal rabbit anti-GFP antibody coupled to protein-G-Sepharose beads. After several washes with NPB, deglycosylation was performed with a fraction of the immunocomplex using PNGase F (New England Biolabs, Schwalbach, Germany) as recommended by the manufacturer. Beads were resuspended in protein sample buffer, boiled 5 min at 95 °C, and proteins were separated by SDS-PAGE and analyzed by autoradiography.

**Protein Sequence Analyses and Transmembrane Segment (TMS) Prediction**—Protein sequence analyses were performed with the following programs: ConPredII, TMAP, TMHMM 2.0, TMpred, and SOSUI.

**RESULTS**

**DV NS4A Localizes to the Putative Sites of Viral RNA Replication**—As a first step in the characterization of DV NS4A, we determined its subcellular localization in infected Huh-7 cells at different time points postinfection (p.i.) by using confocal immunofluorescence microscopy (IFM) and a newly developed DV NS4A-specific peptide antibody (see “Experimental Procedures”). No fluorescence was observed by staining uninfected cells, confirming the specificity of the antiserum (Fig. 1B, panel d). At 14 h p.i. NS4A localizes to cytoplasmic reticular structures that were mainly enriched in the perinuclear region (Fig. 1B, panel a), whereas at 24 h p.i. a dot-like staining pattern was observed in the cytoplasm of the infected cells (Fig. 1B, panel b). A similar pattern was found at 38 h p.i. with a clearly increased number of cytoplasmic foci (Fig. 1B, panel c).

The observed distribution of DV NS4A bears resemblance to the previously described distribution of E, NS3, and NS4B in DV-infected cells (19, 25). These proteins appear to be localized within or in close proximity to the viral RCs. We therefore determined whether NS4A also localizes to these cytoplasmic structures by using colocalization experiments. At 24 h p.i. we described by Griffiths et al. (28). EM specimens were examined using a Zeiss 10C transmission electron microscope.

**Membrane Floatation and Membrane Dissociation Assay**—Cells were transfected or infected as described above. At 8-h post-transfection or 24-h postinfection, cells were harvested, and membrane flotation or dissociation assays were performed as described previously (25). In brief, Nycodenz (Axis Shield, Oslo, Norway) was added to postnuclear cell lysates to a final concentration of 37.5% (w/v), loaded under a 5% to 35% Nycodenz discontinuous gradient, and centrifuged to equilibrium in a Beckmann TLS-55 swing-bucket rotor at 100,000 × g for 20 h. After centrifugation, fractions were collected from the top to the bottom of the density gradient. Equal volumes of samples from each fraction were separated by SDS-PAGE and immunoblotted as described above. For membrane dissociation assays, postnuclear lysates were centrifuged at 20,000 × g for 10 min to pellet membranes and their associated proteins. Pellets were resuspended in phosphate-buffered saline (PBS), 1 mM NaCl, 0.1 mM Na₂CO₃, pH 11.5, or 1% Triton X-100, incubated on ice for 30 min, and fractionated in Nycodenz gradients as described above.

**Western Blot Analysis**—Samples were loaded onto a 12% polyacrylamide-SDS gel, and proteins were electrophoretically separated. After transfer of proteins to nitrocellulose membrane, blots were incubated in blocking solution (5% milk powder and 0.5% Tween 20 in PBS) overnight at 4 °C. Incubation with the primary antibody was performed in blocking solution for 1 h at room temperature. Blots were washed three times for 10 min in washing solution (0.5% Tween 20 in PBS), incubated for 1 h with the secondary antibody in blocking solution, and washed again in the same way. Antibody-protein complexes were detected using the ECL™ plus Western blotting detection system (Amersham Biosciences).
detected NS4A and E in exactly the same cytoplasmic foci, arguing that NS4A could be a component of the viral RC (see Fig. 2, panels p–r). This assumption was corroborated by the fact that the staining pattern observed for NS4A overlapped completely with the dot-like staining pattern of dsRNA (Fig. 2, panels j–l) that is formed as an intermediate of viral RNA replication. dsRNA also colocalizes with the ER marker calnexin in DV-infected cells (Fig. 2, panels m–o), which was otherwise found in cytoplasmic reticular structures in non-infected cells (Fig. 2, panels d–f). These results show that DV2 infection of Huh-7 cells leads to a redistribution of the ER, which did not affect the cis- and trans-Golgi (Fig. 2, panels s–x).

