DISSERTATION

MUTAGENESIS OF THE DENGUE VIRUS ENVELOPE GLYCOPROTEIN GENE CAN SIGNIFICANLTLY ALTER VIRUS INFECTIVITY PHENOTYPES IN CULTURED CELLS AND LIVE MOSQUITOES

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ABSTRACT

MUTAGENESIS OF THE DENGUE VIRUS ENVELOPE GLYCOPROTEIN GENE CAN SIGNIFICANLTLY ALTER VIRUS INFECTIVITY PHENOTYPES IN CULTURED CELLS AND LIVE MOSQUITOES

The dengue virus (DENV) envelope (E) glycoprotein is the primary determinant for initiation of host cell infection. To date, studies investigating the contribution of DENV genetics to mosquito infection are limited. A infectious clone cDNA of DENV type 2 strain 16681 (30P-NBX) provided the ability to introduce site-specific amino acid (AA) mutations into the E protein. The results of the studies herein analyze the effects that AA mutations in the E protein have on infectivity of cultured cells and live mosquitoes.

The ability of 30P-NBX to infect *Aedes aegypti* RexD strain mosquitoes after oral infectious blood-meal was investigated and showed that both 30P-NBX and the parent virus 16681 have low, but equivalent midgut infection rates (MIRs). Mosquito midgut infection with 30P-NBX is not affected by the virus titer in the blood-meal as long as titers are above 6 $\log_{10}$ pfu/ml or 7 $\log_{10}$ $TCID_{50}$/ml. Additionally, multiple experimental repetitions with at least 20 mosquitoes per infectious blood-feed were required to obtain an accurate average MIR for 30P-NBX.

Serial passage of 30P-NBX in RexD mosquito midguts identified a single AA mutation at position 122 in domain II of the E protein from lysine to glutamic acid that

correlated with increased MIRs. Introduction of this AA mutation into the infectious clone (mutant virus K122E) reproduced the results from the serial passage experiment. Compared to 30P-NBX, K122E was not only shown to infect a higher proportion of mosquitoes as early as day 2 post blood-feed, but also to produce a disseminated infection in a higher proportion of mosquitoes by day 6 post blood-feed. Also, K122E consistently produced a midgut infection that spread throughout the entire tissue while 30P-NBX stayed restricted by comparison. Virus attachment to midgut cells was compared and showed that 30P-NBX and K122E could attach with equal efficiencies via our midgut-virus attachment assay. Additionally, incorporation of a single AA mutation into the infectious clone at E protein AA 120 from arginine to threonine significantly enhanced mosquito midgut infection compared to 30P-NBX. This is the first time that mosquito infection determinants have been identified in the DENV E protein.

Amino acid mutations were engineered into the E protein on the lateral ridge of domain III, the fusion peptide at the distal end of domain II, and the molecular hinge region between domains I and II. Mutant virus phenotypes were analyzed in cell culture and live mosquitoes. In contrast to previous suggestions, domain III mutant virus phenotypes showed that the FG loop structure (previously suggested as a mosquito-specific infection determinant) and not the specific AA sequence is important for infection of mammalian cells and live mosquitoes, while the structure and sequence of the FG loop is dispensable for infection of cultured C6/36 cells. Additionally, mutations that remove positively charged residues from the A strand in DIII significantly attenuate infection of mosquitoes after oral infectious blood-meal and completely abrogate

infection in mammalian cells. The results of this study suggest that there may be multiple structures in the E protein that are contributing to virus-receptor interactions.

Viruses with mutations in the fusion peptide and hinge region of the E protein were intrathoracically (IT) inoculated into mosquitoes and showed variable infectivity phenotypes. All of the mutants except for one virus from both the fusion peptide and hinge region viruses attenuated infection of mosquito tissues outside the midgut. Importantly, considering that almost all of these viruses were able to replicate as efficiently as wild type in C6/36 cells, the IT inoculation results provide evidence that C6/36 cells are not a complete surrogate for DENV replication in mosquitoes.

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I would like to extend my gratitude to my committee members, Drs. Carol Blair, John Roehrig, Kenneth Olson, and Chaoping Chen. They have given me valuable guidance during different stages of my graduate school career and have always spoken to me as a scientific equal despite my novice status. The scientific accomplishments of my committee members are staggering and will be a high mark to set for myself in my future career. I thank Dr. Olson for his wit and understanding regarding infection of mosquitoes by arboviruses. I also thank Dr. Chen for becoming a member of my committee despite being in a different department and working on completely disparate subject matter. I hope that this was fulfilling and helped give her ideas for future research.

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I want to mention my appreciation for Drs. Blair, Roehrig, and Huang for allowing me to be a part of their monthly (PDVI/dengue virology) meetings. Despite their distinguished careers and vast scientific experience they were always willing to listen to the thoughts of a lowly graduate student and I am grateful for the opportunity to discuss science with such experts.

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Lastly, though they are again too many to mention, I am tremendously thankful for the friends that I have made since moving to Colorado, especially Erik and Mallory. They have been my family away from home and the love and support they have provided me has been immeasurable. I only hope I have been as good a friend in return and wish them many years of happiness. Through them I have met other amazing people, including Juliet, whom I cherish deeply and look forward to spending many years with by her side.

DEDICATION

I dedicate this dissertation to my parents, Christine and Michael Erb. The person I am today is a direct result of my parent's unconditional love. The trials and tribulations my mother has had to endure and her ability to persevere has been nothing short of miraculous. She is an inspiration to me and my family and is a testament to the strength of the human spirit. Every day my father commits himself and his full support for everyone in the family, including my mother, and including me. His support and advice have always been a part of my decision process and I am a better man for it. With all of my heartfelt sentiment, I appreciate and love them to the fullest capacity.

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CHAPTER 1

LITERATURE REVIEW

The history of arbovirology is linked to the creation of the field of virology itself, when viruses were not yet called viruses but filterable agents with the ability to cause disease; a "contagium vivum fluidum" as described by Martinus Beijerinck in 1898 (Beijerinck, 1898). Two of the first human diseases to be attributed to "filterable agents" were arboviruses, yellow fever and dengue fever. Observations concerning the cause of these diseases led to the inextricable link of disease with the bite of a mosquito (Ashburn and Craig, 1907; Bancroft, 1906; Graham, 1903; Henchal and Putnak, 1990; Reed *et al.*, 1983), and hence arbovirology stems back to the birth of the field of virology.

Arbovirology concerns the study of arthropod-borne viruses (arboviruses). These are viruses that are biologically transmitted via hematophagous arthropod vectors to vertebrate hosts. Blood-feeding has evolved at least 21 separate times in disparate arthropod taxa (Black, 2005) and the exploitation of blood-feeding as a method for virus transmission has likewise arisen numerous times in unrelated virus taxa. Virus families consisting of arboviruses have an assortment of RNA genomes and replication strategies that include single stranded positive sense genomes (*Flaviviridae*, and *Togaviridae*), negative sense genomes (*Bunyaviridae*, *Rhabdoviridae*, and *Orthomyxoviridae*), and double stranded genomes (*Reoviridae*) and one virus with a DNA genome (African swine fever virus, family *Asfaviridae*). Biological transmission requires that virus must infect

and replicate in the invertebrate host (including mosquitoes, biting flies, and ticks) prior to transmission to the vertebrate host (mostly avian and mammalian species). There is a myriad of transmission cycles for clinically relevant arboviruses that include enzootic infection and amplification in intermediate hosts prior to spill over into humans (*e.g.* West Nile virus [WNV]) as well as direct vector to human transmission cycles that eliminate the necessity for intermediate hosts (*e.g.* endemic/epidemic dengue virus). Adding further complexity to arbovirus transmission dynamics, some of these viruses (*e.g.* La Crosse virus) can be transmitted vertically from an infected female to its progeny. The exploitation of hematophagy for virus transmission has had a tremendous impact on public health.

We need only look at yellow fever virus (YFV) and dengue virus (DENV) to appreciate the impact of arbovirology on public health. Both viruses have long been a significant cause of human morbidity and mortality. Devastating epidemics of YFV ravaged a number of American cities prior to 1905 including New York City, Philadelphia, Memphis, Charleston, New Orleans, and one in the lower Mississippi valley that alone claimed 20,000 lives (Barrett and Higgs, 2007). Vector control strategies and the development of YFV vaccines contributed immensely to the suppression of YFV outbreaks after the turn of the century. No vaccines are currently available for DENV and this pathogen is considered the most medically important arbovirus infecting humans today. There are an estimated 50 to 100 million cases a year and ca. 500,000 cases of the more severe manifestation of the disease caused by this virus, dengue hemorrhagic fever (DHF) /dengue shock syndrome (DSS) (http://www.who.int/mediacentre/factsheets/fs117/en/index.html).

Many factors contribute to the emergence and sustained transmission of arboviruses including ecological changes and agricultural development, changes in human demographics and behavior, international travel and commerce, and microbial adaption and change (Morse, 1995). These factors are most often not mutually exclusive. Rising temperatures can enlarge the ecological niches of arthropod vectors, which can in turn increase the opportunity for contact between viruses and previously naïve human populations. Changes in the environment that induce heavy rainfall can raise the water table in areas of eastern Africa that can fill grassland depressions called dambos, thereby triggering the emergence of mosquitoes. These events correlate directly with large outbreaks of Rift Valley fever virus in these regions (Davies, Linthicum, and James, 1985). The creation of dams and modern agricultural techniques including irrigation and flooding produce more breeding grounds for vector mosquitoes and have consequently enhanced Japanese encephalitis virus (JEV) transmission in Asia (Morse, 1995).

Deforestation and human encroachment into areas virgin to human activity facilitates the intersection of humans and vector animals that would otherwise be spatially disconnected. Peridomestic *A. aegypti aegypti* species mosquitoes (the primary vector for DENV) are suspected to have originated in West Africa from forest dwelling *A. aegypti formosus* mosquitoes (Sylla *et al.*, 2009; Tabachnick and Powell, 1979). Urbanization, high human density, and unstable public health infrastructure have produced favorable breeding grounds for *A. aegypti aegypti* mosquitoes, which adopted domestic behaviors and prefers man-made artificial containers holding standing water for reproduction; this further creates situations where large human populations are integrated with high vector populations. International travel and commerce contributes to the

introduction of vectors and viruses into new niche environments. The Asian tiger mosquito, *A. albopictus,* was introduced into the United States via used tires from Asia (Hawley *et al.*, 1987) and is a secondary vector for DENV. WNV was introduced from the Old World into the Americas and generated an unprecedented outbreak in New York State in 1999 (Lanciotti *et al.*, 1999; Nash *et al.*, 2001), which then subsequently proliferated and dispersed across the United States to the West coast. Similarly, a person traveling from India introduced Chikungunya virus (CHIKV) into Italy where a high density of *A. albopictus* mosquitoes facilitated an unexpected outbreak of the virus in a previously naïve population (Bonilauri *et al.*, 2008; Rezza *et al.*, 2007).

Changes in viral genetics and the introduction of new virus strains with high replication and transmission kinetics augment the potential for arbovirus emergence. The introduction of the DENV2 Asian/American genotype in the Americas coincided with increases in DHF cases and the eventual displacement of the DENV2 American genotype with viruses from the Asian/American genotype (Rico-Hesse *et al.*, 1997). A similar phenomenon was recently observed in Vietnam with DENV2 (Ty Hang *et al.*, 2010) and in Sri Lanka with DENV serotype 3 (Hanley *et al.*, 2008). Large scale outbreaks of CHIKV on Reunion island and other Indian Ocean islands between 2004-2006 were instigated by a virus strain with a single amino acid (AA) mutation in its structural E1 protein that facilitated enhanced replication and transmission kinetics in secondary vector *A. albopictus* mosquitoes (Schuffenecker *et al.*, 2006; Tsetsarkin *et al.*, 2007; Vazeille *et al.*, 2007). This was the origin of the CHIKV introduced into Italy as mentioned above. With the constant emergence and sustained transmission of arboviruses throughout the

world, understanding how these viruses emerge, transmit, and cause disease is paramount.

