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# *In Vitro* Reconstitution Reveals Key Intermediate States of Trimer Formation by the Dengue Virus Membrane Fusion Protein[▽]

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**The flavivirus dengue virus (DV) infects cells through a low-pH-triggered membrane fusion reaction mediated by the viral envelope protein E. E is an elongated transmembrane protein with three domains and is organized as a homodimer on the mature virus particle. During fusion, the E protein homodimer dissociates, inserts the hydrophobic fusion loop into target membranes, and refolds into a trimeric hairpin in which domain III (DIII) packs against the central trimer. It is clear that E refolding drives membrane fusion, but the steps in hairpin formation and their pH requirements are unclear. Here, we have used truncated forms of the DV E protein to reconstitute trimerization *in vitro*. Protein constructs containing domains I and II (DI/II) were monomeric and interacted with membranes to form core trimers. DI/II-membrane interaction and trimerization occurred efficiently at both neutral and low pH. The DI/II core trimer was relatively unstable and could be stabilized by binding exogenous DIII or by the formation of mixed trimers containing DI/II plus E protein with all three domains. The mixed trimer had unoccupied DIII interaction sites that could specifically bind exogenous DIII at either low or neutral pH. Truncated DV E proteins thus reconstitute hairpin formation and define properties of key domain interactions during DV fusion.**

Dengue virus (DV) is a flavivirus that is spread by mosquitoes and causes millions of cases of disease each year worldwide (2, 9, 17). DV infection can result in dengue hemorrhagic fever, a more lethal disease that leads to ~500,000 hospitalizations and ~12,500 deaths per year (10, 39). DV is currently endemic in more than 100 countries, including the United States (17), and the World Health Organization estimates that about 40% of the world's population lives in areas where dengue fever is endemic (39). As yet, there is no licensed DV vaccine or antiviral therapy. Studies of the molecular mechanisms of the virus life cycle are important to the development of new antiviral strategies.

Flaviviruses such as DV are small, highly organized enveloped viruses with plus-sense single-stranded RNA genomes (reviewed in references 21 and 25). The flavivirus particle contains 3 structural proteins: a capsid protein, which associates with the genomic RNA to form the viral core, and two membrane proteins, the M protein and the membrane fusion protein E. Like many enveloped viruses, flaviviruses infect cells via endocytic uptake and a membrane fusion reaction triggered by the low pH within endosomes (38). Low-pH-triggered membrane fusion is mediated by conformational changes in the viral E protein, which converts from a prefusion E homodimer to a target membrane-inserted homotrimer. The structure of the DV E ectodomain in the prefusion form shows an elongated finger-like molecule with three domains (DI, DII, and DIII) composed primarily of β-sheets (22, 24, 42) (Fig. 1A; see also Fig. 7). The central DI is connected to DII. The distal tip of DII contains the hydrophobic fusion loop, the region of E that inserts into the target membrane during fusion. On the other side, DI connects via a short linker to DIII, an immunoglobulin-like domain. In the full-length viral E protein, DIII is followed by the stem, which contains 2 helical regions (H1 and H2) connected by a conserved sequence (CS). The stem connects to the C-terminal transmembrane (TM) anchor. The E-protein homodimer is arranged in a head-to-tail fashion, with the fusion loop on DII of each E protein hidden in a pocket formed by DI and DIII of its dimeric E partner.

Upon exposure to low pH, the homodimer dissociates and the E proteins insert their fusion loops into the target membrane and form very stable homotrimers (reviewed in reference 12). The structure of the DV E ectodomain trimer reveals that trimerization is mediated by dramatic domain movements (23, 26). The central region of the trimer is composed of DI and DII. DIII rotates by about 70°, folds back toward the target membrane, and packs against the grooves formed by DI and DII in the central trimer. During this refolding, part of the DI-DIII linker region inserts into a β-sheet of DI. These linker-DI rearrangements produce significant intersubunit contacts at the membrane-distal region of the trimer. The DV E protein stem region is not present in the trimer structure, but its length is sufficient to extend along the central trimer and connect with the TM domain. The final postfusion trimer thus has a hairpin-like conformation with the fusion loops and TM domains at the same end of the molecule. The pre- and postfusion structures of the alphavirus E1 protein (8, 18, 29) are very similar to those of the flavivirus E proteins, suggesting common features of membrane fusion between the two virus groups.

Biogenesis of flavivirus particles occurs by budding into the endoplasmic reticulum (ER) and transit through the secretory pathway. The M protein is synthesized in the ER as a precursor protein termed prM, which forms a heterodimer with the E protein in the ER and on the nascent immature virus particle

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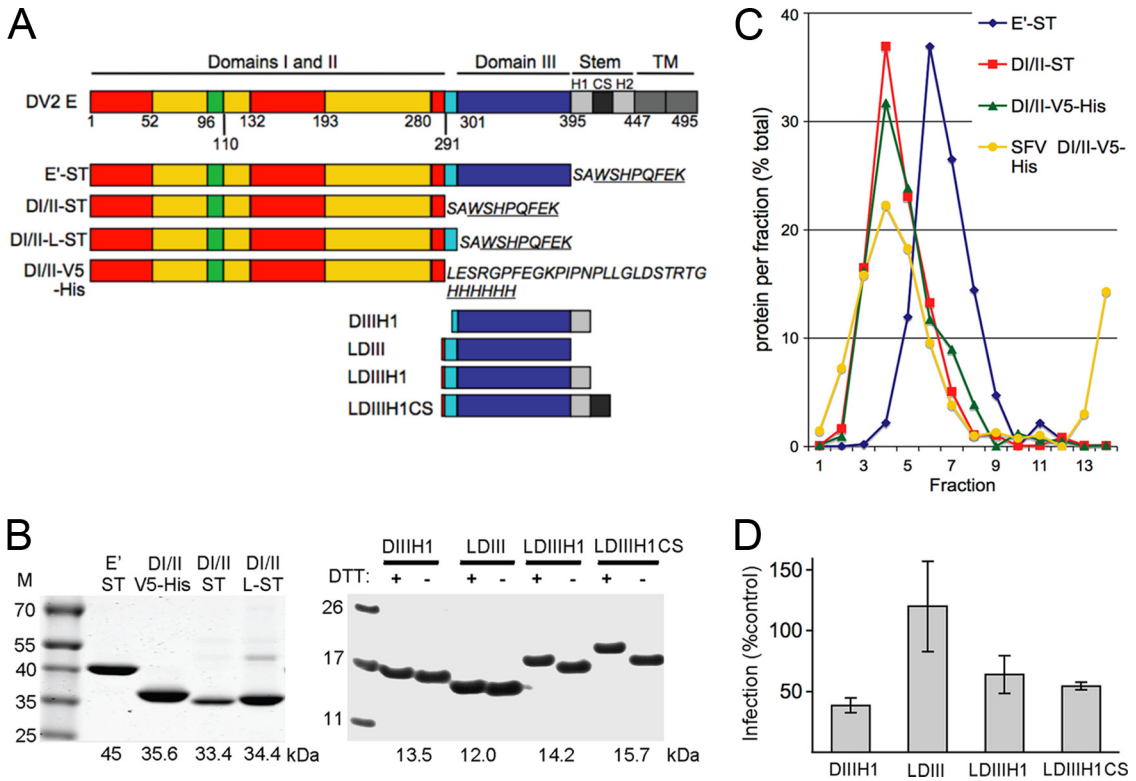


FIG. 1. Production and characterization of truncated DV2 E proteins. (A) Constructs used to express truncated forms of the DV2 E protein. At the top is a linear diagram of the full-length DV2 E protein, with DI indicated in red, DII in yellow, the fusion loop in green, the DI-DIII linker in cyan, DIII in dark blue, and the stem and TM regions in gray. L indicates the linker, and H1, CS, and H2 indicate the stem regions helix1, conserved sequence, and helix2, respectively. The residue numbers of the domain boundaries are listed below the diagram. The four S2 expression constructs primarily used in this work are shown in the middle rows. The E′-ST protein is truncated at residue 395 (DV2-NGC E-protein numbering), DI/II is truncated at residue 291, DI/II-L is truncated at residue 301, and the sequences are joined to the *Strep* or His tag (underlined) used for protein purification. The four DIII constructs are shown in the bottom rows, where LDIII comprises E residues 289 to 395, DIIIH1 residues 296 to 415, LDIIIH1 residues 289 to 415, and LDIIIH1CS residues 289 to 430. (B) Purified truncated E proteins were electrophoresed on SDS gels (left, 4 to 20% acrylamide; right, 10% acrylamide) under nonreducing conditions unless indicated and stained with Coomassie blue. The calculated mass of each protein (without modifications) is shown in kDa below each lane. DTT, dithiothreitol. (C) Sedimentation analysis of E proteins. Samples of purified E proteins were separated on sucrose sedimentation gradients in TAN buffer, pH 8.0, without detergent. Fractions were analyzed by SDS-PAGE, Western blotting, and Licor quantitation, all as described in Materials and Methods. Fraction 1 is the top of the gradient. (D) Inhibition of DV2 fusion by DIII proteins. Serial dilutions of DV2 were bound to BHK cells on ice and treated at pH 5.7 in the presence of the indicated DIII proteins at a final concentration of 50 μM or in buffer alone (control). Cells infected by virus fusion with the plasma membrane were quantitated by immunofluorescence. The data shown are the averages and standard deviations of three independent experiments.