In conclusion, we found that DV NS4A colocalizes with dsRNA and viral proteins to the sites of RNA replication in infected cells. These putative membrane-bound RCs most likely are ER-derived or reside in very close proximity to ER membranes.

**DV NS4A Lacking the 2K Sequence Induces Cytoplasmic Dot-like Structures**—The C-terminal 23 residues of NS4A encode for a signal sequence important for the translocation of NS4B into the ER lumen (21). While this work was in progress, it was reported that expression of uncleaved KUNV NS4A-2K induces membrane rearrangements resembling KUNV-induced structures. Removal of the 2K domain significantly reduced membrane rearrangement and resulted in the redistribution of NS4A to the Golgi apparatus (22).

To analyze if also DV NS4A is able to induce dot-like structures we investigated its subcellular localization in cells expressing a full-length DV NS4A-eGFP fusion protein by using confocal IFM. In the wild-type NS4A-2K-4B precursor, prior processing at the 4A-2K site by the viral NS2B-3 protease is required before the host cell signalase can cleave at the 2K-4B site. To make sure that NS4A in the NS4A-eGFP fusion protein is not cleaved off from the C-terminal reporter by the host signalase, we constructed a fusion protein in which the 2K-4B signalase cleavage site was mutated (designated NS4Amut-eGFP). The comparison of NS4A-eGFP and NS4Amut-eGFP expressed in transfected cells by Western blot analyses revealed that with both proteins the reporter was not cleaved off from the NS4A fusion partner (data not shown). Thus, we used the more authentic NS4A-eGFP protein for all further analyses.

When eGFP was expressed alone in Huh-7/T7 cells it was distributed rather evenly throughout the cell (Fig. 3, panel a). However, when eGFP was fused to the C or N terminus of NS4A, the subcellular localization of eGFP was altered compared with overexpression of eGFP alone, but both fusion proteins exhibited a comparable reticular staining pattern with some enrichment in the perinuclear region (see Fig. 3, panels b and c). The same reticular staining pattern was found in cells analyzed at later time points post-transfection and also with HA- or Myc-tagged NS4A-proteins (data not shown), excluding the possibility that the observed distribution is an artifact because of the fusion to eGFP. Colocalization experiments with cellular marker proteins revealed that NS4A-eGFP colocalized perfectly with the ER marker calreticulin (Fig. 3, panels d–f). However, in contrast to cells transfected with KUNV NS4A (22), no membrane rearrangements were visible in DV NS4A-eGFP-expressing cells.

Because 2K is cleaved off from NS4A during polyprotein processing, we wanted to analyze the subcellular localization of mature NS4A lacking the 2K domain. To this end, we constructed a plasmid encoding for the N-terminal 127 residues of DV NS4A fused to eGFP and analyzed its localization in transfected cells. Surprisingly, the reticular staining pattern was observed very rarely (in less than 5% of transfected cells) and instead nearly all NS4A(-2K)-eGFP was detected in distinct dot-like structures distributed throughout the cytoplasm (see Fig. 3, panel g). The same structures were also found by using an
antibody directed against the ER protein calreticulin, indicating that the expression of NS4A lacking the 2K domain induced rearrangement of ER membranes (Fig. 3, panel h). In contrast, distribution of the trans-Golgi marker p230 and the cis-Golgi marker GM130 was not affected (Fig. 3, panels j–o). The different staining patterns observed in cells expressing NS4A-eGFP and NS4A(-2K)-eGFP were not due to different expression levels of these two fusion proteins as determined by Western blot analyses (data not shown). To make sure that the fusion of eGFP to the C terminus of NS4A(-2K) does not alter the true localization and function of the viral protein, we also constructed an expression plasmid encoding for NS4A(-2K) with an N-terminal eGFP fusion. Cells transfected with this construct showed the same dot-like structures as observed for NS4A(-2K) with the C-terminal eGFP fusion (data not shown). Therefore we concluded that the induction of dot-like structures by NS4A(-2K) is not an artifact of the fusion to eGFP.

