STUDYING THE ROLE OF HIGHLY CONSERVED RESIDUES IN THE STEM REGION OF DENV 1 ENVELOPE PROTEIN ON ASSEMBLY

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ABSTRACT

Dengue virus (DENV), a flavivirus with four serotypes (DENV1 to DENV4), is the leading cause of arboviral diseases worldwide. Despite decades of studies on dengue, no antiviral against DENV is currently available. The envelope (E) protein is involved in virus entry and assembly. The C-terminus of E protein contains two alpha-helices (EH1 and EH2) separated by conserved sequences (CS) in the stem region and two transmembrane domains in the anchor region. Previous cryo-electron microscopic (EM) study revealed that EH1 and EH2 are partially buried in the outer leaflet of viral membrane. The stem region contains 16 residues absolutely conserved among diverse flaviviruses. We hypothesize that the absolutely conserved stem residues are involved in critical steps of virus life cycle including assembly. In this study, we investigated the roles of the absolutely conserved stem residues on virus assembly and the mechanisms involved. Our long-term goal is to explore the stem region of DENV E protein as novel targets for the development of antivirals.

In the first specific aim, we investigated the role of the absolutely conserved stem residues on virus assembly of DENV1 by examining the production of virus-like particles (VLPs). VLPs are similar to infectious virions in the structural, biochemical and antigenic properties, and can be generated by co-expression of precursor membrane (prM) and E proteins. Using a series of DENV1 prM/E expressing constructs containing a single
proline or alanine substitution for each of the 16 highly conserved stem residues by site-directed mutagenesis we were able to examine the role that these play in assembly. After transfection to 293T cells, cell lysates and pellets derived from ultracentrifugation of cultural supernatants were subjected to Western blot analysis and a quantitative capture-ELISA using known concentrations of a recombinant E protein as standard. Compared with those of the wild type (WT), the amounts of E protein in pellets relative to cell lysates were reduced in 15 proline mutants at the EH1, CS and EH2 domains and greatly reduced in 9 mutants at the CS and EH2 domains, suggesting the importance of overall helical structure on assembly and the critical role of residues at the CS and EH2 domains,

In the second specific aim, we investigated the mechanisms of impairment in assembly of DENV1 mutants. Immunoprecipitation assay revealed that alanine substitutions do not affect the prM-E interaction. Enzyme digestion experiment showed that these alanine substitutions do not affect the glycosylation of E protein and both WT and mutant prM/E proteins were primarily located in the ER. Subcellular fractionation experiment revealed that the amounts of prM/E proteins of the alanine mutants in the soluble fraction relative to membrane fraction were either less or greater than that of WT, suggesting that some alanine mutations affect the budding of VLPs from the ER membrane to the lumen of ER, whereas other alanine
mutations affect the release of VLPs from ER lumen or other intracellular vesicles to outside of the cells. We also set up a transmission EM and immuno-gold EM to explore the morphological defects of these mutants.

In summary, our findings indicate several absolutely conserved stem residues are involved in the assembly step of DENV life cycle; in particular, 9 residues in the CS and EH2 domains of the stem region are critical. While considerable efforts have been made to develop antivirals against DENV and other flaviviruses, there are no licensed antivirals against DENV currently available. The absolutely conserved nature of these residues may be potential targets for antiviral strategy to block virus assembly.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADE</td>
<td>Antibody dependent enhancement</td>
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<tr>
<td>DENV</td>
<td>Dengue virus</td>
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<td>DHF</td>
<td>Dengue hemorrhagic fever</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DSS</td>
<td>Dengue shock syndrome</td>
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<td>E</td>
<td>Envelope protein</td>
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<td>EH</td>
<td>Envelope Helix</td>
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<td>EM</td>
<td>Electron microscopy</td>
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<td>Endoplasmic reticulum</td>
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<td>IP</td>
<td>Immunoprecipitation</td>
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<td>JEV</td>
<td>Japanese encephalitis virus</td>
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<td>Kb</td>
<td>Kilo-base</td>
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<td>PCR</td>
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<td>Premembrane protein</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron microscopy</td>
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<tr>
<td>VLPs</td>
<td>Virus-like particles</td>
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<td>WB</td>
<td>Western blot</td>
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<td>WNV</td>
<td>West Nile virus</td>
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<td>YFV</td>
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CHAPTER 1
INTRODUCTION
Dengue virus

Identification and Classification

Flavivirus is a genus that consists of more than 70 viruses with most of them belonging to arthropod-borne human pathogens. Flaviviruses can cause a variety of diseases: fevers, encephalitis, and hemorrhagic fevers. Many of them have become global concerns. These include dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), and yellow fever virus (YFV). Other flaviviruses are of regional or endemic concern. These include Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV), and tick-borne encephalitis virus (TBEV). The decrease in mosquito control in conjunction with societal factors (e.g., increased transportation and dense urbanization) have contributed to the re-emergence of these arthropod-borne viruses in places such as South and Central America, many parts of Asia and the U.S. (1).

With the development of the first live-attenuated flavivirus vaccine, YFV strain 17D, by Max Theiler's in the 1950s, advancement in tissue culture and technology has led to the production of other vaccines, including inactivated TBEV and JEV for use in humans and inactivated WNV, which is issued for use in animals. However, there is still much research and development going on for a safe and effective DENV vaccines that exhibit cross-protection (1).
Epidemiology

DENV is transmitted by mosquito vectors, *Aedes aegypti* and *Aedes albopictus*. It has been estimated that more than 3 billion people in over 100 countries are at risk of infection and more than 50 million DENV infections occur annually, resulting in around 500,000 people being hospitalized with severe dengue worldwide (2, 3). Approximately 2.5% of those affected die and most of them are children (2). Infection is often characterized by fever, headache, pain in various parts of the body, lymphadenopathy and leucopenia (4). However, the more serious symptoms are referred to as Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS). DHF is characterized by abnormal hemostasis and increased vascular permeability, which in some cases can lead to DSS (4). Dengue is not only considered a major health problem in many countries in Asia but is also found in many other areas, particularly those in subtropical and tropical regions. Thus far there are four distinct serotypes of DENV (DENV1-4) with numerous strain variations found worldwide (4). Transmission of the virus is carried out by the mosquitoes *Aedes aegypti* and *Aedes albopictus*. So far the only known hosts for DENV are humans, nonhuman primates and the mosquito vectors. As a result there is no good animal model for which the virus can be studied (1). Still studies are being done on mice and monkeys to find candidates for a suitable dengue vaccine for humans.

Genome Structure and Replication
DENV is a positive-sense, single-stranded RNA virus with a genome of about 10.6 kb. The genome is flanked by a 5’ and 3’ untranslated regions and contains a single open reading frame encoding a polyprotein precursor, which is cleaved by cellular and viral protease into three structural proteins, the capsid (C), premembrane (prM) and envelope (E) proteins; and seven non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (5) (Figure 1).