(19, 40, 41). Exposure to low pH in the trans-Golgi network mediates rearrangement of the viral envelope proteins and allows furin processing of prM to produce pr peptide and the mature M protein (32). The pr peptide remains associated with E throughout the low-pH environment of the secretory pathway, thereby protecting the virus from premature fusion until it is released from the cell (19, 40, 41). In the mature virus particle, the prefusion E homodimers are oriented tangentially to the virus membrane and form a herringbone-like pattern on the virus surface, essentially covering the virus membrane (16, 25).

Thus, extensive structural information is available for both the DV E protein homodimer and the low-pH-induced E homotrimer. In contrast, the intermediates and mechanisms involved in the dramatic conformational transition from prefusion to postfusion E are relatively undefined. Recent studies of the flavivirus West Nile virus (WNV) suggest that an early fusion intermediate involves an extension of the stem region prior to dimer dissociation (15). Studies of the flavivirus tick-borne encephalitis (TBE) virus at pH 10 suggest that initial membrane insertion occurs via an E monomer (36). DV fusion and infection are inhibited by the addition of exogenous DIII during the E conformational change (20), implying that the central trimer region is formed before complete foldback of DIII. The presence of stem peptides can inhibit infection by DV and WNV, indicating the importance of stem interactions during hairpin formation (13). The dissociation of the TBE virus E dimer at low pH is dependent on a key histidine residue on DIII (H323; TBE virus numbering), which also promotes formation of the stable E trimer (5). However, studies of WNV indicate that viral E triggering is not controlled by protonation of a critical histidine residue (27). A better understanding of E-protein conformational changes during trimerization is important to define such intermediate steps and to evaluate their usefulness as targets for fusion inhibitors.

Toward this end, in this study we expressed truncated forms

of the DV E protein and used them to reconstitute steps in the trimerization reaction. This *in vitro* system allowed us to characterize the features of E protein involved in the formation of a stable central trimer and in DIII foldback. Our results suggest that monomeric DI/II proteins insert their fusion loops into target membranes and form a core trimer at either neutral or low pH. This core trimer is relatively unstable and can be stabilized by the binding of DIII, thus reconstituting hairpin formation.

## MATERIALS AND METHODS

**Virus and protein constructs.** Virus experiments and protein constructs were based on DV2 New Guinea C (DV2-NGC) virus (kindly provided by John Roehrig, Center for Disease Control, Fort Collins, CO) and its infectious clone JES18 (28) (kindly provided by Barry Falgout and Jane Byrne, U.S. Food and Drug Administration). To generate the *Drosophila* expression constructs, the sequence of DV2 prM from nucleotide (nt) 439 in the DV2-NGC infectious clone JES18 to the indicated residue of the E protein was PCR amplified and cloned into the expression vector pMT/Bip/V5-His A containing the *Drosophila* metallothionein promoter (Invitrogen, Carlsbad, CA) (31, 37). This plasmid was modified to replace the V5-HisA tag with 1 or 2 *Strep* tags as indicated in the legend to Fig. 1A. DIII constructs were generated by PCR and subcloned into pET-14b (Novagen) as previously reported for DIIIH1 (20). All sequences encoding viral proteins were confirmed by DNA sequencing.

**Protein expression and purification.** Expression of the DV2 prM protein and truncated E proteins in *Drosophila* S2 cells was performed as previously described (14, 31, 37). In brief, the constructs were cotransfected into *Drosophila* S2 cells with either the pCo-BLAST selection vector (Invitrogen) or a puromycin selection vector (a kind gift of Félix Rey), and stable cell populations were selected. Protein expression was induced by treatment of shaking cultures of S2 cells in serum-free medium with 500 to 750 μM copper sulfate for 7 to 9 days. His-tagged E proteins were purified by affinity chromatography on chelating Sepharose, followed by gel filtration on Superdex-200, as previously described (31). The purification of *Strep*-tagged proteins followed the protocol from IBA BioTAGnology (Goettingen, Germany). Briefly, the culture medium was collected, concentrated 10 to 20-fold in some cases using a Vivaflow-200 cassette, adjusted to a final concentration of 100 mM Tris, pH 8.0, and 15 μg avidin/ml, and loaded onto a 1-ml *Strep*-Tactin column from IBA BioTAGnology. The column was washed with buffer W (150 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA) or TAN buffer (20 mM triethanolamine, pH 8.0, 130 mM NaCl), and the protein was eluted with 2.5 mM desthiobiotin in the same buffer.

All of the DIII proteins were expressed in *Escherichia coli*, refolded, and purified by gel filtration as previously described (20). Expression of DIII protein containing the complete stem (H1CSH2) was toxic to bacteria, and thus, this construct could not be produced.

**Fusion infection assay.** The low-pH-triggered fusion of DV with the plasma membrane of BHK target cells was assayed as previously described in detail (20). In brief, serial dilutions of DV were prebound on ice at pH 7.9 to BHK cells cultured on 12-mm coverslips in 24-well plates. Unbound virus was removed by washing, and the cells were treated for 1 min at 37°C in 200 μl pH medium (RPMI without bicarbonate plus 0.2% bovine serum albumin [BSA], 10 mM HEPES, and 30 mM sodium succinate, pH 5.7), with DIII proteins added as indicated. The cells were then incubated at 37°C for 2 days in the presence of 20 mM $NH_4Cl$ to prevent secondary infection, and the infected cells were quantitated by immunofluorescence using mouse hyperimmune ascitic fluid against DV2.

**Liposomes and protein-liposome interaction.** Liposomes were prepared by 10 freeze-thaw cycles, followed by extrusion through 2 stacked 200-nm or 400-nm polycarbonate filters (31). The liposomes were prepared in TAN buffer using a 1:1:1:3 molar ratio of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), sphingomyelin (bovine brain) (Avanti Polar Lipids, Alabaster, AL), and cholesterol (Steraloids, Inc., Wilton, NH), plus trace amounts of [$^3$H]cholesterol (Amersham, Arlington Heights, IL). The liposomes were stored at 4°C under $N_2$ and used within 2 weeks of preparation.

To test protein-membrane interactions, liposomes (~1 mM) were mixed with various truncated DV2 E proteins, typically using concentrations of 50 to 100 μg protein/ml in the presence or absence of DIII proteins. Samples were adjusted to pH 5.75 by the addition of precalibrated amounts of 0.3 M MES (morpholinoethanesulfonic acid), incubated at 25°C for 30 min, and adjusted to pH 8.0 where indicated by the addition of a precalibrated volume of 0.3 M triethanolamine. Samples were then further incubated as described for individual experiments.

**Liposome flotation assay.** Protein-liposome reaction mixtures prepared as described above were adjusted to a final concentration of 20% sucrose, loaded on top of a cushion of 300 μl 40% sucrose in a TLS55 ultracentrifuge tube, and then overlaid with 1.2 ml 15% (wt/wt) sucrose and 200 μl 5% (wt/wt) sucrose in TAN buffer, pH 8.0, or MES buffer, pH 5.5 to 5.75. Gradients were centrifuged for 3 h at 54,000 rpm at 4°C in a TLS55 rotor. The gradients were fractioned by hand into the top 700 μl, middle 400 μl, and bottom 1 ml. The [$^3$H]cholesterol liposome marker was quantitated by scintillation counting; 200-μl aliquots of the fractions were precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE and Western blotting.