For a more detailed analysis of the morphological changes induced in DV NS4A(-2K)-eGFP-expressing cells, immunoelectron microscopy (EM) studies were performed. As expected from the IFM, full-length NS4A was found nearly exclusively in cisternal structures reminiscent of the ER (Fig. 4A, panel a). In

![Figure 3](image-url) **FIGURE 3.** Only NS4A-eGFP lacking the 2K fragment induces cytoplasmic dot-like structures originating from the endoplasmic reticulum. Huh-7/T7 cells were transfected with pTM-eGFP (panel a), pTM-NS4A-eGFP (panels b and d–f), pTM-eGFP-NS4A (panel c), or pTM-4A(-2K)-eGFP (panels g–o). At 8-h post-transfection, cells were analyzed by IFM with a GFP-specific antibody (panels a, d, g, and j, m), and antibodies specific for different cellular marker proteins specified in the lower left of each picture (panels e, h, k, and n). Merged pictures are shown on the right (panels f, i, l, and o). Nuclear DNA was stained with DAPI.

![Figure 4](image-url) **FIGURE 4.** Evidence that fully processed NS4A is responsible for inducing membrane alterations. A, EM analysis of Huh-7/T7 cells expressing DV NS4A-eGFP or NS4A(-2K)-eGFP. Huh-7/T7 cells were transfected with pTM-eGFP (panel a) or NS4A(-2K)-eGFP (panel b) and fixed at 8-h post-transfection. Thawed cryosections of transfected cells were labeled with a GFP-specific antiserum, followed by 10-nm protein A-gold. Full-length NS4A is associated with cisternal structures reminiscent of the ER, whereas NS4A lacking the 2K fragment induces intensive cytoplasmic membrane rearrangements. Bars, 200 nm. B, Huh-7 cells were infected with DV2 (lane 2) or left uninfected (lane 1). In parallel, Huh-7/T7 cells were transfected with pTM constructs encoding for NS4A(-2K) or full-length NS4A, respectively (lanes 3 and 4). 24 h.p.i. or 8-h post-transfection, cells were lysed, and Western blot analysis was performed using the NS4A-specific peptide antiserum.
Characterization of DV NS4A

contrast, NS4A(-2K) was concentrated in large accumulations of intracellular membranes (Fig. 4A, panel b) that were not visible in cells transfected with full-length NS4A. These structures were often found to be surrounded or adjacent to the ER (not shown). A minor portion of NS4A(-2K) was also detectable in ER- and intermediate compartment-derived structures (data not shown). In agreement with the IFM (Fig. 3) these results confirm that expression of DV NS4A lacking its C-terminal 2K fragment is able to induce the formation of cytoplasmic membrane alterations that are likely ER-derived because they contain calreticulin (as seen by IFM). However, in full-length NS4A, the C-terminal 2K domain appears to block the induction of these membrane rearrangements.

Fully Processed DV NS4A Predominates in Infected Cells—Our microscopic studies using recombinant DV NS4A indicate that NS4A is able to induce membrane alterations only after removal of the C-terminal 2K fragment. To investigate whether unprocessed NS4A-2K or processed NS4A lacking the 2K peptide is the predominant species in infected cells, lysates of cells infected with DV2 were analyzed by NS4A-specific Western blot. For comparison, NS4A either containing or lacking 2K was analyzed in parallel on the same gel. The size of NS4A in unprocessed NS4A-2K or processed NS4A lacking the 2K peptide is the predominant species in infected cells, lysates of cells infected with DV2 were analyzed by NS4A-specific Western blot. For comparison, NS4A either containing or lacking 2K was analyzed in parallel on the same gel. The size of NS4A in transfected cells DV NS4A was found exclusively in the LD fractions, indicating its association with cellular membranes (Fig. 5, A, C, and D).