Viral entry is initiated by the binding of E protein to a cellular receptor and receptor-mediated endocytosis (5-8). Low pH environment in the endosome triggers a series of conformational changes of the E protein resulting in fusion of viral and endosomal membranes and release of the nucleocapsid into the cytoplasm (5, 7, 9). Viral RNA is translated as a single polyprotein that is processed by viral and cellular proteases into three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A/B, NS3, NS4A/B and NS5). Viral RNA replication occurs in the replication complexes containing RNA and NS proteins, which are associated with ER membranes (5). After translation and RNA replication, assembly occurs in the membrane structure of the ER. It is here that the immature virions are thought to bud into the lumen of the ER, transport through the secretory pathway where they undergo maturation by furin-mediate cleavage of prM protein to membrane (M) protein and precur (pr) peptide in the trans-Golgi; mature virions are then released outside of the cell (5, 7, 10) (Figure 2).
During viral replication DENV is capable of forming virus-like particles (VLPs), which are smaller and sediment slower than mature virions (5). Studies have shown that co-expression the prM and E proteins are sufficient to generate recombinant VLPs. VLPs are similar to infectious virus in their structural and biochemical properties (11), and has been useful in studying assembly and fusion, serving as non-infectious serodiagnostic antigen and vaccine candidate (12-15).

Clinical Disease

Although most individuals are asymptomatic after DENV infection, there are a plethora of symptoms that follow DENV infection and have come to be described over years of observations since its first discovery in the 1700s. After an incubation period of 5 to 8 days, classical dengue begins to present with an abrupt fever (103 to 106°F) which is accompanied by frontal or retro-orbital headache. From there the host can experience a range of symptoms that include myalgia, chilliness, backache, and malaise. Flushing of the face and a generalized, transient, macular rash that blanches under pressure can be seen at 24 to 48 h of fever. During day 2 to day 6 of fever there can be pronounced anorexia, nausea and vomiting, generalized lymphadenopathy, and cutaneous hyperalgesia. Usually the fever will persist for 4 to 6 days and terminates with a crisis. High viremia generally coincides with fever. Defervescence is usually associated with intense sweating. On the last day of fever or within 24 h, a secondary morbilliform or macropapular rash lasting for 1 to 5 days sometimes appears. With the appearance of the secondary rash, a second rise in temperature can occur, resulting in
a saddleback fever profile. Toward the end of the febrile period or immediately after defervescence, as the generalized rash fades, localized clusters of pinpoint hemorrhagic lesions (petechiae) may appear over the dorsum of the feet, on the legs, hands, or fingers, or occasionally on the mucous membranes of the oral cavity (16). Gastrointestinal bleeding, menorrhagia, and bleeding from other organs have also been described in cases with adults (17). In cases with children they can often present with respiratory symptoms including cough, sore throat, and rhinitis (18).

The more serious illnesses of DHF and DSS are becoming more prevalent in certain regions. In these cases it begins with symptoms indistinguishable from those of classic DF, followed 2 to 5 days later by rapid deterioration, physical collapse, and sometimes death (19, 20). The second phase of the disease coincides with a period of defervescence. Petechiae, easy bruising, bleeding at venipuncture sites, and large spontaneous ecchymoses are frequently observed. Hepatomegaly is occasionally described. Hemorrhage, regardless of site and severity, when not accompanied by thrombocytopenia and hypovolemia, does not satisfy the criteria for DHF. The presence of thrombocytopenia and hemoconcentration differentiates grade I and II DHF from classical DF with hemorrhagic manifestations. Shock is the single criterion for discriminating between grades I and II and grades III and IV (4).

Pathogenesis
The mosquito vector, *Aedes aegypti*, is infected as they feed on humans during the period of viremia, which usually lasts about five days. The virus passes from the mosquito intestinal tract to the salivary glands in a period that takes approximately 10 days (21). Mosquito bites often result in infection, which has been thought to be promoted by the mosquitoes salivary proteins (22). Once injected into the dermis DENV virus will infect immature dendritic cells through ICAM3-grabbing non-integrin (DC-SIGN) (23). As with any other infection the infected dendritic cells undergo maturation and migrate to local or regional lymph nodes where they present viral antigens to T cells and initiate cellular and humoral immunity. Studies have shown DENV replication in the macrophages of lymph nodes, liver and spleen, as well as in peripheral blood monocytes (24). *In vitro* and *in vivo* studies have also shown that macrophages and monocytes cells participate in antibody dependent enhancement (ADE) (25-27). ADE is said to occur when phagocytes are infected via their Fc receptors by the immune complexes that form between DENVs and non-neutralizing antibodies or neutralizing antibodies at suboptimal concentration. Non-neutralizing antibodies are the result of a prior infecting dengue serotype or in the case of infants can come from low concentrations of dengue antibodies from maternal sera (28).

While much of the basic immunology is known there are still several aspects of the immune system and its relationship to DENV infection that have yet to be clarified. So far *in vitro* studies have demonstrated that infected human monocytes and mature dendritic cells can result in increased virus replication when the interferon system is suppressed (29). Type I interferon-associated genes have been found to have
decreased activation in the peripheral blood mononuclear cells of patients with severe dengue disease compared to those with milder disease pathology (30). Any number of situations can occur in an individual infected with DENV. For example if there is an increase in the number of infected cells, which are targeted by CD4+ and CD8+ T cells this can result in large quantities of interleukin (IL)-10, IL-2, interferon (IFN)-γ and TNF that when in combination can contribute to tissue damage and altered hemostasis. In other cases the uptake of NS1 by hepatocytes is said to promote viral infection of the liver (31, 32). With severe cases of DHF complement has also been shown to play a role in pathogenesis. Complement activation of C3a and C5a have been found to correlate with the severity of disease (33). Another hypothesis suggests that secondary T-cell responses are reduced because stimulation of T-cell memory results in the production of heterotypic CD4+ and CD8+ cells that have a diminished capacity to kill but still release inflammatory cytokines that contribute to disease severity (34). Still, others have suggested that there is cross-reactivity between NS1 and human platelets or endothelial cells and that cross-reactive anti-NS1 antibodies can damage these cells (35). But as of now the working hypothesis of dengue pathogenesis that correlates with available evidence is that severe disease in infants with primary infections and in adult individuals with secondary infections is the result of ADE of infection of mononuclear phagocytes. Infection by an antibody–virus complex suppresses innate immune responses, increasing intracellular infection and generating inflammatory cytokines and chemokines that, collectively, result in enhanced disease. Liver infections and a pathogenic role for NS1 add to the complex pathology (1, 29, 36, 37).
DENV produces several symptoms that are conditional by age and immune status. For cases of primary DENV infections in children, most experience subclinical infection or mild undifferentiated febrile syndromes. During secondary DENV infections the pathophysiology of the disease can become severe. At this stage the infected person can have acute and severe vascular permeability syndrome known as DSS. The severity of DSS is dependent on age, where children can experience vascular leakage, a phenomenon that is thought to be related to the intrinsic integrity of the capillaries (38, 39). For adults, primary infections with any of the four DENV serotypes can result in DF (40). In adults there appears to be a tendency for bleeding that can lead to severe hemorrhages (41). As such DENV infections can be life-threatening in individuals with asthma, diabetes and other chronic diseases (42).

Secondary dengue infections in adults can produce DHF or DSS. Studies with secondary infections have observed that the longer the interval between the primary and secondary infection, the more severe the disease (29, 43). Tertiary DENV infections can cause severe disease, but such cases are rare (44).