Dengue viruses

As defined by their antigenic characteristics there are four serotypes of DENV (DENV1-4) that comprise the dengue virus serocomplex in the genus *Flavivirus*, family *Flaviviridae*. There are three other virus serocomplexes in the *Flavivirus* genus that are transmitted by arthropod vectors that include the JEV serocomplex, YFV serocomplex, and the tick-borne encephalitis virus (TBEV) serocomplex (Calisher and Gould, 2003). Generally speaking, the DENVs and YFVs are transmitted by *Aedes* mosquitoes and cause viscerotropic disease while members of the JEV serocomplex of viruses are transmitted by *Culex* mosquitoes and cause neurologic disease (Gaunt *et al.*, 2001; Gubler, 2007). The DENV genome is a single stranded messenger (positive) sense RNA molecule ca. 10.7 kb in length. The genome encodes 10 functional genes (from 5' to 3' directionality) including 3 structural genes (capsid [C], prM/M, and envelope [E]) and 7 nonstructural genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). In addition to a 5' $m^7GpppAmpN_2$ cap, the ORF is flanked with 5' and 3' non-coding regions (NCR) containing conserved secondary sequence structures necessary for replication and translation. The genome does not have a 3' polyadenylate tail. Nucleotide sequence analysis of DENV genomes corroborates their distinction as four separate antigenic types and coupled with phylogenetic analysis, suggests that each serotype emerged independently at different times less than 600 years ago (Rico-Hesse, 1990; Twiddy, Holmes, and Rambaut, 2003; Wang et al., 2000; Weaver and Vasilakis, 2009). Genetic analysis of virus sequences also revealed genotype differences within each of the serotypes (Rico-Hesse, 1990). For example, DENV2 has five endemic/epidemic

genotypes named Asian I, Asian II, Southeastern Asian/American, Cosmopolitan, and American and an additional sylvatic genotype consisting of viruses engaged in a transmission cycle between forest-dwelling mosquitoes and non-human primates (Twiddy *et al.*, 2002). DENV genotype distinctions are based on E gene sequence variations.

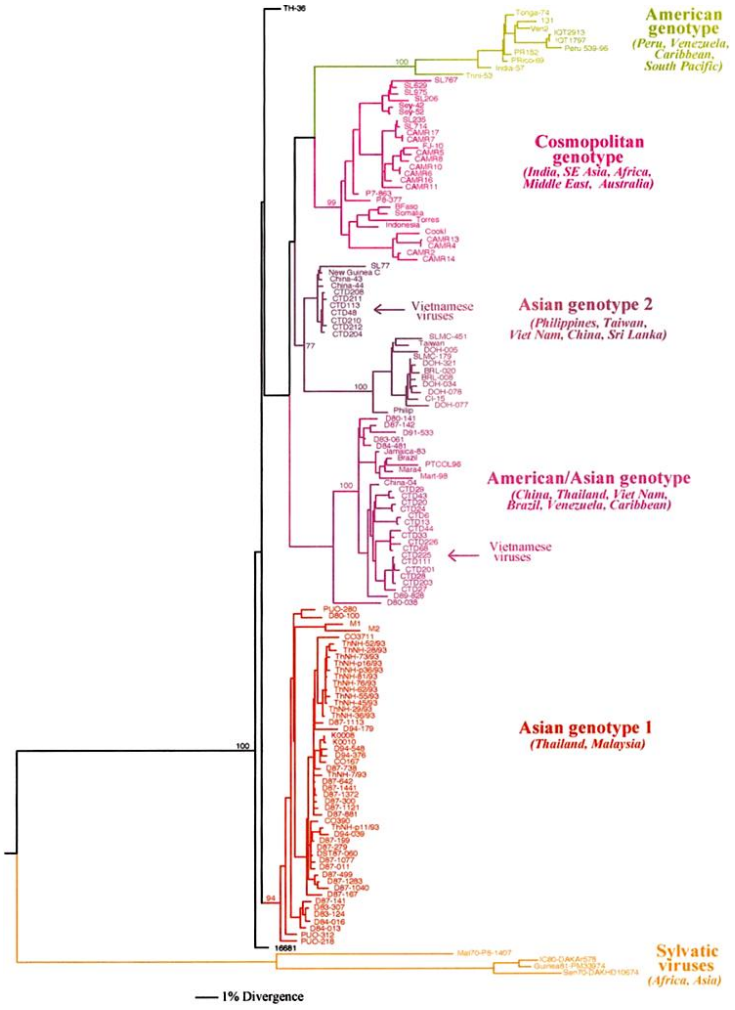

Figure1.1. DENV2 phylogenetic tree. Maximum-likelihood phylogeneic tree of the DENV2 E gene showing genotype distinctions. Figure is borrowed from Twiddy, S. S., Woelk, C. H., and Holmes, E. C. (2002). Phylogenetic evidence for adaptive evolution of dengue viruses in nature. *Journal of General Virology* 83, 1679-1689.

The DENV E glycoprotein is essential for almost all aspects of the virus life cycle and a detailed description of its structure and function is relevant to this dissertation.

DENV virions are characterized by a smooth surface of ca. 500 Å (50 nm) in diameter. The outer shell of the mature virion is composed of 180 copies of the E protein arranged in an icosohedral scaffold of 90 herringbone orientated homodimers that lay extended and parallel to a host cell derived lipid bilayer (Figure 1.1 D); also associated with the lipid bilayer are 180 copies of the M protein (Kuhn *et al.*, 2002; Rey *et al.*, 1995). Within the lipid bilayer is a capsid protein core complexed with the RNA genome. The 2 Å molecular structure of the mature E protein ectodomain of TBEV was first solved in 1995 (Rey *et al.*, 1995) followed by the mature E protein structures for DENV2 (Modis *et al.*, 2003; Modis *et al.*, 2004) (Figure 1.1), DENV3 (Modis *et al.*, 2005), and WNV (Kanai *et al.*, 2006). The E protein crystal structures revealed three distinct structural domains (DI, DII, and DIII), which correlate with monoclonal antibody (MAb) mapping data that defined three antigenic domains (C, A, and B) in the E protein (Guirakhoo, Heinz, and Kunz, 1989; Roehrig, Bolin, and Kelly, 1998). MAb characteristics can be divided into four classifications based on their reactivity to DENV: flavivirus group-specific antibodies recognize multiple viruses in the genus *Flavivirus*, dengue complex-specific antibodies recognize all four serotypes of DENV, dengue subcomplex-specific antibodies recognize some but not all DENV serotypes, and lastly, dengue type-specific antibodies recognize only one serotype of DENV (Henchal *et al.*, 1982).

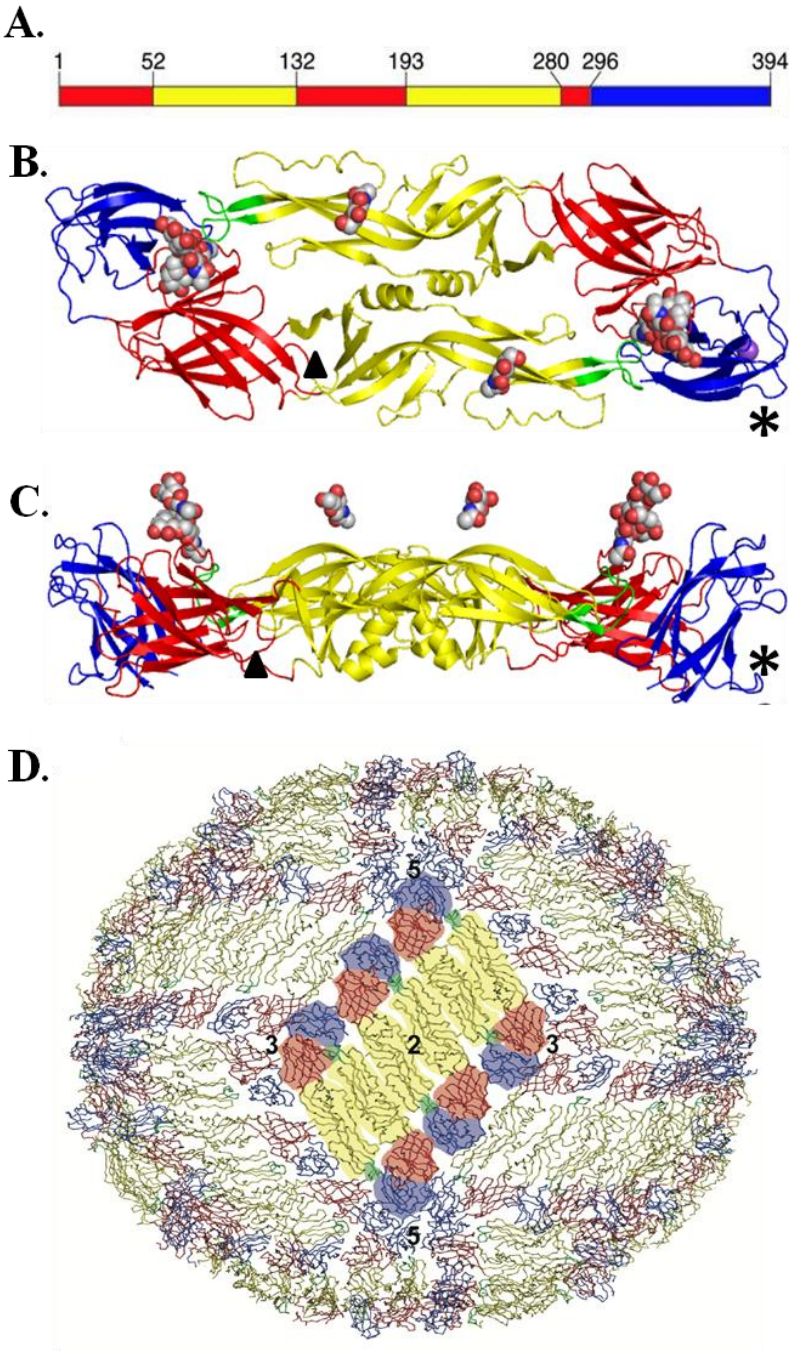

Figure 1.2. Molecular structure of DENV2 envelope glycoprotein. (A) Linear view of E protein monomer with DI in red, DII in yellow, and DIII in blue. (B) Top-down and (C) side view of the DENV2 E protein homodimer with glycans represented as spheres, the fusion peptide (CD loop, AA 98-111) in green, the molecular hinge region between DI and DIII marked by a solid triangle, and the lateral ridge of DIII marked with an asterisk. (D) E protein herringbone arrangement in the DENV2 mature virion. Protein structures were obtained from the protein database bank (DENV2 E protein homodimer ID: 1oan) and were rendered in Polyview-3D (Porollo,

Adamczak, and Meller, 2004). A, B, and C are modified from Modis *et al.* (2004) and D is modified from Zhang *et al.* (2004).

Prior to solving the molecular structure of the flavivirus E protein, MAb were used to determine the antigenic structure of the E protein in a topological model. Murine hybridomas secreting MAbs that target the E protein for TBEV (Guirakhoo, Heinz, and Kunz, 1989; Heinz *et al.*, 1982; Heinz *et al.*, 1983; Mandl *et al.*, 1989), Saint Louis encephalitis virus (SLEV) (Roehrig, Mathews, and Trent, 1983), JEV (Kimura-Kuroda and Yasui, 1983; Kimura-Kuroda and Yasui, 1986), and DENV (Henchal *et al.*, 1982; Henchal *et al.*, 1985) provided antibodies that showed reactivity patterns in hemagglutination inhibition, neutralization, passive transfer, cross-reactivity, and competitive binding assays that helped illustrate the topological relationships between epitopes on the flavivirus E protein. The first E protein topological epitope model was proposed by Heinz *et al.* (1982) for TBEV, and revealed two non-overlapping protein domains, A and B. By using a larger panel of MAb with different functional activities and serological specificities in competitive binding assays, this work was extended and defined the three non-overlapping antigenic domains recognized currently, A, B, and C (Guirakhoo, Heinz, and Kunz, 1989). By considering the locations of conserved disulfide bonds in the WNV E protein (Nowak and Wengler, 1987), locations of epitopes in the antigenic topological model, and MAb reactivity for the E protein after enzymatic treatment and under reducing, and non-reducing conditions (Guirakhoo, Heinz, and Kunz, 1989), a preliminary structural model of the TBEV E protein was proposed (Mandl *et al.*, 1989). Despite the structural inaccuracy of this model compared to the solved molecular structure of the TBEV E protein (Rey *et al.*, 1995), mapped antigenic epitopes corresponded well to the three distinct structural domains. Further confirmation of the

antigenic domains was provided by a more complete and detailed study of the antigenic structure of the DENV2 E protein and showed that again, the three antigenic domains correlated directly with the three structural domains revealed by the crystal structure (Roehrig, Bolin, and Kelly, 1998). Even though the molecular structures currently available are limited to TBEV, DENV2 and 3, and WNV, the locations of flavivirus conserved cysteine residues suggest that all flaviviruses have analogous E protein structures (Rey *et al.*, 1995; Roehrig, 2003), as evidenced by the close similarity between TBEV and DENV E protein structures in spite of having only 37% sequence identity at the AA level (Modis *et al.*, 2003).