To investigate the membrane association of NS4A in more detail, its floatation behavior was examined under conditions designed to remove peripherally associated proteins (29). To this end, postnuclear lysates of cells transfected with the corresponding plasmid were centrifuged at 20,000 × g, and pelleted membranes and their associated proteins were resuspended in PBS, or 1 M NaCl, or 0.1 M Na2CO3 (pH 11.5), or Triton X-100, and fractionated in floatation gradients as described above. Calnexin, used as a control for an integral protein, remained in the membrane fraction even after treatment with 1 M NaCl and 0.1 M Na2CO3 (pH 11.5), whereas most of it was found in the high density (HD) fraction after disruption of the membranes with Triton X-100 (Fig. 5A). In contrast to calnexin, a minor fraction of calreticulin, a nonmembrane protein of the ER, was found in the HD fractions already after NaCl treatment, and this amount increased dramatically after incubation with Na2CO3 (Fig. 5B). Using this assay for the investigation of DV NS4A expressed in infected or transfected cells we found that it remained in the LD fractions after these treatments (Fig. 5, C and D). These results show that NS4A is tightly associated with cellular membranes, most likely because of membrane insertion.

It was reported previously that the last 23 residues of NS4A (the 2K fragment) serve as a signal sequence for the translocation of NS4B into the lumen of the ER. This 2K peptide is cleaved at the N terminus by the NS2B-3 protease on the cytosolic side of the ER membrane, whereas the C terminus is generated by the host cell signalase localized in the ER lumen (21). Thus, 2K has to span the ER membrane and could therefore serve as a membrane anchor of NS4A. To address this assumption we investigated whether NS4A is also tightly membrane-associate d in the absence of 2K by using membrane dissociation assays and flotation gradient analysis of cell lysates containing the NS4A-eGFP fusion protein lacking the 2K sequence. As depicted in Fig. 5E NS4A(-2K)-eGFP sedimented to HD only after pretreatment of lysates with detergent. Thus, NS4A is tightly associated with membranes even in the absence of the 2K fragment, most probably because of at least one additional membrane-spanning region.
Protein Sequences Involved in Membrane Association of NS4A—To find out which regions of DV NS4A are responsible for membrane association, we first developed a consensus model for the potential transmembrane topology of NS4A by *in silico* analysis (Fig. 6, A and B). No TMS was predicted in the region comprising the N-terminal 49 residues, which also has the lowest hydrophobicity. For the rest of the protein, 4 TMSs were predicted at approximately the same positions by all but one program. To discriminate between predicted and experimentally proven TMSs, the first ones are designated potential transmembrane segments (pTMSs) throughout this report.

For experimental evaluation of membrane targeting, we performed deletion mapping analyses of DV NS4A. Plasmids encoding for the individual pTMSs fused in-frame to eGFP (Fig. 6B) were transiently expressed in Huh-7/T7 cells, and 8 h post-transfection, cells were fixed and immunostained with an anti-eGFP antibody (Fig. 6C). As expected, eGFP expressed on its own was distributed evenly throughout the cell. The same staining pattern was observed for the fusion protein containing the N-terminal 49 residues of NS4A, indicating that this part of the protein does not contain membrane-targeting sequences. In contrast, the fusion of each of the other 4 individual pTMs to eGFP led to a reticular staining pattern in the cytoplasm of the transfected cells sometimes enriched in the perinuclear region, indicating that all 4 pTMSs are able to mediate membrane association.