**Sucrose gradient sedimentation assays.** Protein-liposome reaction mixtures prepared as described above were solubilized with 1.5% octyl-β-D-glucopyranoside (OG) (Sigma) for 15 to 60 min at 25°C. Samples were then layered onto discontinuous sucrose gradients consisting of 200 μl 40% (wt/wt) sucrose, 400 μl 20% (wt/wt) sucrose, 400 μl 10% (wt/wt) sucrose, and 200 μl 5% (wt/wt) sucrose in buffers containing 1% OG at pH 8.0 (20 mM triethanolamine, 130 mM NaCl) or pH 5.5 (40 mM MES, 100 mM NaCl) as indicated. The gradients were centrifuged at 4°C for 5 h at 54,000 rpm in a TLS55 rotor. Fourteen 104-μl fractions were collected into tubes containing 10 μg BSA and a final concentration of 1% Triton X-100 to improve protein recovery and analyzed by SDS-PAGE and Western blotting.

Sucrose sedimentation gradients without detergents were used to analyze the oligomeric state of the purified E proteins. Five-microgram samples of the indicated proteins were diluted in TAN buffer to a final volume of 50 μl and layered onto discontinuous gradients as described above but prepared in TAN buffer at pH 8 without OG. The gradients were processed and analyzed as described above.

**SDS-PAGE analysis.** SDS-PAGE analysis was routinely performed on 10 to 11% acrylamide gels using a Bis-Tris buffer system (Invitrogen) or on gradient acrylamide gels where indicated. Proteins were detected by Western blotting using monoclonal antibody 4G2 to the flavivirus E protein fusion loop on DII (4) (a kind gift of Fernando Arenzana-Seisdedos, Institut Pasteur) and a rabbit polyclonal antibody raised against purified DV DIII (31), followed by Alexa-680-conjugated secondary antibodies (Invitrogen). Quantitation of Western blots was performed using an Odyssey Infrared Imaging system and Odyssey InCell Western software (Li-Cor Biosciences).

**Electron microscopy.** Truncated E proteins were mixed with liposomes at a final concentration of 150 μg protein/ml and 0.5 mM lipid. The mixtures were treated at pH 8.0 or pH 5.75 for 30 min at 25°C. A 20-μl drop of sample was then placed on a glow-discharged carbon-coated copper grid and stained with a solution of 3% ammonium molybdate at pH 7.4. Samples were examined on a JEOL 1200EX or a JEOL 100CXII electron microscope operated at 80 kV. Images were recorded at a magnification of ×50,000 and assembled with Adobe Photoshop 7.0.

## RESULTS

**Production and characterization of truncated DV2 E proteins.** Truncated DV2 E proteins were used to analyze the requirements for formation of a stable trimer. We took advantage of a previously described *Drosophila* S2 cell system to inducibly express the chaperone protein prM, along with various forms of the E protein (Fig. 1A) (14, 37). E′-ST contained domains I, II, and III without the stem and TM regions and was thus comparable to the proteins used to determine the pre- and postfusion structures of DV E (22–24, 26, 42). DI/II-ST and DI/II-V5-His contained domains I and II with either the *Strep* or His tag at the C terminus, respectively. DI/II-L-ST contained domains I and II plus the linker region that connects DI to DIII. All of the truncated E proteins were efficiently secreted into the S2 culture medium and were purified by affinity chromatography based on the His or *Strep* tag (see Materials and Methods for details). Using our previously described bacterial expression system (20), we also produced domain III containing various combinations of the DI-DIII linker and the stem region (Fig. 1A). All of the truncated E

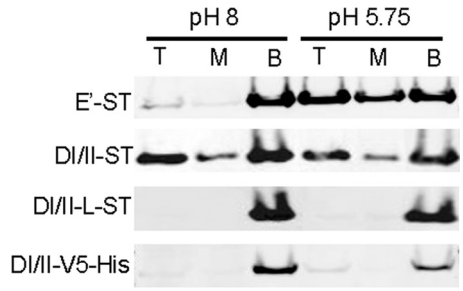


FIG. 2. Membrane interactions of truncated DV2 E proteins. The indicated E proteins were mixed with liposomes at a final concentration of 50 to 100 μg/ml and 1 mM lipid and treated at the indicated pH for 30 min at 25°C. The samples were then adjusted to neutral pH and analyzed by sucrose flotation gradients. The proteins present in the top (T), middle (M), and bottom (B) fractions were analyzed by SDS-PAGE and Western blotting. Shown is a representative example of 2 to 4 experiments.

proteins were of high purity and migrated as predicted in SDS-PAGE (Fig. 1B).

Sucrose gradient sedimentation analysis indicated that purified E′-ST was dimeric at neutral pH (Fig. 1C), consistent with previous studies of the DV2 E protein containing all three domains (22, 42). In contrast, gradient sedimentation assays of the DI/II constructs suggested that these truncated proteins were monomeric at neutral pH. In keeping with this interpretation, the sedimentation profiles of the DV2 DI/II proteins were comparable to that of the previously described monomeric DI/II protein from the alphavirus Semliki Forest virus (SFV) (Fig. 1C) (31). The gel filtration profiles of the DI/II constructs versus the E′ construct also indicated that these proteins are monomers or dimers, respectively (data not shown). Thus, in the absence of DIII, the usual stable DV2 E dimer interaction was not maintained. Nonetheless, the truncated E DI/II protein containing the hydrophobic fusion loop remained soluble in aqueous solution.

Our previous experiments showed that exogenous DV2 DIIIH1 protein can inhibit DV fusion and infection by binding to a trimeric intermediate of the viral E protein during the low-pH-induced conformational change (20). We tested the current four DIII proteins for inhibitory activity (Fig. 1D). A reduction in virus fusion with the plasma membrane was observed for DIIIH1, as expected, and also for the LDIIIH1 and LDIIIH1CS proteins, with somewhat lower efficiencies. No inhibition was observed for LDIII, which does not contain the stem region, suggesting that stabilization of DIII binding by stem interactions may be important. We also tested the truncated DI/II proteins. None of these proteins inhibited DV fusion (data not shown).

**Membrane interactions of DV2 DI/II proteins.** We then tested the truncated E proteins for the ability to interact with target membranes at neutral and low pH. Purified E′-ST and the various DI/II constructs were mixed with liposomes and incubated at pH 8.0 or 5.75, and the liposomes and membrane-bound proteins were separated from unbound proteins by sucrose flotation gradients (Fig. 2). Proteins in the top (liposome-containing), middle, and bottom regions of the gradient were detected by SDS-PAGE and Western blotting. As expected based on previous results (23, 37), E′ protein containing all three domains bound membranes and floated efficiently when treated at low pH, and little binding was observed at neutral pH. The average flotation was ~50% of the total E′ protein after treatment at pH 5.75 and ~2% after treatment at pH 8.0. In contrast, the DI/II-ST protein showed significant membrane interaction when incubated with liposomes at either pH 5.75 or pH 8.0 (average flotation of ~43 to 50%, respectively). This pH-independent membrane association presumably reflects the constitutive exposure of the fusion loop in monomeric DI/II versus its low-pH-dependent exposure in the E′ dimer. We confirmed that membrane association of DI/II-ST occurs through the fusion loop by showing that it was specifically blocked by inclusion of exogenous pr peptide, which associates with the tip of DII and "caps" the fusion loop (A. Zheng and M. Kielian, unpublished data). The efficiency of the membrane interaction of DI/II-ST, which contains a minimal C-terminal tag composed of 10 residues, was thus similar to that observed for various E′ constructs containing all three domains (Fig. 2 and reference 37). However, the substitution of a longer tag on the DI/II protein (DI/II-V5-His) or the addition of either the DI-III linker region (DI/II-L-ST) or a second *Strep* tag (DI/II-STST) led to loss of DI/II membrane coflotation at either pH (Fig. 2 and data not shown). Thus, stable membrane binding could be affected by sequences distal to the fusion loop at the DII tip.

We used electron microscopy (EM) to analyze negatively stained samples of truncated E proteins after incubation with liposomes at neutral or low pH (Fig. 3). Samples were stained under neutral-pH conditions to prevent any effects of low pH during the staining process. As previously observed (23), the E′ protein associated with membranes in a low-pH-dependent reaction (Fig. 3B). Typical homotrimers were observed, with top views showing a donut-shaped morphology with 3-fold symmetry and side views showing conical trimers projecting from the membrane surface. Little membrane-associated E′ was observed at neutral pH (Fig. 3A). In keeping with the liposome flotation results, DI/II-ST associated with membranes at either neutral or low pH, with top views revealing donut-shaped structures with 3-fold symmetry and side views showing conical structures that projected from the membrane (Fig. 3C and D). These DI/II-ST trimers also associated laterally in small patches of hexagonal lattice (Fig. 3C and D). Such lattice formation was previously observed for the alphavirus E1 and DI/II proteins (6, 31). While the DV DI/II-L-ST and DI/II-V5-His proteins did not show significant membrane coflotation, electron microscopy revealed that both proteins associated with membranes and formed trimers (Fig. 3E and F and data not shown). These results suggest that the membrane association of the DV DI/II-L-ST and DI/II-V5-His proteins is less stable than that of E′ or DI/II-ST and is reversible during gradient flotation.

