Cotranslational Membrane Insertion of the Serine Proteinase Precursor NS2B-NS3(Pro) of Dengue Virus Type 2 Is Required for Efficient in Vitro Processing and Is Mediated through the Hydrophobic Regions of NS2B*

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Polyprotein processing of dengue virus type 2, a positive strand RNA virus, is carried out by the host signal peptidase and a novel two-component viral proteinase of the serine proteinase family, NS2B/NS3(Pro), in the endoplasmic reticulum. Using an in vitro processing system, we examined the cis and trans cleavages of the 2B/3 and 4B/5 sites by NS2B/NS3(Pro), respectively. Lysates of BHK-21 cells coexpressing NS2B and NS3(Pro) mediated trans cleavage of the 4B/5 site in vitro, and the protease activity was associated with the membrane fraction. To study the role of membranes in the protease activity of NS2B/NS3(Pro), labeled precursors, NS2B-NS3(Pro), and the mutant ndNS2B-NS3(Pro) in which the functional hydrophilic domain of NS2B was deleted, were analyzed using a coupled in vitro transcription/translation system (TnT). The results showed that cotranslational addition of microsomal membranes to the TnT reaction markedly enhanced the cis cleavage of the 2B/3 site in a dose-dependent manner. NS2B synthesized in the presence of membranes also facilitated trans cleavage of the 2B/3 site in the mutant precursors. The cleavage products, NS2B and NS3(Pro), were membrane-associated. Furthermore, this membrane requirement was dictated by the hydrophobic regions of NS2B. Deletion of hydrophobic regions of NS2B, leaving only the conserved hydrophilic domain of 40 amino acids, resulted in highly efficient processing of the 2B-3 site in vitro in the absence of microsomal membranes.

Dengue virus type 2 (DEN-2), a member of Flaviviridae, has a single-stranded RNA genome of positive polarity. The genomic RNA contains 10,723 nucleotides that contains a single open reading frame encoding a polyprotein precursor of 3391 amino acid residues with a gene order of 5'-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'. (In New Guinea C-strain (1)). The 5'-end of the genomic RNA has a type I cap, and the 3'-end is devoid of a poly(A) tail (for review, see Ref. 2). The polyprotein precursor is processed into three structural proteins that are assembled into the virion (C, prM, and E) and at least seven nonstructural proteins, NS1 to NS5, which are expressed in infected cells (2).

The processing of the N-terminal region of the polyprotein precursor that encodes the structural proteins (C, prM, and E) is carried out by the host signal peptidase associated with the endoplasmic reticulum (3–5). The role of NS3 as the putative viral protease was established subsequent to the identification of a serine proteinase domain within the N-terminal 180 amino acid residues (6, 7). Further studies showed that the protease activity of NS3 was dependent on the presence of NS2B. Moreover, NS2B and NS3 form a complex in virus-infected cells (8–10). This two-component proteinase is required for the rapid cis cleavage of the 2A/2B and 2B/3 sites as well as for trans cleavage of the 3/4A and 4B/5 sites (11–17). In addition, the viral encoded protease mediates cleavages within the C (18–20), NS2A (21), NS3 (8, 14, 22), and NS4A proteins (23). These cleavage sites have the consensus sequence of two amino acids (KR, RR, RK, and occasionally QR) at the −2 and −1 positions, followed by Gly, Ala, or Ser at the +1 position (2). The cleavage that converts prM to M protein occurs at a late stage during viral morphogenesis and is mediated by a cellular protease located in a post-Golgi acidic compartment of the cell (24).

The goal of the current study was to develop an in vitro processing system for characterization of the role of NS2B in the activation of NS3 protease. When NS2B and NS3 were coexpressed in mammalian cells using a recombinant vaccinia virus expression system, the protease activity that cleaved the NS4B-NS5(93 aa) substrate in vitro partitioned with the membrane fraction. Using the coupled in vitro transcription/translation system, we demonstrated that cotranslational insertion of the NS2B-NS3(Pro) precursor into the endoplasmic reticulum membranes markedly enhanced the NS3-dependent cis and trans cleavages. However, this membrane-dependent enhancement was nullified by deletion of the hydrophobic regions of NS2B. Under these conditions, the cleavage of the 2B-3 site in the precursor occurred with increased efficiency in the absence of membranes.