Membrane interaction of the eGFP fusion proteins was characterized in more detail using equilibrium density gradient centrifugation experiments of postnuclear lysates of cells transfected with the corresponding plasmids (Fig. 6D). Exclusive accumulation in the HD fraction was found with eGFP, and the fusion protein expressing the first 49 residues of NS4A fused to GFP, supporting the results of the IFM analyses. As expected, eGFP fused to either pTMS 1 (residues 49–74), pTMS 2 (residues 100–127), or pTMS 3 (residues 100–127) was enriched in the membrane fractions confirming the membrane-targeting capabilities of the fused NS4A fragments. However, the fusion protein comprising pTMS 4 (H11005; residues 127–150) was enriched primarily in the HD fractions. These results suggest that pTMS 4 expressed on its own is able to mediate membrane association of eGFP, but much less efficiently as compared with pTMS 1, pTMS 2, and pTMS 3.

To investigate the stability of membrane association of the eGFP fusion proteins membrane dissociation assays were performed as described under “Experimental Procedures.” Membrane pellets of cells transfected with the corresponding plasmids were either left untreated or treated with 1 M NaCl or 0.1 M Na₂CO₃ (pH 11.5) to remove peripherally associated proteins from membranes. As a control membrane, pellets were pretreated with 0.5% Triton X-100 to disrupt the lipid bilayer. As depicted in Fig. 6E eGFP fusion proteins comprising pTMS 1, pTMS 2, and pTMS 3 were still found in the LD fractions after NaCl or Na₂CO₃ treatment, supporting the notion that these 3
Characterization of DV NS4A

pTMSs are integrated into cellular membranes. In case of pTMS 4, the fraction that is membrane-associated behaved like an integral membrane protein, because it was resistant against NaCl or Na₂CO₃ treatment. This indicates that pTMS 4 also has the ability to associate tightly with cellular membranes.

**Probing NS4A Topology Using Deglycosylation Assays**—With the aim to develop a model of the membrane topology of DV NS4A, we performed glycosylation tagging experiments. To this end we constructed plasmids in which eGFP was fused to the C terminus of given pTMSs of DV NS4A were constructed. White boxes represent the predicted transmembrane segments (pTMSs) of NS4A; black boxes indicate the C-terminal 2K fragment. B, Huh-7/T7 cells were transfected with the constructs specified at the bottom. 4 h post-transfection, cell culture medium was replaced by medium containing [³⁵S]methionine/[³⁵S]cysteine. After 4 h of labeling, cells were lysed, and immunoprecipitation with a GFP-specific antibody was performed. Precipitated immunocomplexes were treated with PNGase F for 2 h, separated by SDS-PAGE, and analyzed by autoradiography.

FIGURE 7. Probing DV NS4A membrane topology by glycosylation tagging. A, schematic representation of the DV NS4A-eGFP fusion proteins used for glycosylation studies. Several plasmids in which eGFP was fused to the C terminus of given pTMSs of DV NS4A were constructed. **White boxes** represent the predicted transmembrane segments (pTMSs) of NS4A; **black boxes** indicate the C-terminal 2K fragment. **B**, Huh-7/T7 cells were transfected with the constructs specified at the bottom. 4 h post-transfection, cell culture medium was replaced by medium containing [³⁵S]methionine/[³⁵S]cysteine. After 4 h of labeling, cells were lysed, and immunoprecipitation with a GFP-specific antibody was performed. Precipitated immunocomplexes were treated with PNGase F for 2 h, separated by SDS-PAGE, and analyzed by autoradiography.