**DIII binding to the DI/II core trimer.** We next asked if the trimers formed by DV DI/II proteins could serve as targets for interaction with purified DIII proteins, thus recapitulating the interaction that occurs during formation of the final E protein hairpin. E′ protein or DI/II-ST was mixed with liposomes at low pH to form membrane-inserted trimers, which were then incubated at low pH with DIII protein containing the linker and stem region (LDIIIH1CS). The membrane-bound proteins were then separated on flotation gradients and detected with domain-specific antibodies. Negligible (~1% of the total)

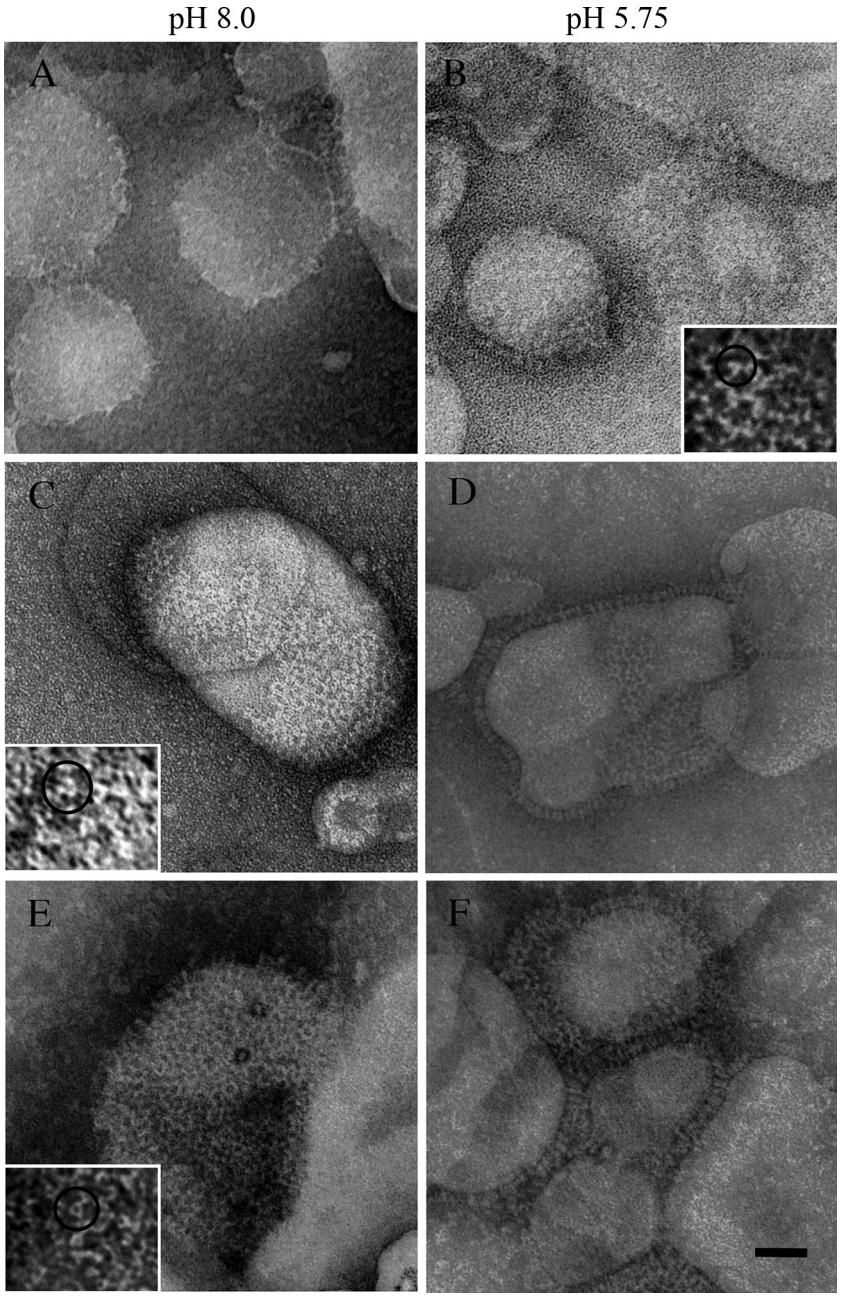


FIG. 3. Electron microscopy analysis of membrane interactions of truncated DV2 E proteins. Truncated E proteins were mixed with liposomes, incubated at pH 8.0 (A, C, and E) or 5.75 (B, D, and F) for 30 min at 25°C, stained with 3% ammonium molybdate at pH 7.4, and analyzed by electron microscopy. Representative images are shown for DV E′-ST (A and B), DI/II-ST (C and D), and DI/II-L-ST (E and F). Except for the insets, all images are shown at the same magnification, with the bar (F) representing 50 nm. The insets show a zoom view with a magnification 3.5 times that of the other panels.

LDIIIH1CS protein floated when incubated with liposomes alone or when incubated with liposomes containing preformed E′ trimers (Fig. 4A, right). The latter result is in keeping with the complete foldback of endogenous DIII on E′, thus "filling" the DIII binding sites. When preformed DI/II-ST trimers were incubated with LDIIIH1CS, however, ~14% of the added DIII floated to the top of the low-pH gradient. Stable binding of LDIIIH1CS required continued maintenance at low pH and was readily reversible by return of the sample to neutral pH (1% flotation on a pH 8 gradient).

The experiments shown in Fig. 2 and 3 indicated that, although various DI/II proteins could associate with membranes as visualized by EM, the membrane interactions of some of these proteins were relatively unstable and were not detected by liposome flotation assays. We therefore tested if DI/II protein-membrane interaction could be stabilized by the addition

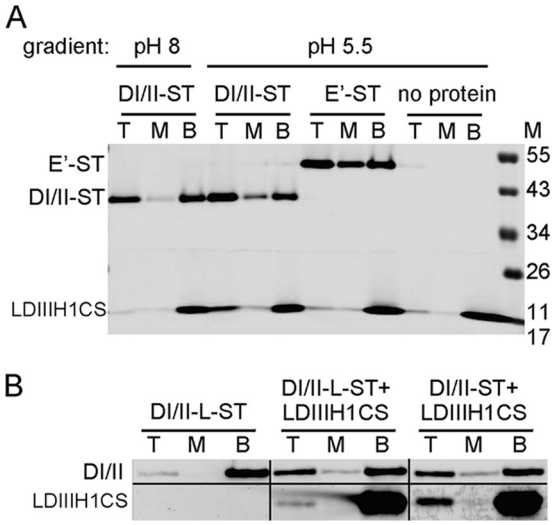


FIG. 4. Interaction of exogenous DIII protein with DI/II proteins. (A) Binding of DIII protein by DI/II trimers. Liposomes were mixed with DI/II-ST, E′-ST, or buffer without added protein, and the samples were treated at pH 5.75 for 30 min at 25°C. LDIIIH1CS was then added (9 μM final concentration), and the incubation at pH 5.75 continued for an additional 30 min at 25°C. The samples were then analyzed on sucrose flotation gradients as for Fig. 2, with the samples either adjusted to neutral pH and separated on a pH 8.0 gradient or maintained at low pH and separated on a pH 5.5 gradient, as indicated. The positions of the DV E proteins are indicated on the left, and the masses of the marker proteins are indicated on the right. (B) Stabilization of DI/II-membrane interaction by binding of DIII protein. Liposomes were mixed with DI/II-ST or DI/II-L-ST protein, and the samples were incubated at pH 5.75 for 30 min at 25°C. LDIIIH1CS was then added (9 μM final concentration) as indicated, and incubation at pH 5.75 continued for an additional 30 min at 25°C. The samples were then analyzed on sucrose flotation gradients at pH 5.5 as for Fig. 2. Shown is a representative example of 4 experiments.

of DIII proteins (Fig. 4B). DI/II-ST or DI/II-L-ST was treated at low pH in the presence of liposomes, and then LDIIIH1CS was added at low pH. Efficient coflotation of DI/II-ST plus LDIIIH1CS was observed, as shown in Fig. 4A. In the absence of added LDIIIH1CS, little flotation of DI/II-L-ST was observed (average, 7%), but significantly more protein was associated with liposomes when LDIIIH1CS was added (average, 33%). Thus, DIII proteins could interact specifically with the core trimer formed by various DI/II proteins, and this interaction stabilized the membrane association of the complex. However, even when the membrane-associated DI/II-ST core trimer was bound with LDIIIH1CS, the complex was not stable to solubilization in nonionic detergents such as Triton X-100 or OG (data not shown). This is in contrast to the E′ homotrimer, which was stable to detergent solubilization and showed essentially irreversible packing of DIII to the core trimer (data not shown and reference 23).