**Diagnosis, Treatment and Control**

Laboratory diagnosis of DENV infection can be made by virus isolation or detection of DENV RNA by nucleic acid amplification in the acute-phase serum, and various tissues postmortem (45). Reverse transcriptase-polymerase chain reaction (RT-PCR) is the most sensitive method for detecting virus (46). Histologic techniques can detect viral
antigen in autopsy tissues (47). Serologic diagnosis of DENV infection is somewhat problematic due to the extensive cross-reactivity among DENV serotypes and with other flaviviruses. However, the most useful assay is the IgM-capture enzyme-linked immunoabsorbent assay (ELISA), which is now commercially available (45). DENV-specific antibodies for IgM are detectable in both primary and secondary infections by day 6 after onset of illness, and wanes by 60 to 90 days. This test is useful to detect recent DENV infection, but it is not confirmatory for current infection. Plaque-reduction neutralization test (PRNT) can detect neutralizing antibodies against DENV but can provide information on the infecting DENV serotype only when performed on primary infection because of the extensive cross-reactivity among four DENV serotypes and flaviviruses in secondary infection (1).

Specific treatment for DENV-infected patients does not exist. Supportive care includes bed rest, antipyretics, and analgesics. Fluid and electrolyte replacement should be carefully managed in the DHF/DSS patients. Aspirin and other salicylates should be avoided in view of diminished platelet numbers and, possibly, function (4).

**Current Antiviral Strategies**

Current antiviral strategies against DENV include entry inhibitors, protease inhibitors, helicase inhibitors, polymerase inhibitors and others. Many groups have studied molecules that interfere with flavivirus entry their antiviral potential. One group demonstrated that a recombinant form of DENV2 E protein domain III (DIII) that
included helix I of the E protein blocked flavivirus entry by specifically inhibiting virus fusion (48). Their findings suggest that exogenous DIII proteins could function as inhibitors of class II fusion mechanisms. Their study also revealed that DIII functioned by binding to fusion intermediates following low pH-induced trimerization and prevented hairpin formation of E protein. Other inhibitory molecules include sulfated polysaccharides, polyoxotungstates, and sulfated galactomannans, sulfated glycosaminoglycans, heparin and suramin (48). The effect of these molecules in their ability to block flavivirus entry is still being studied and improved upon.

For protease inhibitors two main approaches have been taken to develop protease inhibitors. The first one is by using a high throughput screening (HTS) of small molecule libraries, and the other is by designing peptidomimetics, which mimic the natural catalytic substrate. There have been many challenges in targeting the active site of the NS2B–NS3 protease, and yet such work is still in progress (49). Choosing DENV helicases as a target for inhibition is also challenging because crystal structure of DENV NS3 showed that the helicase domain does not have an pockets that are likely to bind a small-molecule inhibitor (49).

Another target for inhibition is the RNA polymerase. For anti-DENV nucleoside analogs, all phosphorylation events must be carried out by host kinases to form the corresponding triphosphate analogs. The nucleoside analog triphosphate then acts as a substrate mimic and chain terminator upon incorporation into the viral genome. Therefore, a selective nucleoside analog must first be recognized by the host
nucleoside/nucleotide kinases and specifically inhibit the viral polymerase over any of the human polymerases (49). Further investigation on this potential antiviral is still in progress.

Structural analysis of the E protein has also provided potential targets for inhibition. One such example is the β-OG pocket. The ligand binding pocket buried at the hinge between domain I (DI) and domain II (DII) of E protein and its movement in the fusion activation of E made it a prime target for the design of compounds that might inhibit required structural transitions of the virus (50). The β-OG pocket forms a channel with open access at both ends which allows linear molecules of varying lengths to fit. The channel is lined by hydrophobic residues that influence the pH threshold of fusion (50).

Other antivirals include ribavirin, interferon-α2b and anti-virus immunoglobulin. Ribavirin has been shown to be work against many DNA and RNA viruses, including flaviviruses like WNV, but the effective concentration in cell culture tends to be very high and has been ineffective in animal studies (51). Interferon-α2b has been shown to have broad antiviral activity in vitro and immunostimulatory effects in vivo, and has been considered as a possible therapy for flaviviral encephalitis but clinical trials to date have been slow and not promising (52).
The E protein of DENV is a class II fusion protein that shares about 40% amino acid sequence homology with other flaviviruses (9, 53, 54). The DENV E protein contains 495 amino acids. The N-terminal ectodomain of E protein contains 395 amino acids, comprising three domains, DI, DII, and DIII (9, 50). DI is located in the center. DII is elongated and mediates dimerization of E protein and membrane fusion through its internal fusion loop that consists of several highly conserved residues (amino acids 98 to 108). DIII has an immunoglobulin-like domain that is predicted to be involved in receptor binding (54, 55) (Figure 3).

DI and DII are connected by four polypeptide chains, while DI and DIII are connected by a single polypeptide linker. Hinge region in both DI–DII and DI–DIII play an important role during the structural rearrangements of the E protein as it transitions between immature, mature and fusion-active forms of the virus (50). While many studies have elucidated the structure of the E protein in different states, many more still focused on designing antivirals or identifying antibodies that can bind to specific areas of this highly antigenic protein.

E protein assumes many different conformational states during DENV life cycle: immature, mature and fusion-active, which play a role in its assembly, maturation and entry (7). During replication virus particles budding into the ER lumen are considered “immature” because of the prM protein that caps the fusion loop of the E protein. Studies have shown that immature virus can take two conformational forms termed “spiky” and “smooth” (56). The oligomeric state of the E protein is controlled by the
intracellular pH environment and the presence or absence of prM protein. Immature virus particles have a diameter of \(~600\) Å and are made up of 180 prM and E proteins heterodimers that are arranged in 60 trimeric spikes. The E trimers are angled in that allows the fusion loop on DII to be at the furthest point from the viral surface and DIII in close proximity to the viral membrane (57).

Recently, the structure of the prM–E heterodimer was solved by X-ray crystallography and depicted the pr peptide of prM protein extends linearly along the E protein surface remaining on the inside edge of the spike. This places the M protein along the dimerization interface on DII, a location where it would prevent homodimerization of E protein (57). The prM protein forms a cap-like structure to prevent premature fusion of the virus with host cell membranes thus allowing the immature “spiky” virus particles transit through the Golgi apparatus and under low pH environment it triggers translational movements within the glycoprotein lattice (57). This transportation result in the transition of the E proteins from prM–E heterodimers to antiparallel E homodimers, which lie flat against the viral membrane. The resulting particle forms a “smooth” topology, but is still considered to be immature due to the presence of prM that protect fusion loop on E from premature fusion. The transition from spiky to smooth topology is reversible but becomes irreversible when furin cleavage of prM occurs. The cleaved pr peptide remains associated with E and is only released from the virus particle following exit into a neutral pH environment outside of cells. Following maturation the virus is released in into the neutral pH environment and does not undergo further re-
arrangement of the E proteins, but instead drives the release of the pr peptide from the virion (56).

Mature virus particles are ~500Å in diameter and have a relatively smooth surface with the lipid bilayer membrane completely covered by the E and M protein shell. This shell consists of 180 copies of the E protein arranged as 90 homodimers forming a herringbone pattern or so-called protein rafts that lie flat on the viral surface. The E protein homodimers within these rafts consist of two monomers associated in an antiparallel orientation with DII forming the primary dimerization interface (9, 50, 58).

