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Insertion of Dengue E into lipid bilayers studied by neutron reflectivity and molecular dynamics simulations

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ABSTRACT

The envelope (E) protein of Dengue virus rearranges to a trimeric hairpin to mediate fusion of the viral and target membranes, which is essential for infectivity. Insertion of E into the target membrane serves to anchor E and possibly also to disrupt local order within the membrane. Both aspects are likely to be affected by the depth of insertion, orientation of the trimer with respect to the membrane normal, and the interactions that form between trimer and membrane. In the present work, we resolved the depth of insertion, the tilt angle, and the fundamental interactions for the soluble portion of Dengue E trimers (sE) associated with planar lipid bilayer membranes of various combinations of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-rac-glycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and cholesterol (CHOL) by neutron reflectivity (NR) and by molecular dynamics (MD) simulations. The results show that the tip of E containing the fusion loop (FL) is located at the interface of the headgroups and acyl chains of the outer leaflet of the lipid bilayers, in good agreement with prior predictions. The results also indicate that E tilts with respect to the membrane normal upon insertion, promoted by either the anionic lipid POPG or CHOL. The simulations show that tilting of the protein correlates with hydrogen bond formation between lysines and arginines located on the sides of the trimer close to the tip (K246, K247, and R73) and nearby lipid headgroups. These hydrogen bonds provide a major contribution to the membrane anchoring and may help to destabilize the target membrane.

1. Introduction

All enveloped viruses have a dedicated protein that promotes fusion between viral and host membranes [1–3]. Fusion proteins are critical for infectivity and are targets of therapeutic intervention. All fusion proteins have a hydrophobic peptide sequence that inserts into the host membrane although the sequences and structures vary considerably among the different enveloped viruses. Although many structures have been solved, the functional mechanisms of fusion proteins and their hydrophobic peptides are still under considerable debate [4,5]. Possible functions include anchoring into the host membrane strongly enough to support the high-energy membrane bending that must occur to form a fusion stalk; [4,6–8] promoting positive curvature in the target membrane and dimple formation, or promoting negative curvature as required to form a fusion stalk; [1,4,9,10] or disrupting the local ordering of the host membrane to facilitate mixing with the viral membrane [1,4,11]. With regard to the latter, prior studies have shown that fusion peptides lower the rupture tension of membranes [12–15]. Molecular simulations have suggested that the fusion peptide of influenza promotes splaying of lipid tails, such that one tail protrudes from the host

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membrane, thereby promoting fusion [16,17]. More work is needed to understand fully the detailed mechanisms by which such proteins promote fusion and the roles of their fusion peptides.

In the present work, we studied the fundamental interactions of the soluble portion of the envelope protein of Dengue virus serotype 2 with lipid membranes. Dengue virus (DV) is a flavivirus that is endemic to tropical and subtropical regions of the world [18,19]. The envelope protein (E) is comprised of three domains and is arranged as a head-to-tail dimer in the mature virus, but reorganizes into head-to-head trimers upon exposure to low pH within endosomes [20]. Residues 98-111 comprise the fusion loop (FL) at the tip of the trimer that inserts into the target membrane. While much prior work has focused on the importance of the FL, we show below that positively-charged arginine and lysine residues (R73, K246 and K247) located on the sides of the trimer (within 10 Å from the FL) are also important in the interaction of Dengue E with the target membrane, as suggested recently for the flavivirus St. Louis encephalitis virus [21]. Our simulations indicate that hydrogen bonds formed between these residues and phosphates in the lipid headgroups provide substantially more enthalpic interaction energy than the interaction of the fusion loop with the membrane.

A great deal of evidence indicates that the sequence of the FL of DV is important for fusion, strongly suggesting that the structure, positioning, and specific interactions of the fusion loop within the bilayer are crucial. The amino acid (AA) sequence of the FL is highly conserved among flaviviruses (Fig. S1) [8,14,15,22]. Mutational studies have shown that fusion efficiency is extremely sensitive to various residues in the FL, especially W101, L107, and F108 [7,8,23]. However, the basis for the extreme sensitivity to these aspects is not yet clear. Residues W101, L107, and F108 have been collectively referred to as a hydrophobic anchor [6–8]. But considering membrane binding energies of a large number of peripheral membrane-associated proteins [24] it is surprising that a protein that associates only with the headgroup region of the outer leaflet and inserts only a few hydrophobic residues into the lipid tails will bind irreversibly and remain anchored in the membrane in the presence of large membrane bending stresses that must occur during formation of the fusion stalk. For comparison, a single myristate group (14 carbons) is known to be insufficient to provide stable anchoring to lipid membranes [25–27]. While hydrophobic interactions between the FL and the lipid membrane may contribute to anchoring, the modest interaction energy afforded by three strongly hydrophobic residues (nine in the trimer) and conservation of the structure and sequence of the FL suggests a critical role beyond this.