**Formation of a stable mixed trimer of E′ plus DI/II proteins.** Reasoning that the presence of subunits containing DIII could confer stability on the DI/II core trimer, we tested the ability of DI/II proteins to form mixed trimers with E′. DI/II-V5-His proteins were mixed with E′-ST and incubated at low pH in the presence of liposomes. The samples were then adjusted to neutral pH and tested for membrane association by sucrose flotation gradients. While the DI/II-V5-His protein alone did not stably

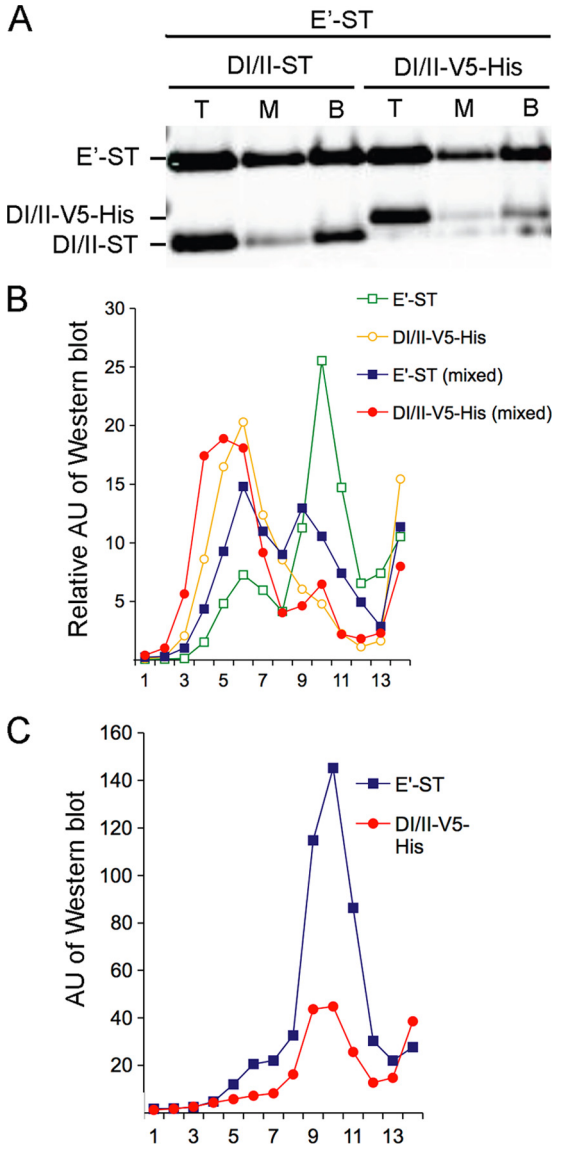


FIG. 5. Formation of stable mixed trimers of E′ and DI/II proteins. (A) E′ protein promotes stable membrane interaction of DI/II proteins. The indicated DI/II proteins (50 μg/ml) were mixed with E′ protein (100 μg/ml), and liposomes were added to a final concentration of 1 mM lipid. The samples were treated at pH 5.75 for 30 min at 25°C, adjusted to neutral pH, separated on sucrose flotation gradients at pH 8.0, and analyzed as for Fig. 2. (B) Sucrose sedimentation analysis of truncated E proteins. E′-ST, DI/II-V5-His (150 μg/ml), or a combination of both proteins (100 μg/ml and 50 μg/ml, respectively) was mixed with liposomes and incubated at low pH as for panel A. The samples were adjusted to neutral pH, solubilized with 1.5% OG, and analyzed by sucrose gradient sedimentation, followed by SDS-PAGE and Western blotting. AU, arbitrary units. (C) Isolation of membrane-bound mixed trimers. E′-ST and DI/II-V5-His (200 and 50 μg/ml, respectively) were mixed with liposomes, incubated at low pH, and separated on sucrose flotation gradients as for panel A. The top 140 μl from the gradient was solubilized with 1.5% OG and analyzed by sucrose gradient sedimentation as for panel B.

bind membranes (Fig. 2), the addition of E′ protein to the reaction resulted in efficient coflotation of both proteins, with ~69% of the DI/II-V5-His in the top, membrane-containing fraction even when the flotation was performed at neutral pH (Fig. 5A).

The increased membrane association of DI/II-V5-His was dependent on the ratio of E′ protein to DI/II protein (2:1 in Fig. 5A). Lower molar ratios, such as 1 E′ protein to 1 DI/II-V5-His protein, strongly reduced the liposome flotation of both proteins in the reaction, suggesting that a critical number of E′ subunits per trimer is required to produce stable membrane association. The presence of E′ also promoted the stable membrane association of the DI/II-ST protein, with ~60% of the protein in the top fraction (Fig. 5A) compared to 40 to 50% in the absence of E′ (Fig. 2).

The coflotation of DI/II and E′ with liposomes suggested that the two proteins were forming a mixed trimer that might have increased stability. We assayed this by treating E′, DI/II-V5-His, or a 2:1 mixture of these proteins at low pH in the presence of liposomes, followed by solubilization in OG and analysis on sucrose sedimentation gradients. Most of the E′ protein sedimented as a trimer after low-pH treatment with liposomes, while the DI/II-V5-His protein sedimented as a monomer (Fig. 5B), in keeping with the detergent lability of the core trimer. Analysis of the mixed-protein reaction showed that a fraction of both E′ and DI/II-V5-His migrated in the trimer position. To determine if this fraction represented the liposome-bound material, we isolated the membrane-associated proteins on flotation gradients, solubilized the membranes in OG, and then analyzed them in a sucrose sedimentation gradient (Fig. 5C). The membrane-bound proteins sedimented as a single peak migrating in the trimer position and containing both E′ and DI/II-V5-His. Thus, the presence of E′ proteins led to the formation of a mixed trimer (referred to here as [E′+DI/II]) that stably associated with membranes and was resistant to treatment with nonionic detergent.

**DIII binding to the mixed [E′-plus-DI/II] trimers.** We then asked if the [E′-plus-DI/II] trimer could serve as a target for binding of exogenous DIII. E′ and DI/II-V5-His proteins were mixed with purified DIII proteins containing the linker region and/or the H1 or H1CS regions of the stem domain (the constructs are shown in Fig. 1A). The protein mixtures were incubated at low pH in the presence of liposomes and then analyzed on sucrose flotation gradients at neutral pH (Fig. 6A). As previously observed (Fig. 5A), the [E′+DI/II] trimers floated in the membrane-containing fraction. When DIII protein was present during formation of the mixed trimer, it was also recruited to the membrane fraction. The flotation of DIII protein with the mixed-trimer-containing liposomes required the presence of both the linker region and the H1stem region. The strongest interaction was observed with LDIIIH1CS. Samples maintained at pH 5.75 during flotation showed identical DIII interaction, as in Fig. 5A (data not shown). Thus, DIII proteins that bound during formation of the mixed trimer at low pH remained stably associated upon return of the complex to neutral pH.

We tested whether DIII could bind to a preformed mixed trimer. The [E′+DI/II] trimer was generated by low-pH treatment in the presence of liposomes. Samples were then either maintained at low pH or adjusted to neutral pH, incubated with LDIIIH1CS, and analyzed by sucrose flotation gradients (Fig. 6B). The LDIIIH1CS protein interacted with the preformed mixed trimer, and binding was independent of the pH. Thus, low pH appears to be involved in initial E-protein rearrangements rather than in DIII interaction with the mixed core trimer.