Several events occur during virus entry into target cells. First, E proteins interact with molecules on the surface of host cells as a point of attachment. Following attachment, specific cell-surface receptors mediate endocytosis of the virus. In the endosome, the fusion loop of E protein becomes exposed at the distal end of the E protein and is inserted into the host membrane. Low pH-induced structural rearrangements within E protein bring the transmembrane domains anchored in the viral membrane closer to the fusion loop, forming a hairpin structure that promotes fusion of the viral and host membranes (7, 59). In the late fusion step the E homodimers within the mature virion dissociate and re-arrange into fusion active homotrimers. In this step the E proteins are in a parallel orientation to one another within the trimer, extending vertically away from the virion surface. This allows the fusion loop on DII to insert itself into the host endosomal membrane (Figure 4).
**Stem region of E protein**

The C-terminus of E protein contains a stem region, which is composed of two $\alpha$-helices (EH1 and EH2) along with a small conserved segment in between. Following the stem region is an anchor region consisting of two transmembrane domains (ET1 and ET2). Cryo-electron microscopic (cryo-EM) study of mature DENV particles has revealed that the stem is structurally distinct from the ectodomain and anchor, with EH1 and EH2 lying on the outer surface of the membrane (57). Other studies using the E protein of tick-borne encephalitis virus (TBEV) have shown that EH1 is involved in the trimerization of soluble E protein under low pH environment while EH2 is involved in the heterodimerization of prM-E proteins (60).

Several recent studies suggest that the stem region is involved in virus assembly. One study reported that substitutions of three residues in the stem region of DENV2 E protein enhanced the production of VLPs (61). In a previous study our group showed that the stem-anchor regions of DENV2 and JEV contained an ER retention signal and affected the production of VLPs (62). Additionally our most recent mutational analysis revealed that most of the proline substitutions which were introduced to each residue at the hydrophobic face of the stem region of DENV4 E protein drastically affect the production of VLPs, suggesting the importance of helical domains at the stem region in assembly - several of these hydrophobic residues are highly conserved by the different flaviviruses (63) (Figure 5).
As a class II enveloped virus, DENV uses its fusion loop to insert into the plasma membrane. From this step it then requires the folding back of the stem to move along a channel located on the intersubunit contacts between domains II (9, 64, 65). The presence of an intermediate step prior to fusion is supported by the observation that recombinant domain III binds to such a fusion intermediate following low pH-induced trimerization and prevents fusion (48, 65). In addition, a peptide derived from the stem region of DENV2 E protein has been observed to block the entry of DENV2 and WNV (66-68). Moreover, the peptide inhibition seems to have a two-step mechanism during virus entry. The first step is a non-specific, membrane binding step followed by a specific interaction with the trimeric intermediate (17-18).

Despite all these mutational studies on the assembly and entry of DENV and other flaviviruses several questions remain largely unknown, such as the mechanism and intermediates of assembly, the intermediate steps of fusion and the mechanism of entry. In addition, the exact residues involved in these steps have yet to be identified.

Based on the amino acid sequence homology of E protein among different serocomplexes of flaviviruses and different serotypes of DENV, the stem region (amino acid residues 396 to 450) of E protein contains 28 highly conserved residues (Figure 5). What is most interesting is that 16 of these are absolutely conserved not only by the four serotypes of DENV but also by different serocomplexes of flaviviruses, including WNV, JEV, YFV and TBEV. This raises the possibility that some of these highly conserved residues may represent ideal targets of antivirals against DENV and other
medically important flaviviruses and potential common antiviral target for all flaviviruses as well.

**HYPOTHESIS AND SPECIFIC AIMS**

The *objective* of the proposed research is to investigate the role of highly conserved stem residues on assembly. The *underlying hypothesis* is that the highly conserved residues in the stem region play important roles in viral assembly. The objective will be achieved by pursuing the following two specific aims:

1. Investigate the role of highly conserved residues in the stem region of the E protein on the assembly of DENV1.

2. Investigate the mechanisms of impairment in assembly of DENV1 mutants.

The rationale is based on mutational studies that suggest the stem region is involved in prM-E heterodimerization, which is believed to be an important step in the assembly of virions and VLPs. And since these residues are highly conserved among all flaviviruses we can gain a better understanding how this family of virus assembles during viral replication. We expect that when these highly conserved residues undergo mutations they will affect the process of assembly and thus demonstrate which residues are crucial to virus production.

**SIGNIFICANCE**
The involvement of the stem region of the E protein in two critical steps of replication (both entry and assembly) and the presence of 28 highly conserved residues, of which 16 are universally conserved, suggest that further characterization of the stem region of E proteins would lead to the identification of novel and common targets for antivirals against both DENV and other medically important flaviviruses. Moreover, studying the mechanism of impairment in assembly of VLPs of these mutants would shed new light on our understanding of the assembly of virions.
Figure 1. Dengue virus genome. Single open reading frame encodes three structural proteins, capsid (C), premembrane (prM) and envelope (E) and seven nonstructural (NS) proteins (NS1 to NS5) (3).
Figure 2. Replication cycle of dengue virus (DENV). (A) DENV binds to its cellular receptor(s) and enters the cell via clatherin mediated endocytosis. (B) Fusion between viral and endosomal membrane occurs at low pH environment in endosome and following fusion viral genome is released into the cytoplasm. (C) Protein translation occurs on ER membrane and produces a polyprotein which undergoes processing. (D) Replication of viral RNA genome on ER membrane. (E) Immature virions assemble on ER membrane and bud into the lumen of ER. (F) Immature virions travel through the trans-Golgi network (TGN), where they are cleaved by furin/furin-like proteases to become mature virions. (G) Mature virions are released outside of the cell via exocytosis (69).
Figure 3. Schematic drawing of dengue virus envelope (E) protein and its conformation states. (A) Linear representation of the E protein, illustrating the regions that fold into the structures shown in B and C. E protein contains 495 amino acids, the N-terminal ectodomain (amino acid residues 1 to 394) contains three domains, domain I, II and III, shown in red, yellow and blue, respectively. At the C-terminus, there is a stem region that contains two α-helices (EH1 and EH2) and a conserved sequence (CS) in between. (B) Structure of E protein in the demerit conformation. (C) E in the trimeric conformation it adopts following a low-pH induced conformational change. Dashed lines indicate the likely position of the stem region (68).
Figure 4. Proposed model of flavivirus membrane fusion reveals that stem region is involved in the fusion step of entry. (A) At low pH (6.2) in endosome, E protein undergoes conformational rearrangement that includes dissociation of the homodimers. (B) A hydrophobic “fusion loop” at one end of the extended E subunit inserts into the outer leaflet of the endosomal membrane. (C) Trimer forms with DIII folding back to DI. (D) Stem zips up along DII. (E) Fusion between viral and endosomal membranes - draw together the fusion loop and transmembrane domains of E protein (70).
Figure 5. Stem region of envelope (E) protein contains two α-helical domains (EH1, EH2) and a conserved sequence (CS) in between. Sequences of the stem region of multiple strains (numbers in parentheses) of each flavivirus including four serotypes of dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), yellow fever virus (YFV) and tick-borne encephalitis virus (TBEV) were obtained from Genbank and analyzed. Single letter designations of amino acids are shown with the numbers above indicating the position of residues and the numbers beneath each amino acid indicating the number of strains containing such residue. ** represents residues that are absolutely conserved by all flaviviruses. * represents residues that are conserved by the four DENV serotypes (71).
References


Halstead SB. In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. J Infect Dis. 1979;140:527-33.


CHAPTER 2

MATERIALS AND METHODS
Plasmid constructs.