**EXPERIMENTAL PROCEDURES**

Materials—Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA). Rabbit reticulo-cyte coupled transcription/translation system and the dog pancreatic...

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microsomal membranes were purchased from Promega (Madison, WI). *trans*-l-[35S]Methionine label (1000 Ci/mmol) was purchased from ICN (Costa Mesa, CA). The PET-PFH-nef vector was a gift from L. J. Zhao (see Ref. 25). The monoclonal antibody against the FLAG epitope was from IBI-Kodak (Kodak Scientific Imaging Systems, New Haven, CT). The pTM1 vector was a gift from B. Moss (26). The pJK3 vector was derived from the pTM1 to include the C-terminal 27 amino acid residues encoding the protein kinase A site, FLAG epitope, and hexahistidine tag (described in the text) is marked with the PFH extension.

**Fig. 1.** DEN-2 expression plasmids. Details of the cloning strategy for each expression construct are described under “Experimental Procedures.” The protease-sensitive cleavage site is shown by an arrowhead. The shaded regions represent the hydrophobic regions flanking the central (conserved) hydrophobic domain of NS2B. The constructs containing a mutation of the catalytic His→Ala is indicated as H→A. The N-terminal deletions in the NS2B are indicated by truncation of the protein, and internal deletions are indicated by lines connecting discontinuous regions of the precursor. The C-terminal modification consisting of a 27-amino acid affinity tag (described in the text) is marked with the PFH extension.
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**RESULTS**

In *vitro* Processing of the NS4B-NS5 Precursor—The goal of this study was to develop an *in vitro* processing system for biochemical characterization of the viral encoded NS2B/NS3 protease. The initial strategy for developing this *in vitro* processing system was to express NS2B and the N-terminal protease domain of NS3 (NS3(Pro)) using the recombinant vaccinia virus expression system. To facilitate purification of NS2B and NS3(Pro), the coding sequences of both proteins were modified by a C-terminal fusion of 27 amino acid residues (PFH) consisting of a protein kinase A phosphorylation site, a synthetic FLAG epitope recognized by a monoclonal antibody, and a hexahistidine tag (25). First, we verified that this C-terminal modification of NS2B and NS3 did not affect their biological activity in the processing of NS3-NS4A-NS4B-NS5 polyprotein processed in *vivo*. When the protease components and the substrate were coexpressed in *vivo*, the efficiency of processing was similar to that of unmodified NS2B and NS3(Pro) reported earlier (16) (data not shown).

To establish an *in vitro* processing system that can carry out *trans* cleavages, BHK-21 cells were infected with recombinant vaccinia viruses encoding the T7 RNA polymerase (vTF7-3), NS2B-PFH, and NS3(Pro)-PFH. The NS2B-PFH and NS3(Pro)-PFH fractionated into cytoplasmic and membrane fractions by centrifugation (30) which require T7 promoter/encephalomyocarditis virus 5′-untranslated region (30) which requires T7 RNA polymerase from vTF7-3 infection for expression. Infected cells were then treated with Triton X-100 lysis buffer, and the lysates were fractionated into cytoplasmic and membrane fractions by centrifugation (15,000 × g). NS2B-PFH and NS3(Pro)-PFH were recovered in the membrane pellet fraction but not with the supernatant fraction (Fig. 2, lanes 3 and 4), and the membrane pellet fraction was recovered in the membrane pellet fraction but not with the supernatant fraction (Fig. 2B, compare lanes 3 and 4).

In this experiment the detection of NS5(93 aa) was obscured by the endogenously produced globin. Since NS4B is a membrane-associated protein, the NS4B-NS5(93 aa)-PFH precursor was synthesized *in vitro* in the presence of microsomal membranes. In this manner, it was possible to make the NS4B-NS5(93 aa)-PFH substrate associated with the membrane and...
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Fig. 3. In vivo and in vitro processing of the NS2B-3(Pro)-PFH precursor. A, HeLa cells were infected with recombinant vaccinia virus, vTF7-3 (to provide T7 RNA polymerase) and transfected with pTM1-NS2B-NS3(Pro)-PFH plasmid as described under “Experimental Procedures.” The total cell extracts were analyzed by SDS-PAGE followed by Western blot analysis using the FLAG monoclonal antibody. Lanes 1 and 2, extracts were prepared from cells infected with vTF7-3 alone (control) or subjected to “infection and transfection” with vTF7-3 and the plasmid. B, NS2B-NS3(Pro)-PFH or NS2B-NS3(Pro)-PFH (which contains the His → Ala mutation in the catalytic site) was expressed in vitro using the TnT reaction. Incubations with or without canine pancreatic microsomal membranes at 30 °C for 90 min are as indicated. The products of processing were analyzed by SDS-PAGE and autoradiography. C, in vitro processing of the precursors were carried out as described in B. Reaction mixtures were then immunoprecipitated with either a mixture of anti-NS2B and anti-NS3 antibodies (lanes 1–3) or anti-NS3 antibody (lanes 4–6). Lane 1, NS2B-NS3*(Pro) (+) microsomal membranes. Lane 2, NS2B-3(Pro)-PFH (−) microsomal membranes. Lane 3, NS2B-3(Pro)-PFH (+) microsomal membranes. Lanes 4–6, same order as lanes 1–3.