DENV2 E protein structure

The flavivirus E protein consists of three distinct structural domains and 12 fully conserved cysteine residues that make up 6 structurally dependent disulfide bonds (Nowak and Wengler, 1987; Roehrig *et al.*, 2004). DI is a linearly discontinuous structure located centrally in the protein monomer (Figure 1.1). It contains two disulfide bonds (4 cysteine residues, cysteine 3 bonded to 30, and 185 bonded to 285), the amino terminus, and AA residues 1-52, 132-192, and 280-295. DI folds into an 8-stranded β-barrel and is connected to DII via four peptide strands that comprise a molecular hinge region (Figure 1.1, solid triangle) that facilitates molecular conformational changes during membrane fusion (Guirakhoo, Bolin, and Roehrig, 1992; Modis *et al.*, 2003; Rey *et al.*, 1995). DI also contains a potential N-linked glycosylation motif (N-X-T/S, where X = any AA) at E protein residue N153. MAb targeted to DI (antigenic domain C) tend to lack biological function and vary in their antigenic specificity (Guirakhoo, Heinz, and Kunz, 1989; Roehrig, Bolin, and Kelly, 1998).

DII is a linearly discontinuous structure connected to DI via the hinge region and is recognized as the dimerization domain (Figure 1.1). It contains three disulfide bonds (6 cysteine residues, cysteine 60 bonded to 121, 74 bonded to 105, and 92 bonded to 116), and is comprised of AA residues 53-131, and 193-279. DII is an elongated finger-like structure with 12 β-strands, 2 alpha helices, and the flavivirus conserved fusion peptide (CD loop, AA 98-111) that is necessary for membrane fusion and is located at its distal end. DII also contains an N-linked glycosylation motif at E protein residue N67, and this glycan can bind to DC-SIGN on monocyte-derived dendritic cells (Navarro-Sanchez *et al.*, 2003; Tassaneetrithep *et al.*, 2003). MAb targeted to DII (antigenic domain A) can recognize the E protein in neutral and low pH conformations, have neutralizing and anti-hemagglutination activity, and can exhibit flavivirus group-specific reactivity (Guirakhoo, Heinz, and Kunz, 1989; Roehrig, Bolin, and Kelly, 1998). Flavivirus group-specific MAbs 4G2 and 6B6C-1 target AA in the fusion peptide (Crill and Chang, 2004; Huang *et al.*, 2010).

DIII makes up the carboxy-terminal end of the soluble E protein and is an immunoglobulin-like structure (Bork, Holm, and Sander, 1994) connected to DI by a single linker peptide (Figure 1.1). It has one stabilizing disulfide bond (cysteine 302 bonded to 333) and is comprised of AA 296-394. One major structural difference in the E protein between DENV2 and TBEV is located in DIII on the lateral ridge (Figure 1.1, asterisk), where DENV2 has an extended loop motif between the F and G beta strands (FG loop) while TBEV does not (Modis *et al.*, 2004; Rey *et al.*, 1995; Zhang *et al.*, 2004). A detailed description of the FG loop and its contribution to DENV2 biology is provided in Chapter 4 of this dissertation. Antibodies with high neutralizing activity have

been mapped to DIII (antigenic domain B) and soluble DIII has been used to block infection of cells with whole virus, both suggesting DIII contains receptor-ligand epitopes (Abd-Jamil, Cheah, and AbuBakar, 2008; Chin, Chu, and Ng, 2007; Chu *et al.*, 2005; Crill and Roehrig, 2001; Huerta *et al.*, 2008; Roehrig, Bolin, and Kelly, 1998).

DENV life cycle and the E protein

The initial events involved with DENV infection of host cells are attachment to a cell-surface receptor, entry into the cell, and virus-mediated cell membrane fusion within host cell endosomes. Several putative cell receptors capable of binding DENV E protein on mammalian and invertebrate host cells have been identified and include glycosaminoglycans (Vero and BHK21) (Chen *et al.*, 1997; Hung *et al.*, 2004; Hung *et al.*, 1999), heat shock proteins (U937 and C6/36) (Salas-Benito *et al.*, 2007; Valle *et al.*, 2005), stress response protein Grp78 (HepG2) (Jindadamrongwech, Thepparit, and Smith, 2004), DC-SIGN (monocyte-derived dendritic cells) (Navarro-Sanchez *et al.*, 2003; Tassaneetrithep *et al.*, 2003), mannose receptor (macrophages) (Miller *et al.*, 2008), prohibitin (C6/36, CCL-125, and *A. aegypti* whole mosquitoes) (Kuadkitkan *et al.*, 2010), and other as yet unidentified proteins of various sizes (Vero, C6/36, and mosquito midgut cells) (Martinez-Barragan and Del Angel, 2001; Mercado-Curiel *et al.*, 2006). The multitude of identified cellular receptors suggests that the DENV E protein is capable of attaching to different receptors via several structural motifs (Erb *et al.*, 2010) (discussed in Chapter 4). An additional mode of virus entry into permissive cells is referred to as antibody-dependent enhancement (ADE) (Halstead, 1970; Halstead and O'Rourke, 1977; Kliks et al., 1988), whereby virus opsinized by subneutralizing concentrations of cross-reactive antibodies can bind to Fc-receptor (FcR) bearing cells

including monocytes, macrophages, dendritic cells, and B cells and enter into these cells via FcR-mediated endocytosis (Goncalvez *et al.*, 2007; Lin *et al.*, 2002; Lindenbach, 2007; Littaua, Kurane, and Ennis, 1990). While earlier studies utilizing electron microscopy suggested that DENV entered cells by direct penetration with the plasma membrane (Hase, Summers, and Eckels, 1989; Lim and Ng, 1999), recent studies have shown that DENV (Acosta, Castilla, and Damonte, 2008; Krishnan *et al.*, 2007; Mosso *et al.*, 2008; van der Schaar *et al.*, 2008), WNV (Chu, Leong, and Ng, 2006; Mizutani *et al.*, 2003), and JEV (Nawa, 1998; Nawa *et al.*, 2003) enters cells via receptor-mediated, clathrin-dependent endocytosis. Single-particle tracking of DENV on infected cells showed that after attachment to a host cell the virus moves through the plasma membrane or rolls over multiple receptors before being captured by a clathrin-coated pit (van der Schaar *et al.*, 2008). Following this endocytic pathway, mature clathrin-coated pits pinch off into the cytoplasm and these vesicles deliver the virus to Rab5-positive early endosomes, which mature into late endosomes by accumulation of Rab7 (Huang *et al.*, 2010; Krishnan *et al.*, 2007; Rink *et al.*, 2005; van der Schaar *et al.*, 2008). DENV2 strain New Guinea C was shown to fuse with early endosomes (Rab5-positive) (Krishnan *et al.*, 2007) in contrast to strain S1, which was shown to fuse with late endosomes (Rab7-positive) (van der Schaar *et al.*, 2008), indicating that DENV2 strains can have different entry characteristics. As the pH of the endosome decreases, virus-mediated cell membrane fusion is initiated, resulting in the release of the virus nucleocapsid into the cytoplasm. Differences in DENV2 fusion pH thresholds may determine at which stage particular virus strains initiate membrane fusion (Huang *et al.*, 2010; Krishnan *et al.*, 2007; van der Schaar *et al.*, 2008).

The flavivirus E protein is a Class II fusion protein and is structurally similar to the E1 surface protein in alphaviruses (Lescar *et al.*, 2001). Solving the crystal structures for DENV2 and TBEV in their post fusion conformations illuminated the mechanism [illustrated in Figure 1.2 (Kaufmann and Rossmann, 2011)] by which these proteins cause membrane fusion (Bressanelli *et al.*, 2004; Modis *et al.*, 2004). Acidification of endosomes causes dramatic molecular conformational and transitional changes to the E protein. Mildly acidic pH (ca. < 6.5) loosens E protein homodimer interactions causing them to dissociate (Figure 1.2 B) (Allison *et al.*, 1995; Gollins and Porterfield, 1986b; Stiasny *et al.*, 1996). Protonation of histidine residues located in the hydrophic pocket between DI and DIII is hypothesized to trigger the initial protein conformational changes for TBEV (Fritz, Stiasny, and Heinz, 2008). However, mutation of relevant histidine residues in the E protein of WNV subviral particles does not support this requirement (Nelson *et al.*, 2009). Nevertheless, upon acidification, the fusion peptide is released from the hydrophobic pocket formed by the opposite monomer between DI and DII, which allows DII to project towards the endosomal membrane by flexing at the hinge region by ca. 37° (Zhang *et al.*, 2004). At the same time, the soluble E-stem region (C-terminal end of the E protein, AA 396-447) extends away from the viral membrane (Kaufmann *et al.*, 2009; Schmidt, Yang, and Harrison, 2010). The dissociation of the homodimers and the insertion of the fusion peptide into the outer leaflet of the endosomal membrane drives the rearrangement and trimerization of E protein monomers into a "pre-hairpin" intermediate structure (Figure 1.2 C) (Modis *et al.*, 2004). Trimer contacts spread from the fusion peptide down towards the base of DI while DIII experiences a significant displacement by ca. 30 Å up towards DII. DIII rotates ca. 20° with its

translocation causing the C-terminus to fold towards the fusion peptide (Modis *et al.*, 2004; Mukhopadhyay, Kuhn, and Rossmann, 2005). This allows the E-stem to zipper up in the hydrophobic groove between DII trimer contacts, bringing the viral and endosomal membranes into close proximity, and thus driving the formation of the fusion pore (Figure 1.2 C-E). The formation of a hemifusion stalk intermediate is necessary for the final configuration of the post-fusion E timer, which is the most energy stable molecular conformation of the E protein (Modis *et al.*, 2004).

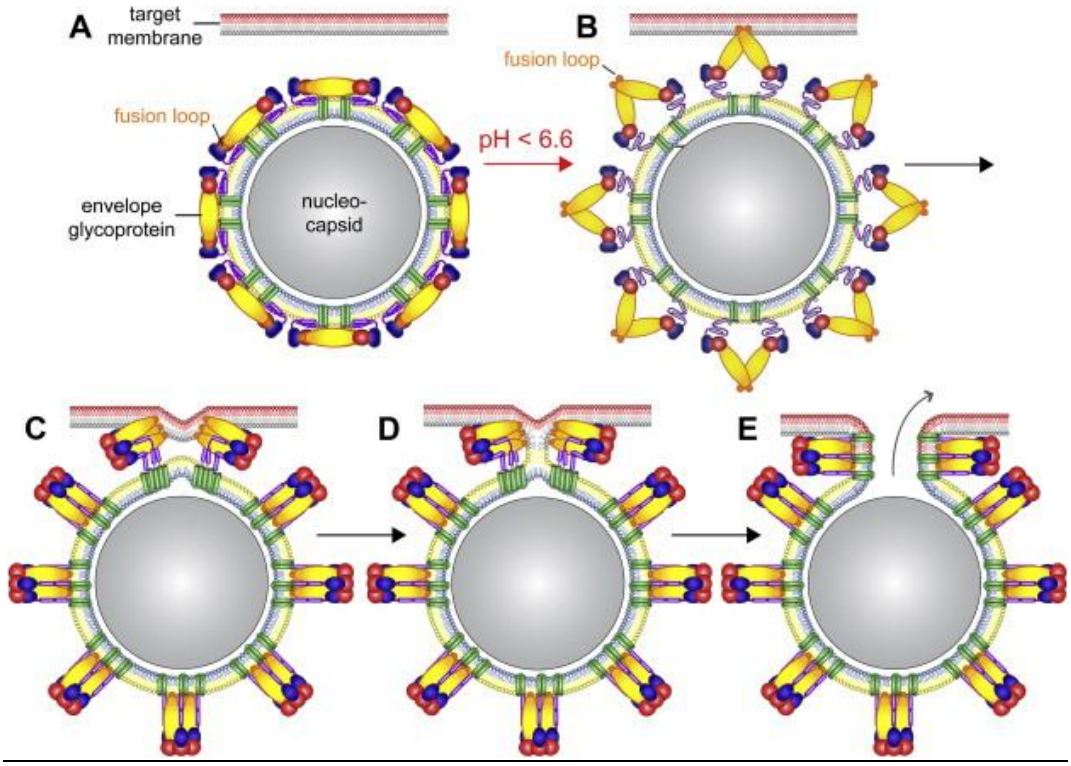

Figure 1.3. Proposed events involved in Class II protein membrane fusion. (A) Mature flavivirus virus prior to conformational changes with DI in red, DII in yellow, DIII in blue, and the E-stem and transmembrane domains in green. (B) Acidic pH causes E homodimers to dissociate, allows the fusion peptide to move into close proximity with the endosomal membrane, and mediates the extension of the E-stem away from the virion membrane. (C) E protein trimerization and the translocation of DIII towards the fusion peptide. (D) Zippering up of the stem region along the DII trimer contacts induces a hemifusion pore. (E) Post fusion E protein trimer opens a fusion pore allowing for the release of the virus nucleocapsid. This figure is borrowed from Kaufmann B., and Rossmann MG. (2011) "Molecular mechanisms involved in the early steps of flavivirus cell entry." Microbes and Infection, 13(1): 1-9.