The prM/E expression construct of DENV1 (pCB-CD1) was described previously (1). To generate mutants in the stem region of E protein, a two-step PCR mutagenesis was performed using pCB-D1 as template and primers (d1S396A, d1G399A, d1T405A, d1G408A, d1R411A, d1W420A, d1D421A, d1F422A, d1S424A, d1G426A, d1G427A, d1S431A, d1G441A, d1F448A, d1G450A, d1S396P, d1G399P, d1T405P, d1G408P, d1R411P, d1A419P, d1W420P, d1D421P, d1F422P, d1S424P, d1G426P, d1G427P, d1S431P, d1G441P, d1F448P, d1G450P), as described previously (2) (The sequences of primers were shown in Table 1). After the second-round PCR, the 596-bp product containing the stem mutation was digested with DraIII and NotI, and cloned back into pCB-D1. All constructs were confirmed by sequencing the entire insert in each construct to rule out a second-site mutation.

Transfection, collection of cell lysates and VLPs

293T cells, prepared in a 10-cm culture dish at 5 x 10⁵ cells per dish 1 day earlier, were transfected with 10 μg of plasmid DNA by the calcium phosphate method (3). At 48 h posttransfection, culture supernatants were collected and cells were washed with phosphate-buffered saline (PBS) and treated with 1% NP40 lysis buffer (100 mM Tris [pH 7.5], 150 mM NaCl, 20 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, and protease inhibitors [Roche Diagnostics]), followed by centrifugation at 20,000 x g and 4°C for 30 min to obtain cell lysates (2).
Culture supernatants were clarified by centrifugation at 1,250 x g for 20 min, filtered through a 0.22-μm-pore-sized membrane (Sartorius), layered over a 20% sucrose buffer, and ultracentrifuged at 65,000 x g and 4°C for 5 h to obtain pellets containing VLPs, which were resuspended in 30 μl TNE (50 mM Tris [pH 8.0], 1M NaCl, 100 mM EDTA) buffer (2).

**Western blot analysis**

For Western blot analysis, cell lysates or pellets were added to nonreducing buffer (2% SDS, 0.5 M Tris [pH 6.8], 20% glycerol, 0.001% bromophenol blue [final concentrations]) and subjected to 12% polyacrylamide gel electrophoresis (PAGE), followed by transfer to a nitrocellulose membrane, blocking, and incubation with sera from confirmed DENV cases or mouse anti-prM monoclonal antibody (mAb), 14.4 (a gift from Dr. Chinhg-Len Liao at the National Defense University, Taiwan) or DM1 (Fischer Scientific) as first antibody and anti-human or anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) as secondary antibody (Pierce) (4). After the final wash, the signals were detected by enhanced chemiluminescence reagents (Perkin Elmer Life Sciences) (1). The ratio of intensity of mutant E or prM band in pellets to that in cell lysates was compared with that of the wildtype (WT).

**Immunoprecipitation.**
293T cells prepared in a 6-well plate were transfected with plasmid DNA by the calcium phosphate method and collected at 48h post-transfection. Cell lysates were precleared and then incubated with mouse anti-prM mAb 14-4 (a gift from Dr. Chinhg-Len Liao at the National Defense University, Taiwan) at 4°C overnight and then with protein A-Sepharose beads (Amersham Biosciences) at 4°C for 6 h. After the beads were washed, they were mixed with 2X sample buffer and heated, and the solubilized fraction was subjected to 12% PAGE (2). The intensities of the prM and E bands were analyzed by a Image J image system (2) and the E/prM index of each each mutant was determined. 

\[
E/prM \text{ index} = \frac{\text{intensity of mutant E band}}{\text{intensity of mutant prM band}} / \frac{\text{intensity of WT E band}}{\text{intensity of WT prM band}}.
\]

**Subcellular fractionation**

293T cells transfected with plasmid DNA were washed 3 times with PBS at 48 h, resuspended in modified buffer B (10% sucrose, 20 mM Tris, 150 mM NaCl, 10 mM magnesium acetate, 1 mM EGTA [pH 7.6]), and subjected to freeze-thaw cycles 8 times, as described previously (5). After the nuclei and debris were cleared by centrifugation at 1,000 x g for 5 min, the membrane fraction was obtained by centrifugation at 20,000 x g for 30 min at 4°C. The resulting supernatants were layered over a 20% sucrose buffer and ultracentrifuged at 246,000 x g and 4°C for 1 h to obtain the pellets of the soluble fraction, which were resuspended in 30 μl TNE buffer. The membrane fraction and pellets of the soluble fraction were subjected to Western blot analysis (2).
Enzyme digestion.

Aliquots from the cell lysates described above were treated with 500 U of endo-\textit{N}\textendash acetylglucosaminidase H (endo H) or peptide \textit{N}\textendash glycosidase F (PNGase F) at 37°C for 1 h, according to the manufacturer's instructions (New England BioLabs), and subjected to Western blot analysis as described previously (2, 6).

Quantitative capture-enzyme linked immunosorbent assay (ELISA).

Four-layer ELISA was performed on total cell lysates, pelleted VLPs, membrane fraction and soluble fractions from transfected 293T cells, and known concentration of recombinant E protein of DENV1 (kindly provided by Dr. Nerurkar Vivek at the University of Hawaii at Manoa) as antigen. A 96-well plate will be coated with anti-E antibodies derived from confirmed human dengue-immune sera overnight at 4°C. After washing with 1X wash buffer (0.5% Tween-20 in 1X PBS) 3 times, blocking buffer (1% BSA in 1X PBS) was added at room temperature for 1 h. After washing 3 times, antigen was incubated at 37°C for 2 h. After washing 3 times primary antibody (mouse anti-E mAb, DA6-7, kindly provided by Dr. Han-Chung Wu at the Academia Sinica, Taiwan) was added and incubated at 37°C for 2 h. After washing 3 times, secondary antibody (anti-mouse IgG conjugated with HRP, Jackson) was added and incubated at 37°C for 1 h. After washing 6 times, TMB was added and incubated at room temperature for about 20 min, followed by adding stop solution (2N H₂SO₄). Absorbance was measured with ELISA plate reader (BioTek ELx50) at wavelength of 450 nm with reference wavelength
of 650 nm. Based on the standard curve derived from recombinant E protein, the concentration of E protein in various antigens was determined using the program GraphPad Prism 5.0 (GraphPad).

Electron microscopy (EM)

293T cells were fixed at 24 h and 48 h post-transfection and processed for EM study. For conventional transmission EM (TEM), cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature then transferred to 4°C overnight. The cells were washed two times with 0.1 cacodylate buffer before being post-fixed with 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.2) for 1 h at room temperature. Then the cells were dehydrated in graded series of ethanol and washed with propylene oxide before being embedded in 1:1 propylene oxide:epoxy resin to incubate overnight at room temperature. The cells were then be embedded with fresh epoxy resin the next day and polymerized at 60°C. Ultrathin (60-80 nm) sections were obtained on a RMC powertome ultramicrotome, double stained with uranyl acetate and lead citrate, viewed on a Hitachi HT7700 TEM at 100 kV, and photographed with an AMT XR41 4 megapixel CCD camera.