The Role of Microsomal Membranes in the Protease Activity of the NS2B-NS3(Pro) Components—The association of the protease activity of NS2B-PFH and NS3(Pro)-PFH with the membrane pellet fraction of recombinant vaccinia virus-infected BHK-21 cell lysates could be explained by two possible scenarios. First, overexpression of proteins in the recombinant vaccinia virus expression system could result in aggregation and partitioning of the protein into the membrane pellet fraction. Second, NS2B-PFH and NS3(Pro)-PFH have intrinsic properties for association with the membranes, and this membrane association is required for optimal protease activity. To investigate these possibilities, the cis cleavage of the 2B/3 site was examined using an expression plasmid encoding the NS2B-NS3(Pro)-PFH precursor.

The NS2B-NS3(Pro)-PFH precursor was expressed in HeLa cells by plasmid transfection followed by infection with vTF7-3 to provide T7 RNA polymerase (30). The processing was monitored by Western blot analysis using the FLAG monoclonal antibody. As shown in Fig. 3A (lane 2), the processing of NS2B-NS3(Pro)-PFH precursor in vitro was very efficient as little unprocessed precursor was detected.

Expression of the NS2B-NS3(Pro)-PFH precursor in vitro using the TnT reaction yielded markedly different results. In contrast to the efficient processing of the NS2B-NS3(Pro)-PFH precursor observed in vivo, only a trace amount of precursor underwent processing in vitro in the absence of microsomal membranes. Therefore, the TnT reactions were conducted in the presence or absence of canine pancreatic microsomal membranes to determine whether membranes influenced the cleavage efficiency. As shown in Fig. 3B, only a faint band was observed in the 36-kDa size range which is the expected size of NS3(Pro)-PFH liberated by cis cleavage of the 2B/3 junction (Fig. 3B, lane 2). However, in vitro processing assays conducted in the presence of microsomal membranes significantly enhanced the efficiency of cleavage of the 2B/3 site (Fig. 3B, lane 3). The possibility that a contaminating protease in the microsomal membrane preparation was cleaving the NS2B-NS3(Pro)-PFH precursor was considered. To eliminate this possibility the precursor NS2B-NS3*(Pro) containing a His51 → Ala mutation within the catalytic triad of NS3 was translated in vitro using the TnT reaction. Incubations with or without canine pancreatic microsomal membranes at 30 °C for 90 min are as indicated. The products of processing were analyzed by SDS-PAGE and autoradiography. C, in vitro processing of the precursors were carried out as described in B. Reaction mixtures were then immunoprecipitated with either a mixture of anti-NS2B and anti-NS3 antibodies (lanes 1–3) or anti-NS3 antibody (lanes 4–6). Lane 1, NS2B-NS3*(Pro) (+) microsomal membranes. Lane 2, NS2B-3(Pro)-PFH (−) microsomal membranes. Lane 3, NS2B-3(Pro)-PFH (+) microsomal membranes. Lanes 4–6, same order as lanes 1–3.

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the His^{31} → Ala mutation was expressed (Fig. 3C, lanes 1 and 4). NS2B and NS3(Pro)-PFH Generated by the Cis Cleavage of the 2B/3 Site in the TnT Reaction Are Membrane-associated—The results obtained thus far established that processing of the NS2B-NS3(Pro)-PFH precursor is markedly enhanced by microsomal membranes. Next, it was examined whether the precursor protein or the products of processing were associated with the membranes. The NS2B-NS3*(Pro) and NS2B-NS3(Pro)-PFH precursors were expressed in the TnT reaction in the presence and absence of microsomal membranes. The reactions were then subjected to centrifugation (15,000 × g) for 20 min, and the supernatant and membrane pellet fractions were analyzed by SDS-PAGE followed by autoradiography.