As is common for enveloped viruses, fusion of flaviviruses has been shown to depend upon the lipid composition of the target membrane. In particular, fusion depends strongly on anionic lipids (AL) and, in the absence of AL, on CHOL [28–31]. Interestingly, the dependence of fusion on CHOL occurs despite lack of a strong association between CHOL and sE [32]. While the viral membrane composition can also impact fusion and will differ for virus produced in different types of cells such as vertebrate and insect cells, we focus here on the composition of the endosomal membrane and its impact on fusion.

In this work, we studied the structure and fundamental interactions of Dengue sE inserted into membranes of 70:30 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC): 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-rac-glycerol (POPG), 49:21:30 POPC:POPG:CHOL, and 49:21:30 POPC:1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE):CHOL by neutron reflectivity (NR). We also studied 70:30 POPC:POPG, 1:1 POPC:POPE, and 1:1:1 POPC:POPE:CHOL by molecular dynamics (MD) simulations. This work was motivated by the desire to understand, for flaviviruses, the fundamental bases for i) the critical role of the fusion loop, as indicated by a high degree of conservation; ii) the critical role of AL to fusion, as reported recently; [33] and iii) cholesterol-enhanced binding of sE [30,32], lipid mixing [29,30,33], and fusion [28] (in absence of AL) despite the lack of a strong specific interaction of CHOL with sE [32].

The combined NR and simulation results resolve the depth of insertion, the orientation of sE with respect to the membrane normal, and the fundamental interactions that occur between sE and the lipids. The results show that positively-charged lysines K246 and K247 and arginine R73 in the vicinity of the FL contribute significantly to the interaction. In particular, K246 and K247 each form multiple hydrogen bonds with phosphates of lipid headgroups that cause the trimer to tilt upon insertion. These hydrogen bonds are promoted by both AL and CHOL, but the effect is much stronger for AL. The results suggest that these hydrogen bonds play an important role in membrane anchoring, along with the FL.

2. Materials and methods

2.1. Materials

POPC, POPG sodium salt, POPE, and CHOL were purchased from Avanti Polar Lipids. The HC18 (*Z*-20-(*Z*-octadec-9-enyloxy)-3,6,9,12,15,18,22-heptaoxatetra-cont-31-ene-1-thiol) tethering compound was synthesized at NIST as previously reported [34].

2.2. Expression and purification of DV2 sE

The DV2 construct (strain NGC) used in this work consists of prM and E ectodomain residues 1-395 with single or double strep tag, and has been described in detail elsewhere [35]. This construct was expressed in S2 cells as follows. S2 cells were grown in SFX-Insect medium (Thermo Hyclone). Initially, we used alternative serum-free media (Insectagro-DS2, Life Technologies) but obtained mediocre growth rates relative to other media and poor protein yield. Shaking cultures in 2 L baffled flasks containing 600 mL of medium were inoculated at 3–6 $\times 10^6$ cells per mL and copper sulfate was added at a final concentration of 1 mM on culture day 0. On days 7–9, culture supernatant was harvested by centrifugation at 13,000 $\times g$, 15 min, 4 °C. For each batch, a total of 8 L of clarified and 0.2 μm-filtered supernatant was then concentrated to 1 L using Vivaflow 200 concentrators (Sartorius, 10,000 Da cutoff). Egg white avidin (Life Technologies) was then added to the supernatant at a final concentration of 15 μg/mL to bind free biotin that would interfere with purification, and the pH was slowly adjusted to 8.0 using 0.5 M NaOH with rapid stirring (M = mol/L). During our efforts, we determined that buffer exchange was unnecessary for binding to streptactin and generally caused formation of a crystalline precipitate that clogged the affinity columns. Supernatant was then passed over two 1 mL streptactin columns (Qiagen) in series at a flow rate of 1 mL/min. Columns were washed with 40 mL PBS pH 8 then eluted using 5 mM desthiobiotin (Sigma) in wash buffer. Buffer exchange (1:100–1:500) and concentration were done using centrifugal ultrafiltration and TAN buffer (20 mM triethanolamine, pH 8.0, 130 mM NaCl). Purified protein in TAN buffer was quantified by UV spectrophotometer (NanoDrop2000) using a calculated molecular weight of 46,906 Da and molar absorption coefficient ε of 59,190. Aliquots were stored at −80 °C.

2.3. Preparation of tethered lipid bilayer membranes

Adsorption of sE to tethered bilayer lipid membranes (tBLMs) was studied by NR. tBLMs were prepared on HC18-self-assembled monolayer (SAM)-coated 3′ diameter Silicon wafers (El-Cat Inc., Ridgefield Park, NJ, USA) assembled in a NCNR reflectometry flow cell [36] as previously described [34]. The tBLMs were completed by liposome fusion. Liposomes of the desired composition were prepared as follows. The lipid mixture in either chloroform (49:21:30 POPC:POPE:CHOL) or 90:10 chloroform:methanol (70:30 POPC:POPG, 49:21:30 POPC:-POPG:CHOL) was dried in a clean glass vial using a steady stream of nitrogen gas and then further dried for a minimum of 4 h under vacuum to remove excess solvent. The lipid mixture was then rehydrated for 10 min in a high salt TAN buffer (20 mM triethanolamine, 500 mM