For immunogold COS-1 cells stably expressing DENV1 prM/E proteins were fixed with %4 paraformaldehyde and %1 gluteraldehyde for 1 hr at room temperature. After fixation they were dehydrated in grade series of ethanol and washed several times with LR? white resin before being polymerized with ultraviolet (UV) light in the freezer.
Ultrathin (60-80 nm) sections were obtained on a powertome ultramicrotome and embedded on nickel grids. Grids were stained with primary antibody 4G2 for 2 hrs, washed three times with 1XPBS, stained with secondary antibody anti-mouse IgG conjugated with 6 nm gold and washed three times with 1xPBS (10 min each) and washed with ddH2O four times (10 min each). The grids were viewed on a Hitachi HT7700 TEM at 100 kV, and photographed with an AMT XR41 4 megapixel CCD camera.
Table 1. Sequences of the primers for PCR and cloning in this study

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References

CHAPTER 3

RESULTS
RESULTS

Specific Aim 1: Investigate the role of highly conserved residues in the stem region of the E protein on the assembly of DENV1

Objective 1: The effect of proline substitutions of 16 absolutely conserved stem residues on the production of VLPs.

Amino acid sequence analysis of the stem region reveals 16 residues that are absolutely conserved among different members in the genus Flavivirus. To examine the roles of these residues site directed mutagenesis was carried out to replace each of the residues with either a proline or alanine on DENV1 prM/E expression construct, pCB-D1 (Figure 1). Substitutions with proline, an α-helix breaker, and alanine are often used to study the effect of disrupting the helical structure and the effect of removing a side chain with the helical structure preserved, respectively.

As shown in Figure 2A, the amounts of prM/E proteins in the 16 proline mutants in cell lysates were generally comparable to those of WT pCB-D1, suggesting that proline substitutions do not greatly affect the expression of prM/E proteins. In contrast, compared with those of the WT the amounts of prM/E proteins in pellets derived from ultracentrifugation of culture supernatants were greatly reduced in most proline mutants except G426P (Figure 2B), which is located in
the CS domain between EH1 and EH2 domains. This finding suggests that compared with the WT most proline substitutions affect the production of VLPs except G426P.

To obtain a quantitative measurement of mutant and WT E proteins in both cell lysates and pellets, we carried out a quantitative capture-ELISA. The concentrations of E protein, [E], in cell lysates and pellets were determined based on a standard curve generated by known concentrations of recombinant E protein in the capture-ELISA (Figure 2C). The ratio of [E] in pellets to [E] in cell lysates for each mutant relative to such ratio of WT was calculated to determine the production of VLPs (Figure 2D). In agreement with the results of Western blot analysis, quantitative capture-ELISA showed that proline substitutions introduced to most residues at either EH1, CS or EH2 domain except A419P and G426P, affect the production of VLPs (relative [E] pellets/[E] cells < 0.4). These findings suggest that the helical structure is important for VLP production.

**Objective 2: The effect of alanine substitutions of 15 absolutely conserved stem residues on the production of VLPs.**

In addition to proline substitutions, alanine substitutions were also introduced into the 16 absolutely conserved residues at the stem region, except residue 419 which is an alanine, in the construct pCB-D1. As shown in Figure 3A, the
amounts of prM/E proteins of the 15 alanine mutants in cell lysates were comparable to those of WT pCB-D1, suggesting that alanine substitutions do not affect the expression of prM/E proteins. However, the amounts of prM/E proteins in pellets were greatly reduced in most alanine mutants in the CS and EH2 domains except G426A, suggesting that most alanine mutants in the CS and EH2 domains severely affect the production of VLPs (Figure 3B).

We also performed a quantitative capture-ELISA to determine the [E] of WT and mutants in cell lysates and pellets (Figure 3C), and calculated the ratio of [E] in pellets to [E] in cell lysates for each alanine mutant relative to such ratio of WT (Figure 3D). Consistent with the results of Western blot analysis, quantitative capture-ELISA revealed that alanine substitutions introduced to most residues at CS or EH2 domain except G426A greatly affect the production of VLPs (relative [E] pellets/[E] cells < 0.2). These findings suggest that residues at the CS and EH2 domains are important for VLP production.

**Specific Aim 2: Investigate the mechanisms of impairment in assembly of DENV1 mutants.**

**Objective 1: Examine the prM-E interaction of stem mutants by immunoprecipitation assay**
Study with TBEV, another flavivirus, has shown that a proper prM-E heterodimeric interaction is important for assembly of VLPs (1). To investigate whether these alanine mutations affect the prM-E interaction, lysates of 293T cells transfected with WT or each of these mutants were subjected to an immunoprecipitation assay using an anti-prM mAb 14-4, followed by SDS–12% PAGE and Western blot using a human dengue-immune serum and anti-prM mAb DM1. As shown in Figure 4A, the amounts of E protein relative to prM protein for each mutant were comparable to those of WT, suggesting that these alanine mutations do not affect the prM-E interaction. We further analyzed the intensities of prM and E protein bands, and calculated the E/prM index for each mutant, which is equal to intensity of mutant E band/intensity of mutant prM band)/(intensity of WT E band/intensity of WT prM band). As shown in Figure 4B, the E/prM indices of all these mutants were higher than 0.6 (most of them were higher than 1.0), suggesting that these mutations do not affect greatly the prM-E interaction.

**Objective 2: Examine the intracellular distribution of prM/E proteins and glycosylation pattern by enzyme digestion**

Trafficking through the ER and Golgi is another step that is important for assembly and producing VLPs. To investigate if glycosylation was affected by these alanine mutants cell lysates were examined by enzyme digestion with
Endo H and PNGase F. PNGase F was used as a control to depict a pattern of deglycosylation of all sugar moieties of either high-mannose or complex form. As shown in Figure 5, a predominantly Endo H-sensitive pattern of glycosylation was found for WT E protein, suggesting that intracellular WT prM/E proteins were mainly present in the ER. Similarly, a predominantly Endo H-sensitive pattern of glycosylation were found for all 15 alanine mutant E proteins, suggesting that these mutations do not affect E protein glycosylation and intracellular localization of prM/E proteins.

Objective 3: Examine the intracellular distribution of prM/E proteins and VLPs by subcellular fractionation assay

To further investigate whether the intracellular prM/E proteins of these alanine mutants are retained in the membrane-bound fraction or form VLPs in the soluble fraction, 293T cells transfected with mock, WT or each mutant construct were washed with PBS, resuspended in modified buffer B, and subjected to freeze-thaw cycles 8 times. After the nuclei and debris were cleared, the membrane fraction and the pellets derived from the soluble fraction by ultracentrifugation were subjected to Western blot analysis using human serum from a dengue case. Quantitative capture-ELISA was also performed to measure the [E] in the membrane and soluble fractions.
As shown in Figure 6A, the amounts of prM/E proteins in pellets of the soluble fraction relative to those in the membrane fraction were reduced for some of these mutants including S396A, R411A, G424A, F408A and G450A, compared with those for the WT. This was further verified by quantitative capture-ELISA to measure the [E] in the pellets of soluble fraction and membrane fraction and calculate their ratios relative to that of the WT (Figure 6B). These findings suggest that these alanine mutations affect the budding of VLPs from the membrane fraction to the soluble fraction, mainly the lumen of ER, since prM/E proteins of alanine mutants primarily reside in ER based on the enzyme digestion experiment (Figure 5).