The results obtained thus far established that processing of the NS2B-NS3(Pro)-PFH precursor was carried out to establish whether NS2B or NS3(Pro)-PFH or both are integral or peripheral membrane proteins. One of the methods used to distinguish between integral versus peripheral membrane association is by treatment of membrane fractions containing the protein(s) of interest with high pH (pH ≈ 11.0) (31). After the \textit{in vitro} processing of the NS2B-NS3(Pro)-PFH precursor by TnT reactions was carried out in the presence of microsomal membranes, the membr-
The NS2B-NS3(Pro)-PFH precursor was expressed in vitro using the TnT system at 30 °C for 90 min in the absence of membranes, the in vivo TnT. However, these results did not show how membranes were influencing the functionality of NS2B or NS3(Pro)-PFH individually using the recombinant vaccinia virus expression system. BHK-21 cells were coinfected with vTF7-3 (to provide T7 RNA polymerase) and vvNS2B-PFH or vvNS3(Pro)-PFH. The membrane pellet fractions isolated from the infected cell lysates were treated with sodium carbonate buffer, pH 11.5. Subsequently, the portions of NS2B and NS3(Pro)-PFH that were solubilized and those remained in the pellet fractions were analyzed by SDS-PAGE and Western blot. The results shown in Fig. 7, B and C, indicate that major portions of both NS3(Pro)-PFH and NS2B-PFH still remained associated with the membrane pellet fractions after the pH 11.5 treatment (Fig. 7B, compare lanes 1 and 2 versus C, lanes 2 and 3). These results independently confirmed the results shown in Fig. 7A.

In Vitro Processing of the ndNS2B-NS3(Pro)-PFH Precursor by Trans Supply of NS2B—The results of this study showed that membranes were required for efficient cis cleavage of the precursor NS2B-NS3(Pro)-PFH and that both NS2B and NS3(Pro)-PFH were associated with the membranes during the course of the in vitro TnT. However, these results did not show how membranes were influencing the functionality of NS2B or NS3(Pro)-PFH or both proteins. To examine the contribution of membrane association of NS2B independent of NS3(Pro) to the processing efficiency, the ndNS2B-NS3(Pro)-PFH precursor was constructed. In this construct the functional domain of NS3(Pro) was deleted requiring addition of the wild type NS2B for the cleavage of the 2B/3 site. The in vitro expression of the ndNS2B-NS3(Pro)-PFH alone in the presence of microsomal membranes did not yield detectable cleavage at the 2B/3 site as expected (Fig. 8, lane 3). This result is consistent with the in vivo studies of mutational analysis of NS2B in the processing of precursor protein (17). Coexpression of the ndNS2B-NS3(Pro)-PFH precursor with NS2B in the absence of microsomal membranes (Fig. 8, lane 5) also did not yield detectable cleavage at the 2B/3 cleavage junction confirming the membrane requirement established in this study (Fig. 3B). When the NS2B was translated in the presence of membranes and the membrane-associated NS2B was incubated with the ndNS2B-NS3(Pro)-PFH substrate expressed in the absence of membranes, no
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processing of the substrate was observed (data not shown). However, if NS2B and the ndNS2B-NS3(Pro)-PFH precursor were coexpressed in the TnT system in the presence of microsomal membranes, processing of the 2B/3 cleavage junction was restored (Fig. 8, lane 4). Similarly, processing of the ndNS2B-NS3(Pro)-PFH precursor was also observed if NS2B was first expressed alone in the presence of microsomal membranes, and the membrane fraction from this TnT reaction was used in a second TnT reaction programmed for expression of the ndNS2B-NS3(Pro)-PFH substrate without additional membranes added (Fig. 8, lane 5). Omission of NS2B + ndNS2B-NS3(Pro)-PFH was carried out in the presence and absence of membranes, respectively.