FIGURE 8. Model for the membrane topology of DV NS4A. The N-terminal one-third of DV NS4A is localized in the cytoplasm; pTMS 1 and pTMS 4 span the membrane from the cytoplasmic to the luminal site, whereas pTMS 3 seems to span the lipid bilayer from the luminal to the cytoplasmic site. Amino acids around positions 76–99 (pTMS 2) most probably do not span the membrane but are closely associated with the luminal side of the lipid bilayer. Suggesting that pTMS 1-mediad membrane translocation of the eGFP reporter. This result clearly shows that pTMS 1 is the first membrane-spanning segment of DV NS4A. Surprisingly, 4A-(1–100)-eGFP-glyc, containing pTMS 1 and pTMS 2, was also glycosylated, indicating that pTMS 2 does not span the membrane despite its tight membrane association (see above). In the case of the fusion protein containing pTMSs 1–3 (residues 1–127), no glycosylation of the C-terminal eGFP-glyc was observed, indicating its cytoplasmic localization. Thus, pTMS 3 seems to be the second membrane spanning segment of DV NS4A. As inferred from the glycosylation of full-length 4A-eGFP-glyc (residues 1–150) pTMS 4 seems to be a third TMS. Similar to that observed for full-length wild-type DV NS4A in infected cells (data not shown), the apparent molecular weight of full-length NS4A fused to eGFP-glyc was slightly smaller than the calculated molecular weight, for reasons we do not know.

Taken together, our data suggest that the N-terminal 49 residues and residues 122–131 of DV NS4A are localized in the cytoplasm (Fig. 8). pTMS 1 and pTMS 4 appear to span the ER membrane from the cytoplasmic to the luminal site, whereas pTMS 3 crosses the lipid bilayer from the luminal to the cytoplasmic site. Residues 73–101 most likely localize in the ER lumen and are tightly associated with the ER membrane.

**DISCUSSION**

In this study, we investigated the membrane topology and subcellular localization of the so far only poorly characterized DV non-structural protein NS4A in detail. Membrane flotation and extraction assays revealed that NS4A is tightly associated with cellular membranes even in the absence of the C-terminal 2K fragment that serves as a signal sequence for the ER translocation of NS4B and is cleaved off by the NS2B-3 protease (21). Based on membrane topology predictions and expression studies, we established a model according to which the N-terminal 49 residues of NS4A are not membrane-associated followed by 4 highly hydrophobic regions acting as pTMSs. These predictions were extended and corroborated by glycosylation studies of engineered NS4A proteins and finally led to the model for the putative membrane topology of NS4A depicted in Fig. 8. The N-terminal 49 residues of NS4A reside within the cytoplasm, where processing by the viral protease at the N-terminal NS3–4A cleavage site occurs. In agreement with this topology Anandarao et al. (30) have shown that sera of DV-infected patients contain NS4A-specific antibodies. All the
epitopes identified in their experiments mapped to the N-terminal third of NS4A, consistent with our finding that this NS4A segment is not integrated into cellular membranes.

Residues 50–74 seem to contain the first TMS of DV NS4A spanning the membrane from the cytoplasmic to the luminal site. In contrast, pTMS 2 (residues 74–100) does not seem to span cellular membranes, although the results of our deletion mapping studies have shown that it is tightly associated with the lipid bilayer. This property is often found with proteins containing amphipathic helices, which are characterized by having one polar (charged) and one hydrophobic side. They bind tightly to the membrane surface with the hydrophobic residues dipping into the hydrophobic phase of the membrane (31–33). Although an axial projection of DV NS4A pTMS 2 did not reveal an amphipathic helix, it is possible that pTMS 2 is rather deeply embedded into the hydrophobic interior of the membrane without spanning the lipid bilayer. Moreover, the predicted very short distance of about two amino acid residues on each side to the adjacent pTMS 5s 1 and 3 most likely has an impact on the topology of pTMS 2 by hindering a membrane passage of pTMS 2 and anchoring it to the ER luminal leaflet of the lipid bilayer. PTMS 3, comprising residues 100–127 of DV NS4A, is the second TMS and spans the lipid bilayer from the luminal to the cytoplasmic site (Fig. 8). Residues 123–130 of NS4A are located in the cytoplasm forming a small loop and exposing the 4A-2K cleavage site that is recognized by the viral protease. The C-terminal 2K fragment spans the membrane from the cytoplasm to the lumen where processing by the host signalase at the 2K-4B cleavage site occurs. Although our glycosylation assays have clearly shown that 2K spans the membrane when expressed in the context of the full-length protein, only a fraction of 2K-eGFP was found to be membrane-associated when expressed individually. Thus, sequences N-terminal of 2K may be required for the efficient membrane insertion of 2K.