After virus entry and membrane fusion the nucleocapsid is disassembled and the virus genome is translated into a large polyprotein that is co- and post translationally processed (polyprotein: NH_2-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH). The C protein contains a hydrophobic signal sequence at its carboxy-terminus that will translocate prM into the ER during translation. prM contains two transmembrane-spanning domains that contain a stop transfer and signal sequence that also allows for the E protein to be translocated into the ER (Lindenbach, 2007;

Mukhopadhyay, Kuhn, and Rossmann, 2005). The E protein contains two transmembrane domains, of which the carboxy-terminal domain acts as a signal sequence for translocation of NS1 protein into the ER lumen. After translocation of prM and E into the ER, the NS2B/NS3 serine protease complex cleaves the carboxy-terminus of the C protein on the cytosolic side of the ER. This step was shown to be critical for the subsequent cleavage of the N-terminus of prM on the luminal side of the ER by host cell signalase (Lobigs, 1993; Lobigs and Lee, 2004; Lobigs *et al.*, 2010; Stocks and Lobigs, 1998). Virus replication complexes containing virus RNA amplification machinery are associated with virus-induced intracellular membrane structures at the rough ER, which localize viral proteins and RNA, and potentially protect the virus genome from being targeted by the host cell interferon pathway and RNAi machinery (Mackenzie, Jones, and Young, 1996; MacKenzie and Westaway, 2001; Welsch *et al.*, 2009; Westaway *et al.*, 1997).

Inside the ER lumen, the prM protein will rapidly fold immediately after signalase cleaves its N-terminus, freeing it up to act as a chaperon protein to assist in the proper folding of the E protein, indicating that E protein folding is dependent on co-expression with prM protein (Courageot *et al.*, 2000; Konishi and Mason, 1993; Lorenz *et al.*, 2002). Proper folding of the prM and E proteins catalyzes the association of prM and E into heterodimers. ER host chaperon proteins BiP, calnexin, and calreticulin are necessary for biosynthesis and assembly of prM and E in the ER lumen (Limjindaporn *et al.*, 2009; Wati *et al.*, 2009). The prM of flaviviruses contains one to three potential N-linked glycosylation sites and at least one of these sites was found to be necessary for productive release of JEV mature virions (Kim *et al.*, 2008). However, it is currently unclear how

these results translate to DENV. The DENV2 E protein has two potential N-linked glycosylation sites at N67 and N153 and individual DENV2 strains vary in their utilization of these two sites. Although glycosylation of N67 was at first found to be critical for virus survival and maturation in mammalian and insect cell cultures (Bryant *et al.*, 2007; Mondotte *et al.*, 2007), it was recently shown that ablation of this glycosylation site can be tolerated for replication in both cell types depending on the AA mutation engineered at this position, albeit with reductions in the amount of virus released from infected cells (Lee *et al.*, 2010). Also, eliminating both glycosylation sites had no effect on infection of adult female mosquitoes after intrathoracic (IT) inoculation (Bryant *et al.*, 2007). It is not yet clear how these glycosylation sites influence DENV infection of midgut cells after oral infectious blood-meal. Virions express structurally different N-linked glycans when produced in either mammalian or mosquito cells. N-linked glycans produced in insect cells have high mannose terminal residues while glycans produced in mammalian cells have more complex terminal sugars with no mannose residues. Despite that DC-SIGN preferentially binds high mannose sugars (Feinberg et al., 2007), DENV virions produced in both C6/36 cells and Vero cells could bind and enter DC-SIGN expressing human monocytes and dendritic cells similar efficiencies (Hacker, White, and de Silva, 2009). The contribution of these glycosylation sites to host cell infection, protein maturation and virus morphogenesis remains unclear.

Nucleocapsid formation is mediated by the association of viral RNA with the highly basic capsid protein on the cytosolic side of the ER membrane, where nucleocapsids bud into the ER and virions acquire their host cell-derived lipid envelope and surface structural proteins (Kiermayr *et al.*, 2004; Welsch *et al.*, 2009). These non-

infectious and immature particles then travel through the ER, Golgi, and trans-Golgi network (TGN) secretory pathway. The immature DENV has 60 prominent spike projections each containing trimers of prM-E heterodimers (Zhang *et al.*, 2003). The prM protein prevents premature fusion of the E protein with intracellular host cell membranes during E protein maturation through the secretory pathway by capping the fusion peptide at the distal end of DII, which projects away from the virion surface by ca. 25° in the immature virion (Guirakhoo, Bolin, and Roehrig, 1992; Zhang *et al.*, 2003). Acidification of vesicles containing virus particles in the TGN induces a conformational change that exposes a furin cleavage site in prM that is cleaved by host cell furin, which releases pr from the M protein (Randolph, Winkler, and Stollar, 1990; Shapiro, Brandt, and Russell, 1972; Shapiro *et al.*, 1997; Stadler *et al.*, 1997; Yu *et al.*, 2008). After furin cleavage, pr stays associated with the E protein, now in its homodimer conformation, to prevent membrane fusion until it disassociates from E when particles are released into the extracellular milieu (Li *et al.*, 2008a; Yu *et al.*, 2008; Zheng, Umashankar, and Kielian, 2010). Immature DENV particles are not infectious for mammalian or insect cultured cells (Zybert *et al.*, 2008) and DENV amplified in insect C6/36 cells produce a higher proportion of immature virions than DENV amplified in Vero cells (Murray, Aaskov, and Wright, 1993). FcR-bearing cells can become infected by prM containing virions by binding to the DENV cross-specific MAb that target the prM protein (Dejnirattisai *et al.*, 2010; Huang *et al.*, 2006). These virions either contain partial amounts of uncleaved prM on their surface (Dejnirattisai *et al.*, 2010; Junjhon *et al.*, 2010) or presumably, upon entry and acidification of the endosome, host cell furin can cleave the prM protein and free up the E protein fusion peptide for subsequent membrane fusion.

DENV pathology in humans

The clinical manifestations of disease caused by DENV range from self-limiting symptomatic DF to more severe disease characterized as DHF and DSS. Following the bite of an infected mosquito, virus is deposited into the skin where local Langerhans dendritic cells (DCs) are the first cell type to become infected (Wu *et al.*, 2000). Infected DCs migrate to regional lymphnodes for antigen presentation to T lymphocytes, where infected cells will release virus that can infect other cell types in the draining lymphnode such as monocyte/macrophages, B cells, and other DCs. From the draining lymphnodes, virus can enter the bloodstream, possibly via infected B cells (Lin *et al.*, 2002), which mediates infection of secondary organs such as the liver, kidneys, endothelium, and spleen (Hall *et al.*, 1991; Jessie *et al.*, 2004). Symptomatic infections leading to DF are preceded by an incubation period generally ranging from 4 to 7 days (WHO, 2009). Clinical features of DF vary according to the age of the patient but manifest as flu-like symptoms marked by sudden onset of fever, severe headache, retro-orbital pain, body aches, joint pains, and rash (Gubler, 1998; Whitehead *et al.*, 2007). The acute phase of illness will last for 3 to 7 days post onset of symptoms and is generally self-limiting (Gubler, 1998; WHO, 2009). Resolution of DENV infection is associated with virus clearance by cytotoxic T cells (Bukowski *et al.*, 1989; Kurane, Meager, and Ennis, 1989; Yauch *et al.*, 2009) and virus neutralization by antibodies that can block virus-mediated cell membrane fusion or virus attachment by targeting DII and DIII of the E protein, respectively (Crill and Roehrig, 2001; Gollins and Porterfield, 1986a; Kaufman *et al.*, 1987; Roehrig, Bolin, and Kelly, 1998; Whitehead *et al.*, 2007). As mentioned earlier, microbial adaptation and change can contribute to the emergence of more virulent strains

of arboviruses and this adaptation can be driven by selective pressure imposed by the human immune response (Roehrig, 2003). Mapping the location of amino acid mutations in the E protein of viruses grown in the presence of neutralizing antibody has identified regions of the flavivirus E protein that are targeted by neutralizing antibodies that are often subtype and type specific; these regions include amino acids pertinent to this dissertation: 123-128, 307-311, and 384-385 (Cecilia and Gould, 1991; Lok, Ng, and Aaskov, 2001; Mandl et al., 1989; McMinn et al., 1995; Roehrig, 2003). In the context of pathogenesis, both the T cell and antibody response can contribute to disease progression from DF to DHF and DSS.

Around the time of defervescence, DF can progress to DHF and DSS, which are characterized by thrombocytopenia, hemorrhagic manifestations, and increased vascular permeability that can lead to hypovolemic shock (Gubler, 1998; Whitehead *et al.*, 2007; WHO, 2009). There are two theories explaining why disease pathogenesis can progress to DHF and DSS, which include ADE and virus genetics that result in increased virulence; these are most likely not mutually exclusive. The ADE hypothesis states that upon secondary infection with a heterologous DENV serotype, pre-existing, sub-neutralizing, and non-protective antibodies will bind to viruses and will enhance their uptake in FcR-bearing monocytic cells, resulting in enhanced infection and greater burden of disease. Concurrent with the antibody response to heterologous infection, activation of memory T-cells specific for the previous infection (original antigenic sin) is postulated to delay viral clearance and increase cytokine production, thereby skewing the immune response away from the current infection (Mongkolsapaya *et al.*, 2003). Memory CD4+ T cells activated by infection can release IFN gamma, which will up-

regulate the expression of FcR on monocytes, and further perpetuate the infection of these cells (Pang, Cardosa, and Guzman, 2007). Infected monocytes release tumor necrosis factor-alpha (TNF-alpha) that in turn can induce vascular leakage by increasing the permeability of endothelial cell monolayers (Espina *et al.*, 2003). TNF-alpha is strongly implicated in DENV pathogenesis (Atrasheuskaya *et al.*, 2003; Prestwood *et al.*, 2008; Shresta *et al.*, 2006). Original antigenic sin is not always associated with ADE disease progression, considering that infants can develop DHF without experiencing a prior infection, suggesting ADE associated disease in infants is mediated primarily by maternal antibodies (Halstead *et al.*, 2002; Kliks *et al.*, 1988).

Virus genetics is also associated with DHF and DSS. The infecting DENV serotype (Balmaseda *et al.*, 2006; Fried *et al.*, 2010) and genotype (Messer *et al.*, 2003; Rico-Hesse *et al.*, 1997; Ty Hang *et al.*, 2010) have been implicated as risk factors for the development of severe disease. Disease severity has been associated with high viremia titers in the infected human (Vaughn *et al.*, 2000) and differences in virus output from infected monocytes has been demonstrated between virus strains (Cologna and Rico-Hesse, 2003; Pryor *et al.*, 2001); aspartic acid at E protein position 390 in conjunction with 5' and 3' NCR specific nucleotide sequences (Leitmeyer *et al.*, 1999) were shown to attenuate DENV2 American genotype virus replication in monocytes compared to viruses in the Asian genotype. AA mutations at N124 and K128 in DII of the E protein were found to be associated with increased vascular permeability in a mouse model (Prestwood *et al.*, 2008; Shresta *et al.*, 2006). The contribution of virus genetics and ADE to the development of DHF and DSS are most likely not mutually exclusive and are important considerations for DENV vaccine design.