Membrane Enhancement of NS2B-mediated Activation of the NS3(Pro) Domain Is Nullified by Deletion of Hydrophobic Regions of NS2B—Since NS2B was associated with the membranes cotranslationally, we sought to determine whether the hydrophobic regions of NS2B played any role in the membrane-dependent enhancement of the activity of NS2B. To address this question, the expression clone pTM1-NS2B(H)-NS3(Pro)-PFH was constructed (Fig. 1, lane F). The processing of this precursor polypeptide was examined using the in vitro TnT system. Microsomal membrane pellet fractions were isolated and treated with either PBS or sodium carbonate buffer, pH 11.5, as described under “Experimental Procedures.” The proteins that were still associated with the membranes or became soluble were analyzed by SDS-PAGE and autoradiography. Lane 1, soluble fraction from the PBS-treated membranes. Lane 2, membrane pellet fraction from PBS-treated membranes. Lane 3, soluble fraction from carbonate-treated membranes. Lane 4, membrane pellet fraction from carbonate-treated membranes. BHK-21 cells were infected with the recombinant vaccinia virus encoding the NS2B-NS3(Pro)-PFH. Cells were lysed in a hypotonic lysis buffer, and the membrane fractions from these lysates were treated with carbonate buffer as described under “Experimental Procedures.” Each fraction was analyzed by SDS-PAGE followed by Western blot analysis using the FLAG monoclonal antibody. Lane 1, membrane pellet fraction from the carbonate-treated membranes. Lane 2, soluble fraction from the carbonate-treated membranes. Lane 3, soluble fraction from the hypotonic lysis. C, BHK-21 cells were infected with the recombinant vaccinia virus encoding the NS2B-NS3(Pro)-PFH. Treatment of the cell lysates was as described in B. Lane 1, soluble fraction from hypotonic lysis. Lane 2, soluble fraction from carbonate-treated membranes. Lane 3, membrane pellet fraction from carbonate-treated membranes.

Fig. 7. Analysis of the nature of membrane association of NS2B and NS3(Pro). A, the NS2B-NS3(Pro)-PFH precursor was expressed in vitro using the TnT system. Microsomal membrane pellet fractions were isolated and treated with either PBS or sodium carbonate buffer, pH 11.5, as described under “Experimental Procedures.” The proteins that were still associated with the membranes or became soluble were analyzed by SDS-PAGE and autoradiography. Lane 1, soluble fraction from the PBS-treated membranes. Lane 2, membrane pellet fraction from PBS-treated membranes. Lane 3, soluble fraction from carbonate-treated membranes. Lane 4, membrane pellet fraction from carbonate-treated membranes. B, BHK-21 cells were infected with the recombinant vaccinia virus encoding the NS3(Pro)-PFH. Cells were lysed in a hypotonic lysis buffer, and the membrane fractions from these lysates were treated with carbonate buffer as described under “Experimental Procedures.” Each fraction was analyzed by SDS-PAGE followed by Western blot analysis using the FLAG monoclonal antibody. Lane 1, membrane pellet fraction from the carbonate-treated membranes. Lane 2, soluble fraction from the carbonate-treated membranes. Lane 3, soluble fraction from the hypotonic lysis. C, BHK-21 cells were infected with the recombinant vaccinia virus encoding the NS2B-NS3(Pro)-PFH. Treatment of the cell lysates was as described in B. Lane 1, soluble fraction from hypotonic lysis. Lane 2, soluble fraction from carbonate-treated membranes. Lane 3, membrane pellet fraction from carbonate-treated membranes.

Fig. 8. Cleavage of 2B/3 site of the ndNS2B-NS3(Pro)-PFH precursor by NS2B supplied in trans is enhanced by membranes. The components of the TnT reactions are as indicated. Total TnT lysates were analyzed by SDS-PAGE followed by autoradiography. In lane 2, the NS2B was expressed in the presence of membranes, and the membrane-associated NS2B was used in a second translation programmed for ndNS2B-NS3(Pro)-PFH without any additional membranes added. In lanes 4 and 5, cotranslation of NS2B + ndNS2B-NS3(Pro)-PFH was carried out in the presence and absence of membranes, respectively.
ies. The origin of the doublet in lane 2 is unknown. The results shown in Fig. 9 taken together indicate that the hydrophilic domain of NS2B alone is sufficient for in vitro cis cleavage of the 2B/3 site in the absence of microsomal membranes.