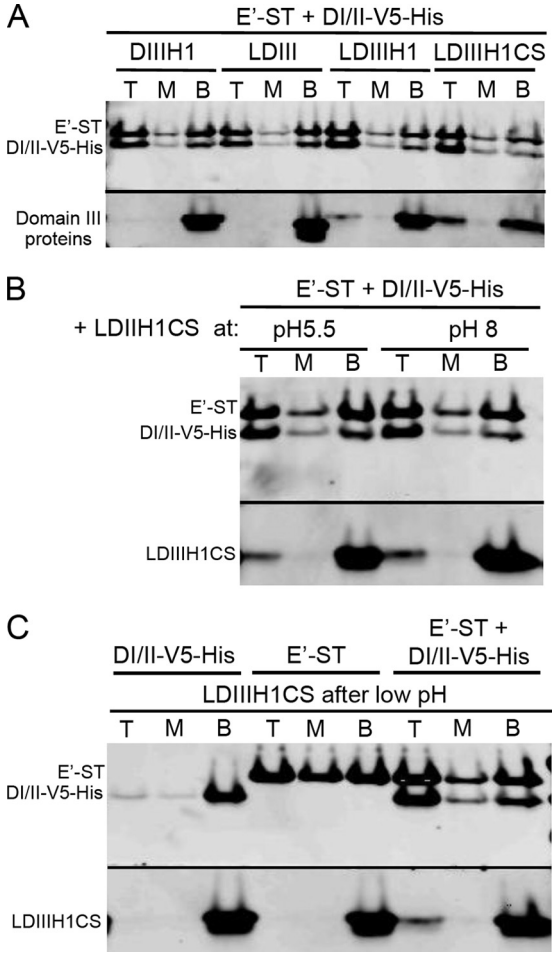


FIG. 6. Interaction of DIII with [E′+DI/II] mixed trimers. (A) DIII protein with linker and stem forms a stable complex with the mixed trimer. E′-ST and DI/II-V5-His (100 μg/ml and 50 μg/ml, respectively) plus the indicated DIII proteins (final protein concentration, 7 μM) were mixed with liposomes, and the samples were incubated at pH 5.75 for 30 min at 25°C. The samples were then adjusted to neutral pH, separated on sucrose flotation gradients at pH 8.0, and analyzed as for Fig. 2. Identical results were obtained if the samples and gradients were maintained at low pH (data not shown). (B) LDIIIH1CS protein binds the preformed mixed trimer in a pH-independent reaction. E′-ST and DI/II-V5-His (100 μg/ml and 50 μg/ml, respectively) were mixed with liposomes, and the samples were incubated at pH 5.75 for 30 min at 25°C. The samples were then adjusted to neutral pH where indicated, LDIIIH1CS was added (7 μM final concentration), and the incubation continued for an additional 30 min at 25°C. The samples were then analyzed on flotation gradients at either low pH or neutral pH as indicated. (C) LDIIIH1CS protein binds specifically to mixed trimers. E′-ST (150 μg/ml), DI/II-V5-His (50 μg/ml), or a mixture of E′-ST and DI/II-V5-His (100 μg/ml and 50 μg/ml, respectively) was mixed with liposomes, and the samples were incubated at pH 5.75 for 30 min at 25°C. The samples were then adjusted to neutral pH, LDIIIH1CS was added (7 μM final concentration), and incubation continued for an additional 30 min at 25°C. The samples were then analyzed on flotation gradients at pH 8.0. In each panel, the positions of the proteins are shown on the left of the gel.

We also evaluated the binding site involved in the DIII interaction. DI/II-V5-His, E′, or a mixture of these proteins

was incubated at low pH with liposomes to form trimers. LDIIIH1CS was then added at neutral pH, and the samples were analyzed by sucrose flotation gradients (Fig. 6C). As observed above, LDIIIH1CS was recruited to the membrane fraction containing the mixed [E′+DI/II] trimer. In contrast, the DI/II-V5-His proteins did not stably bind liposomes, and the added LDIIIH1CS also remained in the bottom of the gradient. Conversely, the E′ protein efficiently floated with the membrane fraction, but no binding of LDIIIH1CS was observed. Thus, our results indicate that LDIIIH1CS recruitment to membranes required both a trimer target and the presence of "empty" DIII binding sites.

## DISCUSSION

Refolding of the flavivirus E protein into a trimeric hairpin drives the fusion of the virus and target membranes. Here, we showed that truncated forms of the DV E protein can recapitulate trimer formation, producing insights into various aspects of trimerization. Similar to the viral E protein (34), the E′ protein was a relatively stable homodimer (23) that required low-pH-triggered dimer dissociation to permit subsequent fusion loop exposure, membrane insertion, and trimerization. In contrast, the DV E DI/II protein was monomeric, and its fusion loop was available to insert into target membranes without a requirement for low pH. Electron microscopy studies indicated that a variety of DI/II protein constructs formed trimers on target membranes at either pH 5.75 or pH 8.0. Stable membrane association and efficient liposome coflotation were observed for the DI/II-ST protein containing a minimal tag on DI. DI/II proteins containing additional sequences at the C terminus formed trimers, but their membrane association was reversible. Truncated DI/II trimers acted as targets for binding of DIII, and this binding stabilized their membrane interaction and coflotation. This is in keeping with the ability of exogenous DIII proteins to block DV fusion and infection, indicating the existence of a core trimer intermediate during virus fusion (Fig. 1D) (20). However, even when bound by DIII, truncated DI/II trimers were less stable than E′ trimers and dissociated when the liposomes were solubilized by nonionic detergents such as OG. Generation of stable trimer targets for DIII binding required a mixture of the E′ and DI/II proteins to produce a prefusion intermediate with unoccupied DIII binding sites. The stoichiometry of E′ and DI/II proteins producing optimal formation of this intermediate suggests that the mixed trimer was composed primarily of 2 E′ plus 1 DI/II proteins.

We used such [E′+DI/II] trimers to evaluate the properties of DIII binding and to gain insights into hairpin formation. Stable DIII binding was detected by liposome coflotation experiments and required the presence of both the DI-DIII linker region and the H1 stem region. LDIIIH1CS binding levels were similar at neutral and low pH, consistent with this late step in hairpin formation being pH independent. We note, however, that since formation of the mixed trimers required treatment at low pH, stabilization of the trimer conformation by foldback of the endogenous DIII could mask low-pH requirements for association of DIII with the core trimer. This may explain why stable binding of LDIIIH1CS to the DI/II-ST trimer was observed only at low pH.

No binding of LDIIIH1CS to the E′ trimer was observed, even though this trimer, which is missing the stem region, contains 3 unoccupied stem binding sites. Thus, in our *in vitro* system, the H1CS stem region acts primarily by stabilizing the binding of DIII, rather than by binding independently to the trimer.

**Properties of E protein-membrane interaction.** The DV E protein fusion loop is normally shielded from solvent, first by the heterodimeric interaction of E with the prM protein (19, 41) and later by the homodimeric interaction between E proteins on the mature virus (22). Our data showed that monomeric E DI/II proteins are soluble in aqueous buffers in the absence of such shielding. This is similar to the solubility of the alphavirus E1 fusion protein monomer (7, 31). In the alphavirus case, it appears that the flexible fusion loop has a somewhat different conformation in the soluble E1 protein than in the virus particle, where it is shielded by the chaperone protein E2 (29). Such a fusion loop conformational alteration may also occur for the DV E DI/II protein monomer and may help to account for its solubility. As the monomeric forms of both the DV and alphavirus fusion proteins efficiently insert into target membranes, any conformational changes resulting from the loss of the dimeric protein partner do not inactivate their fusion loops.

Our results demonstrated stable membrane association of the DI/II-ST core trimer. In contrast, membrane association of DI/II proteins with additional sequences at the C terminus was reversible and could be stabilized by binding of DIII. The presence of 3 fusion loops should have an additive effect in anchoring class II fusion protein trimers. We therefore interpret the reversibility of the membrane interaction of these DI/II proteins as reflecting the reversibility of intersubunit contacts within their core trimers, resulting in the release of DI/II monomers from the membrane. DIII binding would shift the monomer-trimer equilibrium by stabilization of the core trimer and thus indirectly stabilize membrane association by maintaining the cumulative action of three fusion loops.

**The role of low pH in flavivirus fusion protein conformational changes.** Studies of the TBE virus E protein suggest that low pH induces the dissociation of the E dimer (34) and the release of DIII from interaction with DI (36), thus freeing DIII to move toward the target membrane-inserted fusion loop and interact with the core trimer to produce the stable hairpin. Mutagenesis of the TBE virus E protein implicates a conserved DIII histidine residue, H323, in the control of dimer dissociation and DIII release (5). H323 also appears to play a role in promoting E refolding to the final stable trimeric hairpin (5). Recent studies of WNV suggest that, at least for some flaviviruses, the regulation of pH dependence is more complex, without a critical requirement for any individual histidine residue (27).