DENV vaccine considerations

Despite its impact on public health, there are currently no licensed vaccines available for DENV. Efforts to create a DENV vaccine have been hampered by lack of an appropriate animal model that does not include immunocompromised animals or adapted virus strains (Yauch and Shresta, 2008), the need to design four individual vaccines, one against each serotype, and the necessity of producing robust antibody responses against all four serotypes in a tetravalent formulation in order to reduce the risk of inducing ADE by vaccination (Durbin and Whitehead, 2010). The most promising and leading vaccine candidates for DENV are live attenuated virus formulations. Live virus vaccines have the advantage over killed or subunit vaccines because they can induce humoral as well as cellular immune responses that mimic infections by wild type viruses. Additionally, live vaccines require fewer booster immunizations than their counterparts (Whitehead *et al.*, 2007). Favorable characteristics for live-attenuated DENV vaccines include balanced replication kinetics between each virus serotype in the tetravalent vaccine formulation so that the immune response is targeting and creating sufficient levels of neutralizing antibody against each virus equally; defined genetic mutations that confer attenuating phenotypes that are stable during replication in humans and can be monitored during vaccine production; and the inability of each virus to infect mosquitoes. An excellent example of a successful live attenuated arbovirus vaccine is YFV 17D. Highly virulent YFV strain Asibi was passaged 176 times in mouse brain and chicken tissue to produce the 17D strain (Theiler, 1937a; Theiler, 1937b). Vaccination with 17D results in viremia titers less than 10^2 pfu/ml, produces a robust and long-term neutralizating antibody response that is protective against all known YFV strains for

decades (Barrett and Teuwen, 2009), and is unable to disseminate from mosquito midguts to secondary mosquito tissues (McElroy *et al.*, 2006; Miller and Adkins, 1988; Whitman, 1939), thereby precluding transmission of the vaccine strain to naïve individuals. Despite the tremendous success of the YFV 17D vaccine, viscerotropic and neurological disease has been associated with YFV 17D vaccination (Barrett and Teuwen, 2009; Engel *et al.*, 2006; Guimard *et al.*, 2009; Jennings *et al.*, 1994; McMahon *et al.*, 2007). Augmented neurovirulence of one of the 17D vaccine strains was mediated by a single AA mutation in DIII of the E protein; however, this particular mutation did not provide the virus with the ability to disseminate from mosquito midguts (Jennings *et al.*, 1994). Understanding how mutations affect virulence in humans as well as in mosquitoes is important to the design of safe and efficacious live attenuated virus vaccines.

Infection of mosquitoes with DENV

DENV are maintained in nature in a mosquito-human-mosquito transmission cycle and *A. aegypti* mosquitoes are the primary vector (Bancroft, 1906; Gubler and Rosen, 1976). *A. albopictus* and *A. polynesiensis* are also capable vectors for DENV (Gubler, 1988). As a highly domesticated mosquito, *A. aegypti* prefers to breed in artificial containers and feed almost exclusively on humans, often more than once during a single gonotrophic cycle (Scott *et al.*, 2000; Scott *et al.*, 1993), a trait that has been associated with higher reproductive rates for this species and for the viruses it transmits (Scott *et al.*, 1997). *A. aegypti* is susceptible to each DENV serotype and co-circulation of multiple serotypes in the same region is common (Gubler, 1998).

Mosquitoes become infected with DENV when feeding on a viremic host. After an extrinsic incubation period (the length of time it takes for virus to reach the salivary

glands and be transmissible [EIP]) of ca. 7 to 14 days, the mosquito can infect a naïve host upon subsequent blood-feeding (Gubler and Rosen, 1976; Salazar *et al.*, 2007; Watts *et al.*, 1987). The length of the EIP can vary for individual mosquito and virus strains. Also, new experimental (Irma Sanchez-Vargas, personal communication) and field based evidence is accumulating to suggest that DENV can transmit vertically from infected females to their progeny (Arunachalam *et al.*, 2008; Gunther *et al.*, 2007; Vilela *et al.*, 2010). The epidemiological implications of this phenomenon are unclear.

Upon blood-feeding, the blood-meal is deposited into the posterior midgut of the mosquito (Figure 1.3). The mosquito midgut is the site of blood-meal digestion and absorption, and the nutrients obtained from vertebrate blood are necessary for the process of egg production (Pennington, 2005). The *A. aegypti* midgut is composed of a single layer of epithelial cells surrounded by a basement membrane and muscle fibers. The cell types that make up this layer include columnar epithelial cells that are extensively microvillated, secretory cells, and endocrine cells. Each is involved with blood-meal digestion and is thought to be polarized, consisting of an apical cell surface exposed to the midgut lumen and a basal surface that faces the porous basal lamina. Zieler and colleagues (2000) examined the luminal surface of the *A. aegypti* midguts pre- and post blood-meal by scanning electron microscopy and showed that the surface of this tissue is covered by a dense layer of branching fibers they identify as the microvilli-associated network (MN). The majority of cells on the luminal surface of the midgut are extensively covered by the MN except for a few that account for approximately 1% of all midgut cells; these were designated as bare cells. The MN is distinct from the peritrophic matrix (Zieler *et al.*, 2000). The peritrophic matrix is a semi-permeable extracellular layer

comprosed of chitin, proteins, and proteoglycans that surrounds a blood-meal to protect the epithelial cells against pathogens, abrasion, and toxic compounds in the vertebrate blood (Davenport, 2005; Richards and Richards, 1971). It is formed ca. 4 to 8 hours post blood-feeding (Perrone and Spielman, 1988). Furthermore, the midgut is separated from the mosquito hemolymph by the basal lamina, which serves as a barrier for the movement of macromolecules between these two areas. Molecules no larger than 15 nm can move through this barrier structure (Reddy and Locke, 1990), which suggests that DENV (ca. 50 nm) would not be able to passively escape the midgut and penetrate the hemolymph directly through this barrier. Virus has also been detected in secondary tissues without establishment of infection in the midgut following an infectious blood-feed (Hardy *et al.*, 1983; Richardson *et al.*, 2006; Weaver and Scott, 1990; Weaver *et al.*, 1991), although this may be an artifact of challenging mosquitoes by artificial means.

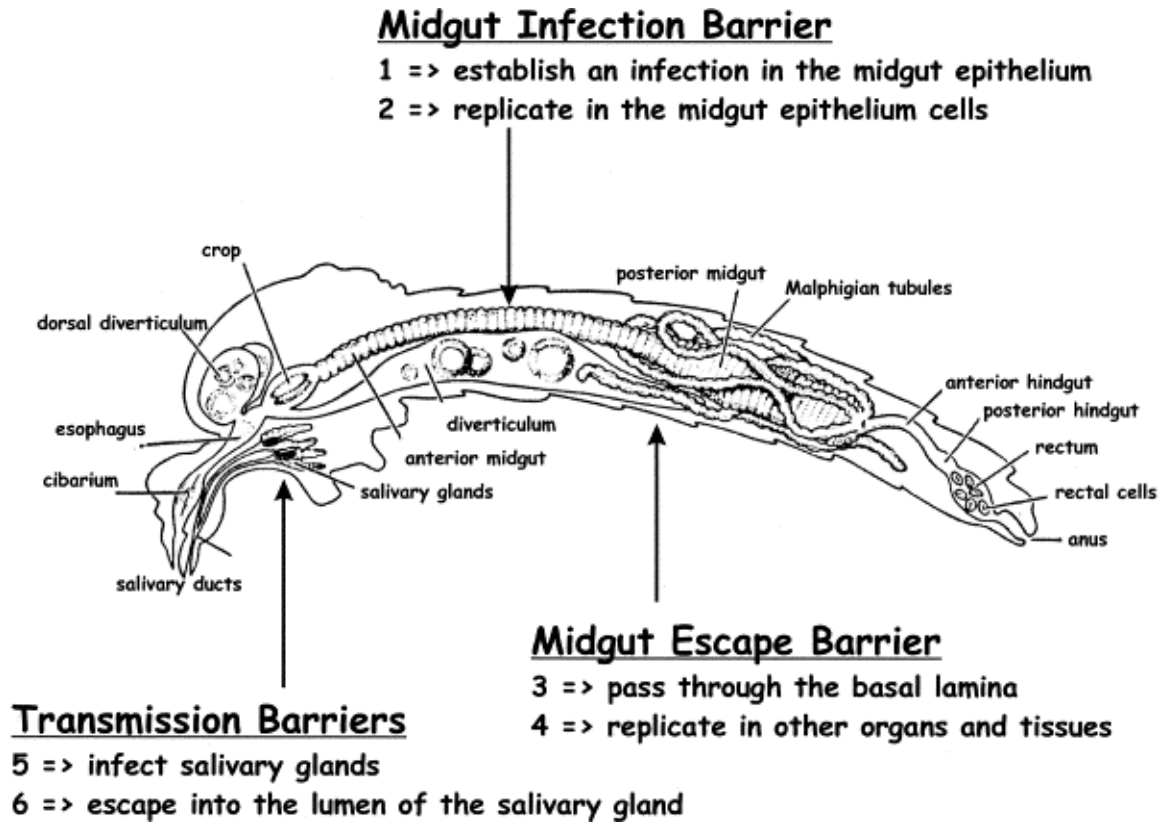

Figure 1.4. Mosquito anatomy and barriers to infection by arboviruses. Borrowed from Black *et al.* (2002).

Arboviruses are biologically transmitted by their vectors and this requires that virus must first infect and replicate in the invertebrate host prior to transmission to the vertebrate host. The steps involved and the barriers to vector transmission of arboviruses are outlined in Figure 1.3. Barriers to infection by arboviruses include the midgut infection barrier (MIB), midgut escape barrier (MEB), and two transmission barriers called the salivary gland infection barrier (SIB) and the salivary gland escape barrier (SEB). Vector competence is the intrinsic permissiveness of a vector for infection, replication, and transmission of a virus and *A. aegypti* strains vary in their vector competence for DENV (Bennett *et al.*, 2002; Black *et al.*, 2002; Bosio, Beaty, and Black, 1998; de Oliveira *et al.*, 2003; Failloux, Vazeille, and Rodhain, 2002; Gubler *et al.*, 1979;

Tardieux *et al.*, 1990; Vazeille-Falcoz *et al.*, 1999). Similarly, DENV strains also vary in their ability to successfully be transmitted by mosquitoes (Armstrong, 2001; Armstrong and Rico-Hesse, 2003; Lambrechts *et al.*, 2009). Vector competence is affected by both extrinsic environmental (discussed in Chapter 2) and intrinsic genetic factors (Bosio, Beaty, and Black, 1998). Quantitative genetic studies analyzing the ability of *A. aegypti* to become infected with and transmit DENV identified quantitative trait loci (QTL) that condition DENV infection of mosquito midguts and dissemination to secondary tissues (Bennett *et al.*, 2005; Bosio, Beaty, and Black, 1998; Bosio *et al.*, 2000; Gomez-Machorro *et al.*, 2004). Known mosquito genes associated with QTL regions are carboxypeptidase, apolipophorin 2, early trypsin, and late trypsin. Currently, only the contribution of trypsin enzymes to DENV infectivity has been investigated experimentally by QTL mapping.

Trypsins are integral for blood-meal digestion in the mosquito midgut. How these enzymes affect DENV infectivity of midguts remains unclear. Proteolytic processing of LACV structural genes in the mosquito midgut is necessary for enhancement of midgut infectivity (Ludwig *et al.*, 1989; Ludwig *et al.*, 1991), and initially early trypsin was found to enhance DENV infection of mosquito midguts (Molina-Cruz *et al.*, 2005). A subsequent study using RNAi knockdown of trypsin showed that suppression of early trypsin had no effect on DENV midgut infectivity, and that late trypsin actually reduced DENV midgut infectivity (Brackney, Foy, and Olson, 2008; Lu *et al.*, 2006). Furthermore, association mapping showed that there were no consistent associations between the segregating sites in the early trypsin gene and DENV susceptibility (Gorrochotegui-Escalante *et al.*, 2005). QTL encode hundreds of genes that could

contribute to DENV2 susceptibility and although not entirely conclusive, these studies analyzing trypsin and mosquito vector competence are the first to investigate the QTL data.

Mosquito midgut protein R67/R64 was shown to bind DENV2 and is also suggested to be a marker of vector competence (Mercado-Curiel, Black, and Munoz, 2008). Comparison of midgut R67/R64 protein expression between highly susceptible and refractory mosquito strains showed that the refractory strain expressed significantly less protein then its counterparts. However, DENV protein expression in each mosquito strain was equivalent at 5 hours post blood-feed until it decreased significantly in the refractory strain compared to the susceptible strains (Mercado-Curiel, Black, and Munoz, 2008), suggesting that despite the difference in R67/R64 expression between the mosquito strains, a step downstream from initial attachment to a host cell receptor in the midgut lumen is influencing refractoriness.