Other mutants including G398A, T405A, G408A, F422A, G426A and G427A showed an increase in the amounts of prM/E proteins in pellets of soluble fraction relative to those in the membrane fraction when compared with that of the WT (Figures 6A and 6B), suggesting these mutations do not affect the budding of VLPs from the membrane fraction to the lumen of ER but affect the release of VLPs from the lumen of ER and/or intracellular vesicles to outside of the cells, thus causing the accumulation of VLPs in the soluble fraction. Taken together these findings suggest that certain residues in the stem region are involved in the budding of VLPs from the ER membrane to the lumen of ER while other stem residues are involved in the release of VLPs from the lumen of ER and/or intracellular vesicles to outside of the cells.
Objective 4: Examine intracellular VLPs by EM

The ER is the assembly site for flaviviruses before they form mature infectious virions. But much about the morphological details of this assembly process is still unknown because the assembly proceeds very quickly and no intermediate of the assembly process has not been visualized thus far. In order to better understand the morphological features during assembly, we used TEM to observe 293T cells transfected with our WT or stem mutant constructs that had impairment in the assembly or production of VLPs based on our biochemical assays. As shown in Figure 7, there were several dense spherical particles together with some protein material within the intracellular vesicles of WT-transfected cells (Figure 7B to 7H). There was no dense spherical particles within the intracellular vesicles of mock-transfected cells (Figure 7A). Consistent with previous reports and unpublished data from our lab, the sizes of the dense spherical particles within intracellular vesicles fall into two categories, large VLPs with a mean diameter of 50 nm and small VLPs with a mean diameter of 30 nm (2, 3). Similar to WT-transfected cells, dense spherical particles with protein material were found within the intracellular vesicles of mutant S424A-transfected cells (Figure 7I to 7K). From our results we could not find distinct assembly defects in our S424A mutant when compared to WT under TEM.
In order to confirm if these potential VLPs contained E protein we used immunogold labeling. Because we knew that our transfected cells would express E protein less efficiently, we used the COS-1 cells that stably express DENV1 prM/E proteins (kindly provided by Dr. Jeff Chang at the CDC Fort Collins, Colorado) to ensure that there was a sufficient amount of antigen that would bind to our mAb (4G2). We fixed the cells with 4% paraformaldehyde plus 1.0% gluteraldehyde and imbedded them an LR white. Samples were cut via ultramicrotome and imbedded on nickel grids. The grids were incubated with primary mouse mAb 4G2 (1:10) and secondary anti-mouse IgG conjugated with gold (6 nm) (1:20). Results showed that gold particles were found both inside and outside intracellular vesicles of these COS-1 cells but not in the control, where secondary antibody was not added. COS-1 (Figure 8). This suggests that 4G2 could bind to E protein in these intracellular compartments, but it was difficult to determine if they indeed bound to VLPs or not because no dense particles with discernible size can be identified under the staining condition. Thus there is still much we need to improve upon if we wish to view an assembly intermediate by immunogold EM.
Figure 1: Schematic drawing of DENV1 prM/E expression construct pCB-D1 (4) The C terminus of E protein contains two α-helical domains (EH1 and EH2) and a stretch of CSs in between, followed by two transmembrane domains (ET1 and ET2). Single-letter designations of amino acids are shown with numbers above indicating the positions of residues and the numbers beneath each amino acid indicating the number of DENV1 strains containing such residue. ** represents 16 residues that are absolutely conserved by all flaviviruses. * represents 12 residues that are conserved by the four DENV serotypes.
Figure 2. Expression and production of VLPs of proline mutants in the stem region of E protein and quantitative capture-ELISA to determine the amount of E protein. (A and B) At 48 h post-transfection of 293T cells, cell lysates and pellets derived from culture supernatants of WT pCB-D1 or mutants were subjected to Western blot analysis using human serum from a dengue case. The long-exposure gel for prM bands in cell lysates is shown below. Arrow heads indicate E and prM protein. The sizes of the molecular weight markers are shown in kDa. (C) Known concentrations of recombinant DENV1 E protein were subjected to a capture-ELISA to generate a standard curve. (D) Aliquots of diluted cell lysate and pellets were subjected to the capture-ELISA in parallel and the concentrations of E protein, [E], in cell lysates and pellets were determined. The ratio of [E] in pellets to [E] in cell lysates for each mutant relative to such ratio of WT was calculated. Dotted line indicates relative [E] pellets/[E] cells < 0.4.
Figure 3. Expression and production of VLPs of alanine mutants in the stem region of E protein and quantitative capture-ELISA to determine the amount of E protein. (A and B) At 48 h post-transfection of 293T cells, cell lysates and pellets derived from culture supernatants of WT pCB-D1 or mutants were subjected to Western blot analysis using human serum from a dengue case. The long-exposure gels for prM bands in cell lysates and pellets are shown below. Arrow heads indicate E and prM protein. The sizes of the molecular weight markers are shown in kDa. (C) Known concentrations of recombinant DENV1 E protein were subjected to a capture-ELISA to generate a standard curve. (D) Aliquots of diluted cell lysate and pellets were subjected to the capture-ELISA in parallel and the concentrations of E protein, [E], in cell lysates and pellets were determined. The ratio of [E] in pellets to [E] in cell lysates for each mutant relative to such ratio of WT was calculated. Dotted lines indicate relative [E] pellets/[E] cells < 0.4 or < 0.2.
Figure 4. Interaction between prM and E proteins examined by Immunoprecipitation assay. (A) 293T cells were transfected with WT or each of the 15 alanine mutants. At 48 h post-transfection, cell lysates were immunoprecipitated with anti-prM MAb 14-4, and subjected to SDS–12% PAGE and Western blot analysis using blot using a human dengue-immune serum and anti-prM mAb DM1. The sizes of the molecular weight markers are shown in kDa. (B) The intensities of prM and E bands were analyzed by a Image J image system (5). The E/prM index of a mutant is equal to (intensity of mutant E band/intensity of mutant prM band)/(intensity of WT E band/intensity of WT prM band). Data are means and standard deviations from two experiments.
Figure 5. Glycosylation pattern of WT and mutant E proteins. 293T cells were transfected with WT or each of the 15 alanine mutants. At 48 h post-transfection, cell lysates were digested with Endo H (H) or PNGase F (F), and subjected to Western blot analysis using a human dengue-immune serum (5). Arrowheads indicate E or deglycosylated E (dE) protein. The sizes of the molecular weight markers are shown in kDa.
Figure 6. Subcellular fractionation experiment of alanine mutants in the stem region of E protein. (A) 293T cells were transfected with mock, WT or each of the 15 alanine mutants. At 48 h post-transfection, cell lysates were washed with PBS, resuspended in modified buffer B, and subjected to freeze-thaw cycles 8 times (5). After the nuclei and debris were cleared, the membrane fraction and the pellets derived from the soluble fraction by ultracentrifugation were subjected to Western blot analysis using human serum from a dengue case. Arrow heads indicate E and prM protein. The sizes of the molecular weight markers are shown in kDa. (B) Known concentrations of recombinant DENV1 E protein were subjected to a capture-ELISA to generate a standard curve. (C) Aliquots of membrane fraction and pellets from soluble fraction were subjected to the capture-ELISA in parallel and the concentrations of E protein, [E], in cell lysates and pellets were determined. The ratio of [E] in pellets to [E] in cell lysates for
each mutant relative to such ratio of WT was calculated.