**DISCUSSION**

Flavivirus NS2B/NS3 proteinases belong to the class of two-component proteinases that includes the adenovirus cysteine proteinase (32), hepatitis C virus serine proteinase (33), and the mitochondrial processing peptidase of *Neurospora crassa* (34). However, flavivirus NS2B/NS3 proteinases bear some resemblance to the α-lytic serine proteinase (35) and the serine proteinase subtilisin (36) as well. The propeptide of subtilisin and α-lytic proteinases is essential for production of active enzyme in vivo and is autoprocessed from the precursor of the mature protease by a cis cleavage. In vitro experiments demonstrated that the propeptide need not be physically linked to the protease domain. It was suggested that they function as an intramolecular chaperone assisting in the folding of the protease domain (35–37). The requirement of flavivirus NS2B for generating an active NS3 protease in vivo, the physical link of NS2B to the protease domain of NS3 (N-terminal to the NS3 protease), and the property of NS2B to activate NS3 in trans are analogous to the subtilisin and α-lytic proteinase systems. However, how NS2B activates the protease domain of flavivirus NS3 is currently unknown.

The hydrophobicity plot of NS2B using the Kyte-Doolittle program (38) shows that NS2B contains a central hydrophilic domain flanked by two hydrophobic domains at the N terminus (I and II) and a single hydrophobic domain (III) at the C terminus of NS2B followed by a 10-aa region upstream of the 2B/3 cleavage site (Fig. 10). The central hydrophilic region contains 40 amino acids which is conserved among flaviviruses. Deletion analysis of the NS2B region in DEN-4-encoded NS2B-NS3(Pro)-PFH precursor and transient expression using the recombinant vaccinia virus expression system further supported by the results shown in Fig. 8. Only when both NS2B and ndNS2B-NS3(Pro) precursor were cotranslationally inserted into membranes were they active in the cleavage of the 2B/3 site. This precursor could not undergo cis cleavage of the 2B/3 site because of the deletion of the hydrophilic domain which is required for NS2B activity. It should be noted that the ndNS2B-NS3(Pro)-PFH precursor contains the entire hydrophobic domain III (Fig. 10). Even in the presence of membranes the efficiency of cleavage of the 2B/3 site in the ndNS2B-NS3(Pro)-PFH precursor by trans supply of NS2B was significantly reduced when compared with the cis cleavage of the 2B/3 site in the NS2B-NS3(Pro) precursor. It should be noted that the membrane requirement for NS2B function in the activation of NS3 protease is not conserved in different flaviviruses. In vitro processing studies of tick-borne encephalitis virus and yellow fever virus (YF) revealed that the
NS2B-NS3 precursors undergo processing in vitro in the absence of membranes (11, 39, 40). However, the in vitro processing of a precursor polyprotein NS2A-NS2B-NS3 encoded by West Nile virus requires microsomal membranes (15, 42). These studies indicate that the membranes were targeting the NS2A or NS2B/NS3 components for efficient processing was not established. In this regard it is worth noting that NS2A is an integral membrane protein (43).

It is possible that the conformation of the DEN-2 NS2B-NS3(Pro) precursor might be different from that of YF precursor which does not require membranes. In support of this view, pulse-chase analysis of YF and DEN-2 precursors revealed that YF precursors were processed first at the 2B/3 site followed by cleavage of the 2A/2B site, whereas in the DEN-2 precursor, cleavage of the 2A/2B site preceded the cleavage of the 2B/3 site (11, 12).

The results of this study showed that even though the hydrophobic domain is the only region required for processing at 2B/3 site in vitro, the three hydrophobic regions of the wild type NS2B are likely to be involved in anchoring the NS2B into membranes in vivo. This membrane insertion could facilitate the interaction of the hydrophobic region with the NS2 protease domain. Moreover, another possible function of the membrane-anchored NS2B via its hydrophobic regions is the localization of NS3 to the membranes through protein-protein interaction. Thus the membrane-localized NS2B-NS3 complex could additionally recruit NS5 to the membranes as part of an RNA replication complex where RNA replication process is localized (2, 44). NS3 is a multifunctional protein that contains the protease and the NTPase/RNA helicase domains. It was previously reported that NS3 and NS5 exist as a complex in extracts from DEN-2-infected cells (27). Additional support for the notion that the hydrophobic regions of NS2B may play a role in viral replication comes from the evidence that small deletions in both the N- and C-terminal hydrophobic domains of YF NS2B when introduced in an infectious clone were found to be deleterious for viral replication (9). Thus, the hydrophobic regions of NS2B, which are conserved in position but not in amino acid sequence in different flaviviruses, may play a role in viral replication.

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