Mosquito innate immunity has also been shown to affect infection of mosquitoes by DENV. Host cell RNAi machinery recognizes flavivirus double stranded RNA intermediates produced during replication in infected cells and then targets specific virus sequences for degradation. The RNAi pathway has been shown to have anti-viral activity against infection by DENV2 in *A. aegypti* (Barbosa-Solomieu *et al.*, 2007; Franz *et al.*, 2006; Sanchez-Vargas *et al.*, 2009; Sanchez-Vargas *et al.*, 2004), O'nyong nyong virus in *Anopheles gambiae* (Keene *et al.*, 2004), and WNV in *Culex pipiens quinquefasciatus* (Brackney, Beane, and Ebel, 2009). Specific virus genome regions are targeted by the RNAi machinery at higher frequencies than others and this has been suggested to drive genetic diversification of viruses (Brackney, Beane, and Ebel, 2009). Invertebrate C6/36

cells are often used as a surrogate for studying mosquito-virus interactions. These cells were originally isolated from Singh's *A. albopictus* larval line (Singh, 1967) for their ability to replicate DENV and CHIKV to high titers (Igarashi, 1978). Recent evidence has shown that these cells have a defective RNAi pathway (Brackney et al., 2010; Scott et al., 2010). C6/36 cells infected with different arboviruses including DENV (Scott et al., 2010), WNV, Sindbis virus, and LACV do not effectively cleave double stranded RNA due to inefficient Dicer 2 activity, a protein integral to the RNAi pathway. While C6/36 cells remain an important tool for amplifying viruses to high titer, their status as an surrogate for studying molecular interactions between arboviruses and mosquitoes is coming into question (Brackney et al., 2010; Scott et al., 2010). The mosquito Toll pathway (Ramirez and Dimopoulos, 2010; Xi, Ramirez, and Dimopoulos, 2008b) and JAK-STAT pathways (Souza-Neto, Sim, and Dimopoulos, 2009) have also been shown to induce anti-virus responses in the mosquito after infection by DENV2. Research investigating the importance of these two pathways to DENV infectivity of mosquitoes is in its infancy.

The replication and tropisms of DENV2 in orally infected *A. aegypti* mosquitoes have been described thoroughly (Richardson *et al.*, 2006; Salazar *et al.*, 2007). DENV2 antigen is detected in midguts as early as day 2 post blood-feed (pbf) and virus titers and antigen expression continue to increase until ca. day 10 pbf, when they start to decline. While virus titers and antigen expression decreases in the midgut after day 10 pbf, viral RNA levels stay relatively high, suggesting that while viral RNA can persist in midgut cells, these cells begin to repress virus translation (Richardson *et al.*, 2006; Salazar *et al.*, 2007). Virus dissemination kinetics varies by virus and mosquito strain but can occur as

early as day 2 pbf. The tracheal system has been implicated as a conduit for virus to escape from the midgut (Romoser *et al.*, 2005; Romoser *et al.*, 2004; Salazar *et al.*, 2007), although in general, virus dissemination from mosquito midguts is not well understood. Secondary tissues that have been shown to express virus antigen are fat body in the abdomen, dorsal diverticulum, cardia, crop, hemocytes, nervous system tissue, and salivary glands (Figure 1.3) (Salazar *et al.*, 2007).

Dissertation Project

An area of DENV research that has not received sufficient attention is the contribution of virus genetics to mosquito infection. Studies have been limited to comparing mosquito infectivity between virus strains and mosquito strains and have not specifically identified virus genetic factors that determine mosquito infectivity phenotypes. The overall goal of this dissertation was to investigate the contribution of the E protein to infection of mosquitoes.

A infectious clone cDNA of DENV2 strain 16681 (30P-NBX) provides the ability to introduce site-specific AA mutations into the E protein. The ability of 30P-NBX to infect mosquito midguts was investigated in Chapter 2, showing that this virus has a low infectivity phenotype for infection of midguts in multiple mosquito strains. In Chapter 3, 30P-NBX was serially passaged in mosquito midguts and adaptive mutations in the E protein that enhance midgut infection were identified. AA mutations identified in the serial passage experiments were introduced into the infectious clone and the increased infectivity phenotype was confirmed, showing for the first time that DENV2 mosquito infection determinants are located in the E protein. In Chapter 4, the effect of E protein DIII FG loop mutations were analyzed in cell culture and in mosquitoes, and in Chapter 5

the effect of E protein fusion peptide and hinge region mutations on the infectivity of mosquitoes after IT inoculation were analyzed. The work described herein significantly enhances our knowledge of flavivirus E protein biology and mosquito interactions.

CHAPTER 2

INFECTION OF *AEDES AEGYPTI* BY DENGUE VIRUS TYPE 2 STRAIN 16681

Introduction

Dengue viruses (DENVs) are the most medically important arthropod-borne viruses infecting humans today. The DENVs comprise a serocomplex in the family *Flaviviridae*, genus *Flavivirus* that includes DENV serotypes 1-4 (DENV1-4) and are the etiological agents of dengue fever and dengue hemorrhagic fever/dengue shock syndrome. Approximately one third of the world's population is at risk of becoming infected by DENV due to the distribution of their primary vector, *Aedes aegypti* (Gubler, 1998). DENVs are maintained in nature via a human to mosquito transmission cycle. The first mosquito tissue to become infected after a mosquito feeds on a viremic host is the midgut epithelium. Amplification of the virus in midgut cells can result in dissemination to other tissues where the virus will amplify in cells in the hemocoel, fat body, and finally the salivary glands. The transmission cycle is continued when a mosquito with a salivary gland infection feeds on a new host. The two most important barriers to infection and dissemination in the mosquito that affect vector competence include the midgut infection barrier (MIB) and midgut escape barrier (MEB) (Bennett *et al.*, 2002; Black *et al.*, 2002; Bosio, Beaty, and Black, 1998). If a virus is able to overcome both barriers, virus transmission can occur. While there are licensed vaccines for other medically important flaviviruses such as Japanese encephalitis virus (JEV),

yellow fever virus (YFV), and tick-borne encephalitis virus (TBEV), no currently licensed vaccines or antiviral drugs are available for DENV.

Serial passage of DENV2 strain 16681 in primary dog kidney cells resulted in a virus (DENV2 PDK-53) with attenuated phenotypes in cell culture, AG129 mice, mosquitoes, and humans and provides the nonstructural gene backbone for a promising live-attenuated tetravalent DENV vaccine candidate (Butrapet *et al.*, 2000; Huang *et al.*, 2000; Huang *et al.*, 2003; Khin *et al.*, 1994; Kinney *et al.*, 1997b; Vaughn *et al.*, 1996; Yoksan S, 1986). A favorable characteristic of a live-attenuated flavivirus vaccine is its inability to infect and disseminate in its primary vector and it is vital that all precautions should be made to prevent such an event. One of the most successful arbovirus vaccines, yellow fever 17D strain, has a MEB phenotype, which terminates the transmission cycle of the virus should a mosquito feed on a viremic vaccinee (Barrett and Higgs, 2007; Jennings *et al.*, 1994). This feature should be mirrored in dengue vaccine candidates. Domain III of the yellow fever virus E protein is suggested to be a determinant of dissemination and seems to work in close synergism with the nonstructural genes and the 3'NCR of the virus (McElroy *et al.*, 2006). This indicates that there is no one determinant of mosquito dissemination in flaviviruses and analysis of all attenuating mutations should be closely analyzed in invertebrate systems as well as mammalian systems. Evaluation of DENV attenuation in mammalian or mosquito cell culture via temperature sensitivity, fusion inefficiency, and plaque phenotype do not always translate to reliable biological markers for attenuation in mosquitoes (Erb *et al.*, 2010; Huang *et al.*, 2010). Some dengue vaccine candidate viruses have been found to replicate in mosquitoes as efficiently as the parent (Schoepp, Beaty, and Eckels, 1990; Schoepp,

Beaty, and Eckels, 1991). Therefore, investigating virus attenuation in mosquitoes is an important facet to vaccine candidate development and the necessity of including multiple attenuating mutations into the candidate virus without compromising vaccine antigenicity should not be overlooked.

As stressed above, it is important to investigate the ability of vaccine candidate viruses to infect their vector mosquitoes. In this study, virus derived from vaccine candidate DENV2 parent strain 16681 infectious cDNA clone 30P-NBX was characterized for its ability to infect *Aedes aegypti* mosquito midguts. To date, there has been only one report regarding the infection of mosquitoes with this virus and our results show 16681 is less efficient at infecting mosquito midguts than previously reported (Khin *et al.*, 1994). Furthermore, our results suggest that this phenotype is most likely determined by the virus itself. Published literature regarding DENV genetic determinants for infection of mosquitoes is lacking. The low mosquito infection rate of strain 16681 and the availability of an infectious clone for this virus presented us with the opportunity to investigate if mosquito infection determinants can be identified in the DENV E protein.

Methods and Materials

Cell Culture and viruses

C6/36 cells used for virus propagation and assays were grown at 28°C in Leibovitz L-15 medium with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin (p/s), and 2mM of L-glutamine (L-glut). C6/36 cells used for virus propagation were maintained in L15 infection medium with 2% FBS, 1X non-essential amino acids, 100 U/ml of penicillin and 100 μg/ml of streptomycin, and 2 mM

L-glut or YE-LAH/BSS (YE-LAH) medium with 2% FBS (2.0 g/100 ml yeast extract, 10.0 g/100 ml lactalbumin hydrolysate mixed with Earle's Balanced Salts Solution). C6/36 cells used for titration were maintained in DMEM with 2% FBS, 1X non-essential amino acids, 100 U/ml of penicillin and 100 μg/ml of streptomycin, 2 mM L-glut, and 3% (35.0 g/L) sodium bicarbonate. LLCMK2 cells used for titration were grown in DMEM with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin, 2 mM L-glut.

DENV2 strain 16681 (16681) was first isolated from serum of a dengue hemorrhagic fever/dengue shock syndrome patient in Bangkok, Thailand in 1964 (Halstead and Simasthi.P, 1970). 16681 is a member of the DENV2 Asian 1 genotype and has been extensively passaged since isolation. 16681 has been passaged multiple times in BS-C-1 cells, six times in LLC-MK2 cells, once in a rhesus macaque monkey, and twice in *Toxiorhynchites amboinensis* mosquitoes (Halstead and Simasthi.P, 1970; Kinney *et al.*, 1997b). After these initial passages, the virus was passaged once in Vero cells, twice in LLC-MK2 cells, and four times in C6/36 cells (Kinney *et al.*, 1997b).

Construction of DENV2 strain 16681 infectious clone virus has been described previously and the virus used in this study was derived from a modified version of infectious clone D2/IC-30P-A (Huang *et al.*, 2010; Kinney *et al.*, 1997b). To introduce select amino acid mutations into the E gene of DENV2 strain 16681 infectious cDNA clone for E protein mutagenesis studies, pD2/IC-30P-A was modified to contain a BspE1 restriction site that results in silent mutations for introduction of unique cloning sites. The insertion and change of BspE1 site between the E gene and NS1 gene at genome nucleotide position 2425 splits pD2/IC-30P-A into two intermediate clones, pD2/I-

5'NBAX and pD2/I-3'ABX, containing the structural genes and nonstructural genes, respectively. Ligation of these two intermediate clones yields the full length modified infectious clone pD2/IC-30P-NBX (Chapter 3, Figure 3.1). The clone derived virus, 30P-NBX, was passaged once in C6/36 cells and exhibits the same phenotypes in mammalian and invertebrate cell cultures as parent 16681.

DENV2 strain Jamaica 1409 (J1409) was isolated from a human with DF in 1983 and is a member of the American/Asian genotype. J1409 was first plaque purified in LLC-MK2 cells and then passaged extensively (> 25 times) in C6/36 cells (Deubel, Kinney, and Trent, 1986; Deubel, Kinney, and Trent, 1988; Pierro *et al.*, 2006) before construction of the infectious clone (J1409-ic) by Pierro and colleagues. After transfection and virus recovery in C6/36 cells completed by Pierro and colleagues, J1409-ic derived virus was passaged once in C6/36 cells for our use.