Our results with truncated DV E proteins have not addressed specific residue requirements for low-pH dependence, but they support the general steps in regulation of E protein trimerization identified in the TBE virus system. Thus, in the absence of homodimerization, DV DI/II proteins insert into membranes without any requirement for low pH. Trimer cores are formed on the membrane, but their stabilization requires interaction with DIII, either from an E′ protein in the trimer or from exogenous DIII. Low pH is needed to generate mixed

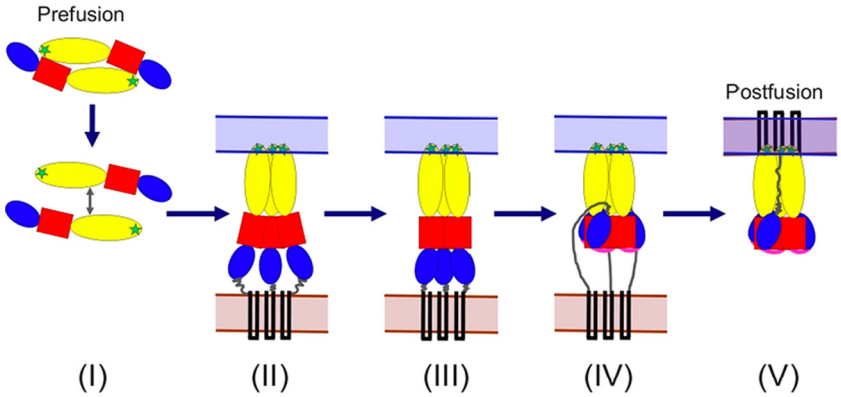


FIG. 7. Model for the steps in rearrangement of the dengue virus E protein during membrane fusion. DI, DII, and DIII are colored red, yellow, and blue, respectively. The hydrophobic fusion loop at the tip of DII is shown as a green star. The stem region is shown in gray and the TM domains in black. The virus membrane is shown in pink and the target membrane in blue. (I) At the top is shown the prefusion E-protein dimer, with the orientation looking down on the virus membrane. During the initial step of the fusion protein conformational change, the dimer dissociates upon exposure to low pH (bottom). (II to V) Side views of the trimerization reaction with the target membrane at the top. (II) The E fusion loops insert into the target membrane, and initial trimerization occurs between the DII tips. (III) Trimerization continues with contacts between DI and the β-strand exchange reaction. (IV) The DI-DIII linker inserts into the groove formed by strand exchange. DIII folds back against the core trimer, locking the linker into place. The trimer is now irreversible and stable in detergent. (V) In the final postfusion trimer, the stem has packed against the core trimer. The exact disposition of the fusion loops versus the stem and TM domains is not known, except that they are at the same end of the trimer, as shown in the model.

trimers containing E′ proteins, as E′ requires low pH for both dimer dissociation and release of DIII from the DI interface. In contrast, exogenous DIII can bind to preformed [E′+DI/II] trimers without a low-pH requirement, since DIII binding is downstream of both dimer dissociation and DIII-DI interface release. Studies of TBE virus suggest that initial insertion of the fusion protein into the target membrane is mediated by E-protein monomers (36). Our results suggest that there may be a monomer-core trimer equilibrium on the target membrane, which could explain the process of initial E membrane insertion and its subsequent stabilization. Our results also illustrate some limitations of biochemical assays of trimerization, since core trimers may be dissociated by detergent solubilization and may not produce stable membrane interaction.

**Comparison of the flavivirus and alphavirus fusion protein trimerization reactions.** We previously characterized truncated forms of the structurally related E1 fusion protein from the alphavirus SFV (31). The SFV E1 DI/II construct contains the DI-DIII linker region and was tested with and without various affinity tags. These SFV DI/II proteins cofloat with liposomes and form a DI/II core trimer that is stable in detergent. There is no requirement for mixed trimers or DIII interaction for stability, and there is no apparent effect of the tag sequence. Membrane coflotation and stable trimer formation require initial low-pH treatment but are then maintained upon shift to neutral pH. Similar to the DV proteins, the preformed SFV core trimer specifically binds DIII at either neutral or low pH. EM analysis of DI/II proteins on liposomes showed that the SFV core trimers interact to form hexagonal lattices. These SFV DI/II proteins show membrane-bending activity and generate elongated membrane tubules from the liposomes. While the DV DI/II proteins form trimers that interact in small patches of hexagonal lattice, membrane tubule formation was not observed with any of our DV constructs. These functional differences in membrane-bending activity of the SFV and DV core trimers could be due to the lower stability of the DV core trimer and/or to differences in the membrane interactions of the SFV and DV fusion loops.

Membrane fusion of both the alphaviruses and the flaviviruses is very rapid, reaching completion within seconds of low-pH treatment at 37°C (1, 3). Previous studies from our laboratory showed that exogenous DIII can specifically inhibit SFV and DV fusion and infection when present during low-pH treatment of virus prebound to the plasma membranes of cells (20). Inhibition is due to a block in final hairpin formation by the binding of exogenous DIII. Our current liposome coflotation results indicate that LDIIIH1CS shows the most stable binding to the DV core trimer (Fig. 6A), although this does not produce an increase in fusion inhibition (Fig. 1D). Thus, the outcome of the fusion infection assay is determined by more than the binding affinity of exogenous DIII with the core trimer. Longer DIII protein constructs may have higher affinity for the binding site but may also have more difficulties in gaining access to their target positions on the rearranging virus glycoprotein shell.

Experiments with both viruses showed that no inhibition of fusion was observed in the presence of exogenous DI/II proteins (reference 31 and this study). Thus, the foldback of DIII against the extended trimeric intermediate may be a more accessible target for inhibition than the initial formation of the core trimer. Fusion of both alphaviruses and flaviviruses normally occurs in the intracellular low-pH environment of the endosome, and the rapidity and intracellular location suggest that these fusion reactions may be relatively difficult to inhibit. The development of stable trimeric targets that bind DIII *in vitro* opens the possibility of screens for small-molecule inhibitors of alphavirus and flavivirus hairpin formation and their potential development as inhibitors of virus fusion and infection.

**Model for the steps in flavivirus fusion protein trimerization.** Our results and many structural and functional studies from a number of other laboratories (reviewed in references 11, 25, 30, and 35) suggest a model for the flavivirus fusion reaction (Fig. 7).

In step I, low pH triggers the dissociation of the E-protein dimer, releasing DIII from interaction with DI and allowing a change in the hinge angle between DI and DII. Step I is low pH dependent and reversible by a return to neutral pH (33). In step II, E proteins insert their fusion loops into target membranes in a pH-independent reaction and then interact via the fusion loops/DII to form an unstable trimer. While the initial E-membrane interaction is reversible, upon trimerization, it is stabilized by the formation of a hydrophobic patch consisting of 3 fusion loops. Step III involves the formation of stronger trimer contacts between DI of the 3 E proteins. This is due to a β-strand exchange reaction in DI, which generates annular contacts at the membrane-distal side of DI and a binding site for the DI-DIII linker region (23). These interactions are sensitive to the presence of exogenous sequences at the end of DI. In step IV, the DI-DIII linker fits into the groove formed by the strand exchange reaction. DIII and the stem region fold back to pack against the core trimer, locking the linker into position and preventing reversal of DI contacts. Concomitantly, these interactions generate the stable hairpin and drive membrane fusion. Step V shows the completed fusion reaction with the stem fully zipped up along the core trimer. Since *in vivo* the core trimer intermediate very rapidly transitions to the final postfusion conformation (20), *in vitro* reconstitution is an important tool to dissect this conformational state. Our data, in particular, highlight the reversibility of the initial membrane insertion of the fusion loop, the stepwise formation and stabilization of the core trimer, and the role of low pH in initial triggering versus in promotion of the final hairpin. Further studies of the formation of the outer layer of the DV trimer will define the importance of the linker, DIII, and stem region and determine if their binding to the core trimer occurs in stepwise fashion to drive specific stages of membrane fusion.