Figure 7. Electron microscopy (EM) of 293T cells transfected with WT and alanine mutant S424A. 293T cells were transfected via lipofectamine with WT pCB-D1 or mutant construct. Sections were cut with an ultramicrotome and examined under transmission EM (A) Mock. (B and C) WT. (D,E,F) enlarged view of vesicles in B. (G,H) enlarged view of vesicles in C. (I) S424A mutant. (J,K) enlarged view of vesicles in I. Arrows point to VLP structures with the sizes labeled next to them.
Figure 8. Immuno-gold staining of COS-1 cells stably expressing DENV1 prM/E proteins. Cells were fixed and imbedded in LR white before cutting with ultramicrotome. Stained with anti-E mAb (4G2) at 1:10 dilution for 2 hr. Washed several times before staining with anti-mouse IgG conjugated with gold (6 nm) at 1:20 dilution
References

CHAPTER 4

SUMMARY AND DISCUSSION
Overview

In this study, we investigated the roles of highly conserved residues in the stem region of DENV1 E protein on the assembly of DENV by using site-directed mutagenesis on a DENV prM/E expression construct and different functional assays on the assembly of VLPs and the mechanism involved. Analysis of VLPs revealed that most of the proline mutations in either EH1, CS or EH2 affected VLP production, suggesting the importance of the helical structures of EH1, CS and EH2 in the assembly of VLPs. Alanine mutations in the same residues showed a severe decrease in VLP production especially 9 residues in the CS and EH2 domains, suggesting the importance of these residues on virus assembly.

Previous studies with TBEV E protein using C-terminal truncational constructs revealed that the prM-E interaction was reduced by truncation of ET1 and abolished by truncation of EH2, whereas assembly of VLPs was abolished by truncation of EH1 (1). It was concluded that EH2 and ET1 are required for prM-E interaction and assembly, respectively, and that proper prM-E heterodimerization is important for assembly. Using immunoprecipitation assay to investigate the prM-E heterodimerization of our alanine mutants we found no effect of alanine substitutions on the prM-E heterodimerization including those alanine mutations at the SC and EH2 domains that greatly affect the production of VLPs. These observations suggest differential effect of truncation and substitution in the stem region.
region on prM-E interaction. Moreover, alanine substitutions in the CS and EH2 domains may affect the assembly of VLPs without affecting the prM-E interaction.

Neither was the glycosylation of E protein affected by the alanine stem mutants when tested by enzyme digestion with Endo H. This finding suggests that these alanine mutations do not affect the intracellular localization of prM/E proteins. We further performed a subcellular fractionation experiment to explore the intracellular localization of prM/E proteins and VLPs. Our results showed that some alanine mutations (S396A, R411A, G424A, F448A and G450A) affected the budding of VLPs from the membrane of ER to the lumen of ER and thus the production of VLPs, whereas other alanine mutations (G398A, T405A, G408A, F422A, G427A) affected the release of VLPs from the lumen of ER or intracellular vesicles to outside of cells. The high resolution structure of DENV2 virions depicted by cryo-EM revealed that the N-terminus of EH1 domain and the entire EH2 domain are partially buried in the outer leaflet of viral membrane (2). It is conceivable that substitutions introduced to EH2 domain might affect the curving and bending of the membrane lipid bilayer during the assembly in the ER.

To further explore the morphological details of the assembly process and examine the possible morphological defects of the stem mutants that had impairment in assembly of VLPs based on our biochemical analysis, we turned to set up the TEM analysis and immuogold EM to examine the VLPs in 293T cells.
transfected with mock, WT or mutant construct. Looking at 293T cells 24 h post-transfection, we found that within the intracellular vesicles there was much protein material combined with dense spherical particles with sizes compatible with VLPs reported previously (3, 4). In agreement with previous reports and unpublished data from our lab, the sizes of the dense spherical particles within intracellular vesicles fall into two categories, large VLPs with a mean diameter of 50 nm and small VLPs with a mean diameter of 30 nm.

In an attempt to further clarify if the protein material inside the intracellular vesicles contained actual VLPs, we used immunogold labeling to tag the E protein inside the cells. Using COS-1 cells stably expressing DENV1 prM/E proteins as a test, we were not able to visualize spherical shaped dense particles within intracellular vesicles. However our mouse anti-E mAb (4G2) bound with anti-mouse IgG conjugated with 6 nm gold particles appeared to recognize some E proteins within intracellular vesicles. These observations of E-specific signal without spherical shaped dense particles in the intracellular vesicles suggest that VLPs are likely to be present in the intracellular vesicles but the staining and other conditions of the immunogold EM need to be optimized. The improvement in immunogold EM and TEM would be important for further morphological studies of the assembly process, identification of intermediates during assembly and possible assembly defects of our stem mutants.

Limitations and future plans
There are several limitations of this study. First, the assembly of DENV virion was investigated by studying the assembly of VLPs. This is based on the similarity in the morphological, biochemical and antigenic properties between virions and VLPs and the assumption that the assembly of virions and VLPs is similar. The critical residues for assembly of VLPs identified by prM/E expression construct in this study need to be further tested in the context of CprME constructs or full-length infectious clone to verify for their roles in the assembly of replicon particles or virions. Second, the mechanisms of impairment in VLP assembly by our stem mutants were primarily examined by biochemical assays, however, the morphological features of the defects of these stem mutants remains to be investigated. As discussed above, further improvement and optimization in the immunogold EM and TEM would be important. Third, since only single alanine substitution mutants were used in the EM study, the possibility that double or triple alanine substitution mutants may have more severe morphological phenotypes that could be observed by EM cannot be completely ruled out. Therefore, including double or triple stem mutants in the future EM study is needed.

While DENV assembly still remains to be investigated, we could use our results to postulate the idea of exploring the stem region as a novel target for antivirals. Thus far the current strategies of antivirals against DENV target the following steps or proteins: entry, fusion, protease/helicase, and RNA polymerases. We
wondered if it is possible to target the critical stem residues (in the CS and EH2 domains) identified in this study to develop assembly inhibitors. While there is no studies that have tried this approach yet, Schmidt et al. (5-7) have used a stem peptide inhibitor that can block DENV fusion to develop a binding assay that could be used to screen for small molecule inhibitors that can block such binding and thus fusion of DENV. In this way we could follow a similar approach to develop an assay that could be used to screen for potential assembly inhibitors of DENV.

**Concluding summary**

In summary, we used the approach of site-directed mutagenesis in a DENV1 prM/E expression construct which produces VLPs to investigate the roles of 16 highly conserved residues in the stem region of DENV1 E protein on the virus assembly and the mechanisms involved. Most of the proline substitutions in either EH1, CS or EH2 affected the production of VLPs, suggesting the importance of the helical structures of EH1, CS and EH2. Alanine substitutions of residues in the CS or EH2 domains greatly affected the production of VLPs, suggesting the importance of these residues on assembly. Immunoprecipitation assay revealed that these alanine mutations do not affect the prM-E interaction. Enzyme digestion experiment showed that these mutations do not affect the glycosylation of E protein and the intracellular localization of prM/E proteins. Subcellular fractionation experiment suggested that some alanine mutations
affect the budding of VLPs from the ER membrane to the lumen of ER, whereas other alanine mutations affect the release of VLPs from ER lumen or other intracellular vesicles to outside of the cells. We also set up a TEM and immuno-gold EM to explore the morphological defects of these mutants. While considerable efforts have been made to develop antivirals DENV, there is no licensed antivirals against DENV currently available. We have identified 9 residues in the CS and EH2 domains of the stem region critical for assembly of VLPs. The absolutely conserved nature of these residues may be potential targets for antiviral strategy to block virus assembly.

References