***Aedes aegypti* mosquito midgut infection by bloodfeeding**

Aedes aegypti RexD strain laboratory colony mosquitoes (RexD) originating from Rexville, Puerto Rico, and a DENV2 susceptible strain, D2S3 (Bennett, Beaty, and Black, 2005), were reared from eggs and maintained as adults at 28^{o}C, 80% relative humidity with a photocycle of 12h light: 12h dark. Adult female mosquitoes were maintained in one-pint cartons with organdy covering, and given water and sugar until infection. To obtain DENV2 for infectious blood-feeds, C6/36 cells were infected at a MOI of 0.001 in L15 or YE-LAH infection medium and maintained for 12-14 days with a medium change at 7 days pi (no medium change was performed for YE-LAH grown virus). Infected cells were scraped into the medium, mixed with an equal volume of defibrinated sheep blood, and then supplemented with ATP to a final concentration of 1

mM. Adult female mosquitoes 4-6 days post-emergence were starved for 24 hours, deprived of water for 4 hours, and then exposed to the infectious blood-meal for 45 minutes using a 37^{o}C water-jacketed glass feeding device with a hog gut membrane. Virus titers in the blood-meals ranged from 6.3 to 8.2 $\log_{10}$ pfu/ml or 7.45 to 9.45 $\log_{10}$ $TCID_{50}$/ml. Fully engorged mosquitoes were selected and maintained for 7 days, when midguts were dissected in PBS, fixed in 4% paraformaldehyde in PBS overnight, and analyzed for virus antigen via immunofluorescence (IFA) to determine midgut infection rates. Each bloodfeed experiment was repeated at least three times with a minimum of 19 mosquito midguts analyzed per experiment.

Indirect immunofluorescence assay for mosquito tissues

Midgut IFAs were performed as described previously (Brackney, Foy, and Olson, 2008). Virus antigen in midguts was detected using flavivirus E protein DII group-reactive mouse MAb 4G2 (HB-112, ATCC, Manassas, VA) in wash buffer (PBS, 0.05% TritonX-100). Secondary antibody was ImmunoPure biotin-labeled goat anti-mouse IgG (Thermo Scientific, Waltham, MA) with 0.005% Evan's Blue counter-stain, followed by streptavidin-fluorescein (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). MIRs and head squash infectivity rates were determined by dividing the number of virus antigen-positive midguts or head squashes by the total number analyzed. Student's t tests (p-value ≤ 0.05) were performed using Excel 2007.

Virus titration by plaque assay or 50% tissue culture infectious dose

Plaque assays were performed on LLC-MK2 cells according to methods described previously (Sanchez-Vargas *et al.*, 2009). Briefly, confluent LLC-MK2 cell monolayers in 12-well plates were infected with 10-fold serial dilutions of virus, overlaid with

agarose (1%) nutrient medium, and maintained for 12 days at 37°C. Wells were stained with 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution, incubated for 4 hours, and plaques were counted to determine plaque-forming units (pfu) per ml.

Infectious virus $TCID_{50}$ titers were determined by titration on C6/36 cells and detection of virus antigen by ELISA as described previously (Bryant *et al.*, 2007). C6/36 cells were seeded into the inner 60 wells of a 96 well flat-bottom plate at 1.2×10^5 cells/well. Cells were infected the next day with 10-fold serial dilutions of virus and maintained for 7 days at 28°C with 5% CO_2. Cells were fixed with 85% cold acetone overnight at 4°C and then wells were blocked with blocking buffer (3% goat serum in PBS with 0.1% Tween20). Virus antigen was detected by ELISA using DENV2 strain New Guinea C M30197 polyclonal antiserum at 1:8000 in wash buffer (PBS with 0.05% Tween20), goat anti-mouse alkaline phosphatase conjugate (Jackson Laboratory, Bar Harbor, ME) in blocking buffer, and Sigma 104 substrate (Sigma, St. Louis, MO) in 1 M Tris-HCl pH 8.0. Reactions were stopped with 3M NaOH. Absorbences were read at 405 nm and 630 nm and the difference was determined to obtain the delta optical density (ΔOD). ΔOD values greater than 2-fold of the negative control ΔOD values were considered positive. Virus titers were calculated by the method of Reed and Muench (Reed and Muench, 1938).

Results

30P-NBX midgut infection of Aedes aegypti mosquitoes

Kinney *et al.* (1997) constructed a full-length infectious cDNA clone of DENV2 strain 16681 designated D2/IC-30P-A (30P-A). In order to make the introduction of select amino acid (AA) mutations into the E gene easier, a secondary infectious clone, D2/IC-30P-NBX (30P-NBX) was derived from 30P-A and virus produced from this infectious clone was used for all subsequent studies. Although 30P-NBX cell culture phenotypes were found to be equivalent to the parent strain 16681, 30P-NBX infection of *Aedes aegypti* RexD strain mosquitoes has not yet been elucidated and are expected to be similar to the parental strain.

Before adult female mosquitoes received an infectious blood-meal, 30P-NBX was amplified in C6/36 cells using YE-LAH or L15 infection medium. Virus was treated the same way for both infections except virus cultures grown in L15 medium had the medium changed at day 7 post infection (pi) while virus cultures grown in YE-LAH did not. It was assumed that virus grown in YE-LAH medium did not need a medium change because the pH was stable throughout the infection compared to L15 virus cultures. 30P-NBX has a low midgut infection rate (MIR) in RexD mosquitoes (Figure 2.1). Cumulative data for infection of RexD mosquitoes with virus grown in either YE-LAH and L15 gave a MIR of 20.4% (1160 mosquitoes total) (cumulative data from Figure 2.1 A). Virus grown in L15 (29.6%) has a significantly higher MIR compared to virus grown in YE-LAH medium (6.7%) (Figure 2.1 A). Differences in virus titers in the blood-meals ranging from 6 to 8 $\log_{10}$ pfu/ml did not affect midgut infection for virus grown in L15

and YE-LAH as evidenced by no significant differences in MIRs between each titer range (ANOVA and individual student's t tests) (Figure 2.1 B-D).

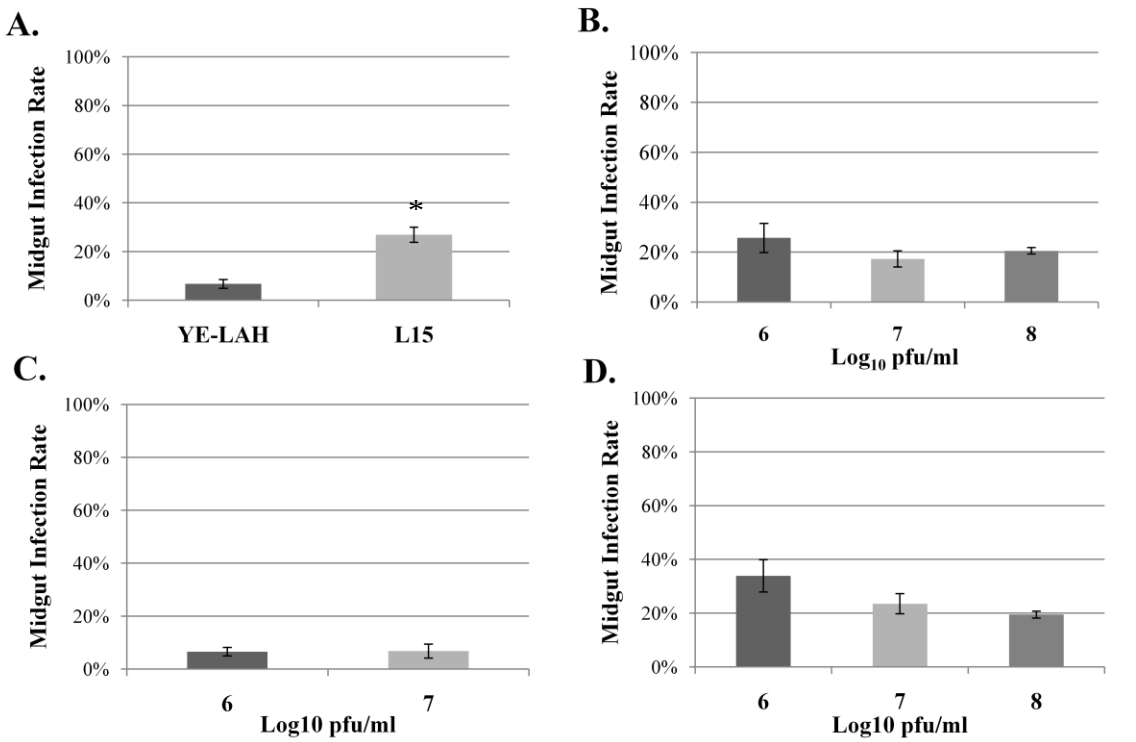

Figure 2.1. MIRs for 30P-NBX grown in YE-LAH and L15 infection media in RexD strain mosquitoes. *Aedes aegypti* RexD strain mosquitoes were given an infectious blood-meal with 30P-NBX cultured in C6/36 cells in either L15 or YE-LAH infection medium, maintained for 7 days when midguts were dissected, and MIRs were determined. MIR data for virus cultured in YE-LAH and L15 (A). Cumulative data from YE-LAH and L15 (A) are separated by virus blood-meal titer (log_{10} 6: 6.0-6.99; 7: 7.0-7.99; and 8: 8.0-8.99) versus MIR (B). Virus blood-meal titer versus MIRs for virus cultured in YE-LAH (C) and L15 (D) infection medium.

Given the low MIR of 30P-NBX in RexD mosquitoes, an infectious blood-meal of 30P-NBX amplified in either L15 or YE-LAH medium was given to D2S3 highly susceptible to DENV2 strain mosquitoes with expectations that virus MIRs would be higher in this strain. Cumulative data for both medium types showed there was a significantly higher MIR in D2S3 mosquitoes compared to RexD mosquitoes; however,

the MIRs were still very low, especially when compared to another DENV2 strain used routinely in our laboratory, Jamaica 1409 (Bennett, Beaty, and Black, 2005). MIRs for virus grown in YE-LAH medium were also significantly higher in D2S3 mosquitoes compared to RexDs in contrast to virus grown in L15 infection medium, which had no significant difference between the two mosquito strains. The inability of 30P-NBX to infect a high proportion of D2S3 or RexD mosquitoes suggests the virus and not the mosquito may be the determining factor for inefficient midgut infection.

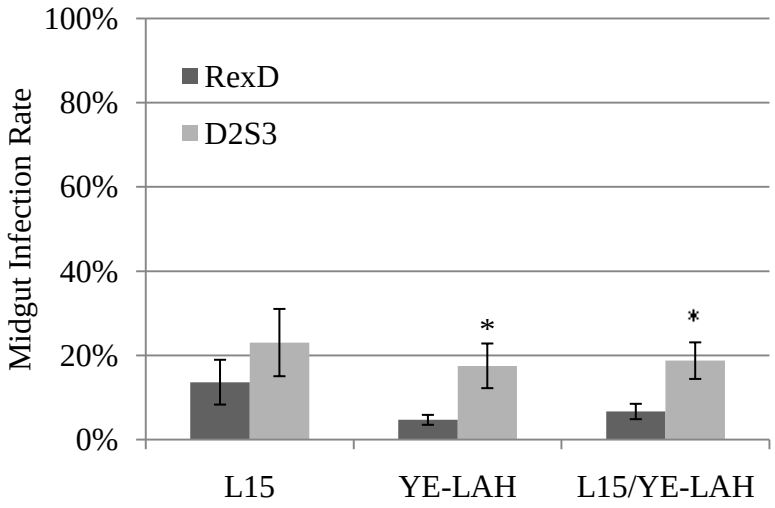

Figure 2.2. 30P-NBX MIRs in RexD and D2S3 strain mosquitoes. *Aedes aegypti* RexD and D2S3 strain mosquitoes were given an infectious blood-meal with 30P-NBX cultured in either L15 or YE-LAH infection medium, maintained for 7 days when midguts were dissected and MIRs were determined. MIR data are separated by media type, with L15/YE-LAH representing the average cumulative MIR for both media types combined.

During the early bloodfeed experiments with 30P-NBX, a C6/36 cell ELISA-based $TCID_{50}$ assay became available to titrate DENV2 blood-meal samples and this assay was used for all subsequent blood-meal titrations. 30P-NBX infectious virus titer by $TCID_{50}$ tends to be 1 log higher than titer by plaque assay. There is no significant difference between MIRs whether virus blood-meal samples are titrated by plaque assay

or $TCID_{50}$ (Figure 2.3 A). Given the previous infection medium comparison, a standardized protocol was established for infection of *Aedes aegypti* mosquitoes by oral infectious blood-meal with 30P-NBX. C6/36 cells were infected using L15 infection medium with a medium change on day 7 pi, and harvest of infected cells and medium at 12 to 14 days pi were titrated by $TCID_{50}$. After this standardization, a data set representing all values for MIRs for 30P-NBX in RexD mosquitoes was accumulated and is shown in Figure 2.3 B. The average MIR for this data set is 33.29% (17 individual experiments, blood-meal titer range 7.45 to 9.45 $\log_{10}$ $TCID_{50}$/ml, with 517 mosquitoes total) and is the representative MIR for 30P-NBX in RexD mosquitoes. Breaking down the data in Figure 2.3 B into blood-meal titer versus MIR shows that the virus titer in the blood-meal does not seem to affect the MIR (Figure 2.4). This data set was used for all subsequent MIR statistical comparisons between 30P-NBX and other viruses in RexD mosquitoes. Additionally, to examine the effect of virus titer on midgut infection, 10-fold serial dilutions of 30P-NBX were included in infectious blood-meals for RexD mosquitoes and the results show that infection rates decreased with titer, but with dilutions greater than 1:10 (Table 2.1).