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## REFERENCES

1. **Bron, R., J. M. Wahlberg, H. Garoff, and J. Wilschut.** 1993. Membrane fusion of Semliki Forest virus in a model system: correlation between fusion kinetics and structural changes in the envelope glycoprotein. EMBO J. **12:**693–701.
2. **Clarke, T.** 2002. Dengue virus: break-bone fever. Nature **416:**672–674.
3. **Corver, J., A. Ortiz, S. L. Allison, J. Schalich, F. X. Heinz, and J. Wilschut.** 2000. Membrane fusion activity of tick-borne encephalitis virus and recombinant subviral particles in a liposomal model system. Virology **269:**37–46.
4. **Crill, W. D., and G. J. Chang.** 2004. Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes. J. Virol. **78:**13975–13986.
5. **Fritz, R., K. Stiasny, and F. X. Heinz.** 2008. Identification of specific histidines as pH sensors in flavivirus membrane fusion. J. Cell Biol. **183:**353–361.
6. **Gibbons, D. L., I. Erk, B. Reilly, J. Navaza, M. Kielian, F. A. Rey, and J. Lepault.** 2003. Visualization of the target-membrane-inserted fusion protein of Semliki Forest virus by combined electron microscopy and crystallography. Cell **114:**573–583.
7. **Gibbons, D. L., B. Reilly, A. Ahn, M.-C. Vaney, A. Vigouroux, F. A. Rey, and M. Kielian.** 2004. Purification and crystallization reveal two types of interactions of the fusion protein homotrimer of Semliki Forest virus. J. Virol. **787:**3514–3523.
8. **Gibbons, D. L., M.-C. Vaney, A. Roussel, A. Vigouroux, B. Reilly, J. Lepault, M. Kielian, and F. A. Rey.** 2004. Conformational change and protein-protein interactions of the fusion protein of Semliki Forest virus. Nature **427:**320–325.
9. **Gubler, D. J.** 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol. **10:**100–103.
10. **Halstead, S. B.** 2007. Dengue. Lancet **370:**1644–1652.
11. **Harrison, S. C.** 2008. Viral membrane fusion. Nat. Struct. Mol. Biol. **15:**690–698.
12. **Heinz, F. X., and S. L. Allison.** 2000. Structures and mechanisms in flavivirus fusion. Adv. Virus Res. **55:**231–269.
13. **Hrobowski, Y. M., R. F. Garry, and S. F. Michael.** 2005. Peptide inhibitors of dengue virus and West Nile virus infectivity. Virol. J. **2:**49.
14. **Ivy, J., E. Nakano, and D. Clements.** October 2000. Subunit immunogenic composition against dengue infection. U.S. patent 6,165,477.
15. **Kaufmann, B., P. R. Chipman, H. A. Holdaway, S. Johnson, D. H. Fremont, R. J. Kuhn, M. S. Diamond, and M. G. Rossmann.** 2009. Capturing a flavivirus pre-fusion intermediate. PLoS Pathog. **5:**e1000672.
16. **Kuhn, R. J., W. Zhang, M. G. Rossman, S. V. Pletnev, J. Corver, E. Lenches, C. T. Jones, S. Mukhopadhyay, P. R. Chipman, E. G. Strauss, T. S. Baker, and J. H. Strauss.** 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell **108:**717–725.
17. **Kyle, J. L., and E. Harris.** 2008. Global spread and persistence of dengue. Annu. Rev. Microbiol. **62:**71–92.
18. **Lescar, J., A. Roussel, M. W. Wien, J. Navaza, S. D. Fuller, G. Wengler, and F. A. Rey.** 2001. The fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH. Cell **105:**137–148.
19. **Li, L., S. M. Lok, I. M. Yu, Y. Zhang, R. J. Kuhn, J. Chen, and M. G. Rossmann.** 2008. The flavivirus precursor membrane-envelope protein complex: structure and maturation. Science **319:**1830–1834.
20. **Liao, M., and M. Kielian.** 2005. Domain III from class II fusion proteins functions as a dominant-negative inhibitor of virus-membrane fusion. J. Cell Biol. **171:**111–120.
21. **Lindenbach, B. D., H.-J. Thiel, and C. M. Rice.** 2007. Flaviviruses: the viruses and their replication, p. 1101–1152. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields' virology, 5th ed. Lippincott, Williams & Wilkins, Philadelphia, PA.
22. **Modis, Y., S. Ogata, D. Clements, and S. C. Harrison.** 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc. Natl. Acad. Sci. U. S. A. **100:**6986–6991.
23. **Modis, Y., S. Ogata, D. Clements, and S. C. Harrison.** 2004. Structure of the dengue virus envelope protein after membrane fusion. Nature **427:**313–319.
24. **Modis, Y., S. Ogata, D. Clements, and S. C. Harrison.** 2005. Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. J. Virol. **79:**1223–1231.
25. **Mukhopadhyay, S., R. J. Kuhn, and M. G. Rossmann.** 2005. A structural perspective of the flavivirus life cycle. Nat. Rev. Microbiol. **3:**13–22.
26. **Nayak, V., M. Dessau, K. Kucera, K. Anthony, M. Ledizet, and Y. Modis.** 2009. Crystal structure of dengue virus type 1 envelope protein in the postfusion conformation and its implications for membrane fusion. J. Virol. **83:**4338–4344.
27. **Nelson, S., S. Poddar, T. Y. Lin, and T. C. Pierson.** 2009. Protonation of individual histidine residues is not required for the pH-dependent entry of West Nile virus: evaluation of the "histidine switch" hypothesis. J. Virol. **83:**12631–12635.
28. **Polo, S., G. Ketner, R. Levis, and B. Falgout.** 1997. Infectious RNA transcripts from full-length dengue virus type 2 cDNA clones made in yeast. J. Virol. **71:**5366–5374.
29. **Roussel, A., J. Lescar, M.-C. Vaney, G. Wengler, G. Wengler, and F. A. Rey.** 2006. Structure and interactions at the viral surface of the envelope protein E1 of Semliki Forest virus. Structure **14:**75–86.
30. **Sánchez-San Martín, C., C. Y. Liu, and M. Kielian.** 2009. Dealing with low pH: entry and exit of alphaviruses and flaviviruses. Trends Microbiol. **17:**514–521.
31. **Sánchez-San Martín, C., H. Sosa, and M. Kielian.** 2008. A stable prefusion intermediate of the alphavirus fusion protein reveals critical features of class II membrane fusion. Cell Host Microbe **4:**600–608.
32. **Stadler, K., S. L. Allison, J. Schalich, and F. X. Heinz.** 1997. Proteolytic activation of tick-borne encephalitis virus by furin. J. Virol. **71:**8475–8481.
33. **Stiasny, K., S. L. Allison, A. Marchler-Bauer, C. Kunz, and F. X. Heinz.** 1996. Structural requirements for low-pH-induced rearrangements in the envelope glycoprotein of tick-borne encephalitis virus. J. Virol. **70:**8142–8147.
34. **Stiasny, K., S. L. Allison, J. Schalich, and F. X. Heinz.** 2002. Membrane interactions of the tick-borne encephalitis virus fusion protein E at low pH. J. Virol. **76:**3784–3790.
35. **Stiasny, K., and F. X. Heinz.** 2006. Flavivirus membrane fusion. J. Gen. Virol. **87:**2755–2766.

36. **Stiasny, K., C. Kossl, J. Lepault, F. A. Rey, and F. X. Heinz.** 2007. Characterization of a structural intermediate of flavivirus membrane fusion. PLoS Pathog. **3:**e20.
37. **Umashankar, M., C. Sanchez San Martin, M. Liao, B. Reilly, A. Guo, G. Taylor, and M. Kielian.** 2008. Differential cholesterol binding by class II fusion proteins determines membrane fusion properties. J. Virol. **82:**9245–9253.
38. **van der Schaar, H. M., M. J. Rust, C. Chen, H. van der Ende-Metselaar, J. Wilschut, X. Zhuang, and J. M. Smit.** 2008. Dissecting the cell entry pathway of dengue virus by single-particle tracking in living cells. PLoS Pathog. **4:**e1000244.
39. **WHO.** 2009. Dengue and dengue haemorrhagic fever. WHO fact sheet 117. WHO, Geneva, Switzerland.
40. **Yu, I. M., H. A. Holdaway, P. R. Chipman, R. J. Kuhn, M. G. Rossmann, and J. Chen.** 2009. Association of the pr peptides with dengue virus at acidic pH blocks membrane fusion. J. Virol. **83:**12101–12107.
41. **Yu, I. M., W. Zhang, H. A. Holdaway, L. Li, V. A. Kostyuchenko, P. R. Chipman, R. J. Kuhn, M. G. Rossmann, and J. Chen.** 2008. Structure of the immature dengue virus at low pH primes proteolytic maturation. Science **319:**1834–1837.
42. **Zhang, Y., W. Zhang, S. Ogata, D. Clements, J. H. Strauss, T. S. Baker, R. J. Kuhn, and M. G. Rossmann.** 2004. Conformational changes of the flavivirus E glycoprotein. Structure **12:**1607–1618.