The function of NS4A for the DV replication cycle is as yet not known. However, Lindenbach and Rice (20) described an interaction between NS1 and NS4A that appears to be critical for replicase function and provides the first genetic data suggesting that NS4A is involved in RNA replication. In agreement with these results, we observed a clear colocalization of NS4A with dsRNA as well as with the viral protein E. NS3 and NS4B were previously found to colocalize with E and dsRNA (25). Moreover, NS4A localizes to reticular structures and large cytoplasmic foci in DV-infected cells, which were either originating from or associated with the ER, arguing that NS4A is part of the viral RC corresponding to the cytoplasmic foci. Similar cytoplasmic structures have been described for other flaviviruses. In KUNV-infected cells, the cytoplasmic foci detected by IFM correspond to accumulations of viral proteins on distinct membrane alterations. The structures are induced by the viral infection and seem to originate from different cellular organelles (for review see Ref. 34). So called convoluted membranes and paracrystalline structures are putative sites of viral polyprotein processing, whereas proliferating ER and vesicles of about 100 nm in diameter (designated smooth membrane structures) may represent the sites of viral RNA replication (10, 35–38). Earlier studies revealed that KUNV NS4A localizes within these virus-induced membranes and interacts with most of the other viral non-structural proteins, including NS3 and NS5 (9). Thus, it is well possible that the dot-like structures found in our studies represent DV RCs.

Recently, it was published that a regulated cleavage at the KUNV NS4A-2K-NS4B junctions is important for the rearrangement of cytoplasmic membranes. Interestingly, expression of full-length KUNV NS4A led to membrane alterations similar to those induced by KUNV infection, but removal of the 2K fragment impaired the ability of NS4A to induce membrane rearrangements and led to a Golgi localization of this protein (22). In contrast to these results, we found that expression of full-length DV NS4A did not induce cytoplasmic foci but rather reticular structures indicating that membrane alterations were not induced. However, expression of DV NS4A lacking the 2K fragment (representing the fully processed NS4A) induced cytoplasmic membrane rearrangements and led to a redistribution of the ER as observed in infected cells. These results provide strong evidence that in case of DV processing at the 4A-2K site is required for the induction of cellular membrane alterations. Apart from possible biological differences between KUNV NS4A and DV NS4A we note the different experimental set-ups. While we used a T7-polymerase-based expression system and a cell line that stably expresses the T7-RNA polymerase, et al. (22) employed the Semliki Forest Virus (SFV) expression system, which can induce membrane alterations on its own that differ, however, in morphology from the structures induced by KUNV (39). It is also possible that the observed differences between KUNV and DV are due to the different cell lines utilized in the study by Roosendaal et al. (Vero cells) and us (Huh-7 cells). Further studies will be required to clarify the contribution of NS4A processing for induction of membrane rearrangements with different flaviviruses.

The topology model described here sheds some light on the possible mechanism by which NS4A induces membrane curvature. In the simplest scenario the insertion of pTMS 2 into the luminal leaflet of the ER membrane may act like a wedge resulting in a curvature toward the cytosol. This effect may be amplified by NS4A oligomerization similar to what has been described for HCV NS4B (40) and several other membrane curvature-inducing proteins (32, 41). So far, it is not known whether DV NS4A forms homo-oligomers or hetero-oligomers with other viral or cellular proteins. However, there is evidence that KUNV NS4A is able to homo-oligomerize (9), and we obtained preliminary evidence that the same occurs with DV NS4A (data not shown). Taken together, our data provide the first detailed characterization of the DV NS4A protein. The topology model established here may serve as a frame to unravel the function of NS4A in the DV replication cycle.

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