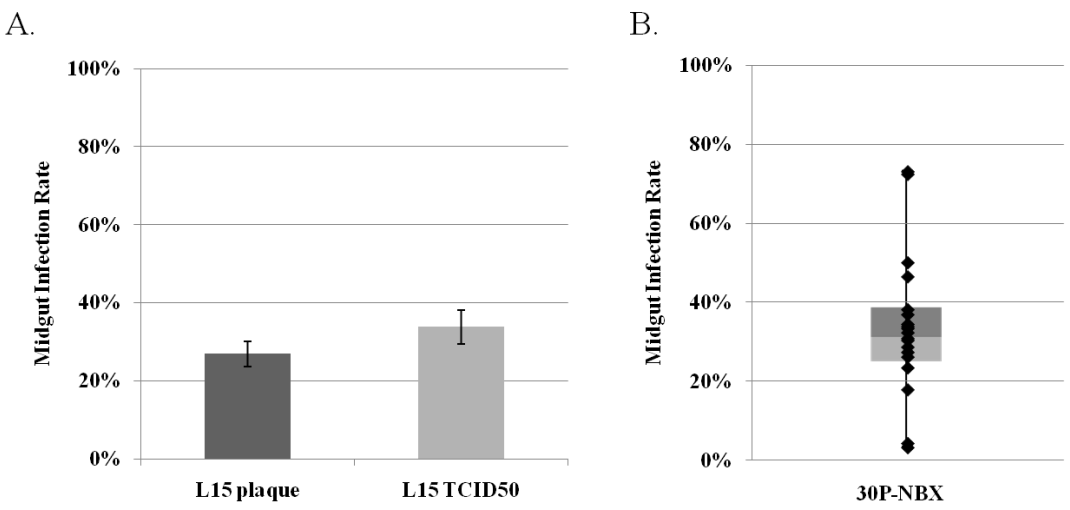

Figure 2.3. 30P-NBX blood-meal titration method versus MIR comparison in RexD mosquitoes, and representative MIR. *Aedes aegypti* RexD strain mosquitoes were given an infectious blood-meal with 30P-NBX cultured in L15 infection medium, maintained for 7 days when midguts were dissected and MIRs were determined. Average MIR for virus blood-meals titrated by plaque assay or $TCID_{50}$ (A). Box plot showing MIRs from 17 individual blood-feed experiments that generate the average MIR for 30P-NBX (B).

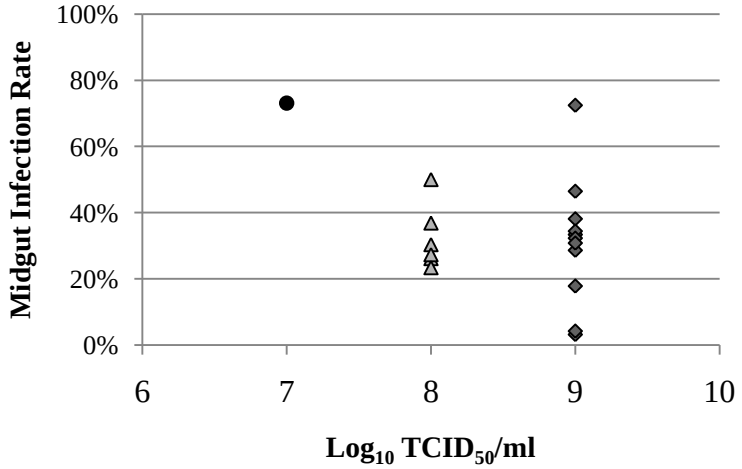

Figure 2.4. 30P-NBX MIRs in RexD mosquitoes versus blood-meal virus titer. *Aedes aegypti* RexD strain mosquitoes were given an infectious blood-meal with 30P-NBX cultured in L15 infection medium, maintained for 7 days when midguts were dissected and MIRs were determined. MIRs versus virus blood-meal titer are from the 17 individual blood-feed experiments that generate the average MIR for 30P-NBX shown in Figure 2.3 B.

Table 2.1. MIRs of RexD mosquitoes orally infected with serial dilutions of 30P-NBX.

Blood-meal Dilution[a]	Midgut Positive	Total Midguts	MIR
1:1	7	30	23.33%
10^{-1}	6	28	21.43%
10^{-2}	2	31	6.45%
10^{-3}	1	36	3.85%

[a]Blood-meal 1:1 titer = 8.2 $\log_{10}$ $TCID_{50}$/ml

Finally, parent strain 16681 and clone-derived 30P-NBX were compared for their ability to infect RexD mosquito midguts. Similar to their phenotypes in cell culture, there was no significant difference in MIR between the parent and clone-derived virus. However, DENV2 strain J1409-ic had a significantly higher MIR than both viruses (Figure 2.4), further highlighting that 16681 is inefficient at infecting RexD mosquito midguts.

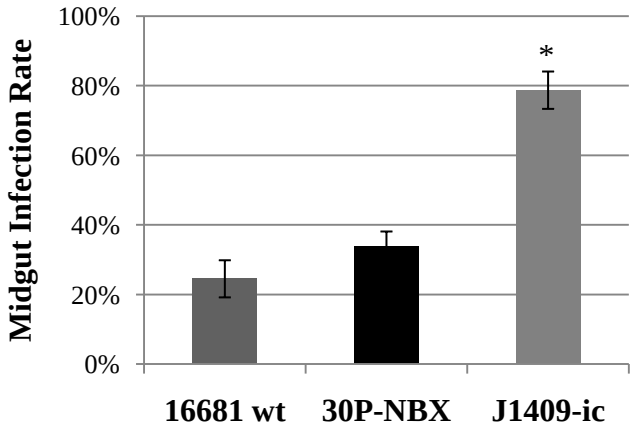

Figure 2.5. DENV2 strains J1409-ic, 16681, and 16681 clone-derived 30P-NBX MIRs in RexD mosquitoes. *Aedes aegypti* RexD strain mosquitoes were given an infectious blood-meal with virus cultured in L15 infection medium, maintained for 7 days when midguts were dissected and MIRs were determined. Significant differences were determined by comparison with 30P-NBX via student's t test (*p value < 0.05).

Discussion

The results of this study show that strain 16681 is not very efficient at infecting *A. aegypti* mosquitoes and in combination with the availability of an infectious clone for this virus, provides us with the opportunity to investigate whether the low infectivity can be attributed to virus genetics. There has been only one report concerning infection of *Aedes aegypti* mosquitoes by DENV2 strain 16681 (Khin *et al.*, 1994) in which the authors report an infection rate of 59% (114/194 mosquitoes) via whole body squash, which is in contrast to the 33.79% (517 total mosquitoes analyzed) average MIR for infectious clone 30P-NBX determined in this study. Infectious clone 30P-NBX was found to have comparable infection rates to the parent virus strain 16681 in our system as expected. Two notable differences that exist between methodologies used by Khin *et al.* (1994) and those used in this study include how the virus was prepared and the specific mosquito strain used.

Khin *et al.* (1994) used virus amplified in LLC-MK2 cells and/or virus amplified in *Toxorhynchites splendens* mosquitoes but do not distinguish which preparation gave the higher infection rates. Virus cultures prepared in mammalian cells tend to give lower infection rates compared to virus cultures prepared in insect cell cultures (Miller *et al.*, 1982) so it is interesting that the average infection rate in our study with virus grown in mosquito cell cultures is lower by comparison. However, when 30P-NBX was amplified in Vero or LLC-MK2 cells and then included in an infectious blood-meal for RexDs we obtained lower MIRs compared to virus amplified in C6/36 cells (20.52% for Vero and 4.44% for LLC-MK2 cells). An evaluation of mosquito infection rates using virus from three different preparations, virus amplified in C6/36 cells for 7 days, virus amplified in

C6/36 cells for 14 days with a medium change at day 7 p.i., and virus amplified in *Toxorhynchites splendens* mosquitoes showed that virus grown for 14 days with a medium change at day 7 pi gave the highest infection rates (Black, 2010; Schoepp, Beaty, and Eckels, 1990). The protocol in our study that gave the highest MIR utilized a similar virus preparation. Virus that was amplified in YE-LAH medium gave significantly lower MIRs compared to L15 infection medium (Figure 2.1A). This difference may be the result of not changing the YE-LAH infection medium at 7 days pi and not a difference between virus production, considering both medium types gave comparable virus titers. Twelve day growth curve experiments in C6/36 cells show that 30P-NBX reaches peak infectious titers between days 5 and 8 pi and then begins to decline (Chapter 3, Figure 3.9). It is unclear why virus harvested at day 7 pi infects a lower proportion of mosquitoes than virus harvested on day 14 that had a prior medium change. Replacing medium could remove harmful byproducts produced by dead and infected cells that can adversely affect mature virus or resupplying cultures with fresh nutrients may help infected cells produce higher quality virus. Midgut epithelial cell susceptibility to DENV may be more sensitive to harmful cellular byproducts than cultured cells or to slight changes in the proportion of mature virus produced by C6/36 cells, which could reduce MIRs.

Khin *et al.* (1994) used a laboratory colonized *Aedes aegypti* mosquito strain from Bangkok, Thailand and our study utilized a strain originating from Rexville, Puerto Rico. It is not unusual to find variation in the susceptibility of geographically disparate strains of *Aedes aegypti* to DENVs (Armstrong, 2001; Armstrong and Rico-Hesse, 2003; Bennett et al., 2002; Gubler et al., 1979; Lambrechts et al., 2009; Tardieux et al., 1990).

DENV2 strain 16681 was isolated from a DHF/DSS patient in Bangkok, Thailand so both the virus and the mosquito used in the study by Khin *et al.* (1994) are from the same geographical region, which may account for the higher infection rate compared to our infection rate in mosquitoes colonized from the Caribbean. However, we found that the average MIR for 30P-NBX in an *Aedes aegypti* mosquito strain isolated in Thailand in 2004 (Higgs *et al.*, 2006) is 43.63%; although results using these mosquitoes were highly variable and had a standard deviation of 38.41% (Appendix 6.1). In addition, when these mosquitoes were orally challenged with J1409, a strain of virus isolated in the Caribbean, the average MIR was 94.44% (2 repetitions, 120 mosquitoes total). Furthermore, variation in vector competence for J1409 was found among 24 collections of *Aedes aegypti* mosquito strains isolated throughout Mexico (Bennett *et al.*, 2002), highlighting that variations in vector competence are a product of the combination between vector genotype and virus genotype (Lambrechts *et al.*, 2009). Despite its limitations (see below), without directly testing vector competence in the laboratory, it is difficult to make predictions about vector susceptibility. The importance of this suggestion is exemplified by 30P-NBX's inability to efficiently infect DENV2 highly susceptible D2S3 strain mosquitoes (Figure 2.2). D2S3 mosquitoes were selected for their high susceptibility to DENV2 (Bennett, Beaty, and Black, 2005). Despite there being a statistical difference between infection of RexD and D2S3 mosquitoes with 30P-NBX, the infection rate is far below those achieved for other DENV2s and other dengue serotypes (Bennett, Beaty, and Black, 2005), further showing that viruses within the same serotype vary in their ability to infect mosquitoes (Armstrong, 2001; Armstrong and Rico-Hesse, 2003; Hanley *et al.*, 2008; Lambrechts *et al.*, 2009). The low efficiency for

infection of *Aedes aegypti* mosquitoes by 30P-NBX seems to be determined by the virus and not the mosquito strain used in the analysis. Furthermore, in addition to mosquito genetics, differences in vector competence and virus infectivity as determined in the laboratory by artificial systems are under the influence of uncontrollable experimental variables influenced by environmental factors.

There is a multitude of extrinsic environmental factors and intrinsic genetic factors that affect the vector competence of mosquitoes for DENV (Black, 2010; Black *et al.*, 2002; Bosio, Beaty, and Black, 1998). Bosio *et al.* (1998) concluded that approximately 60% of the variance in vector competence of *Aedes aegypti* for DENV can be attributed to environmental factors, leaving the remaining 40% of the variance being attributed to the genetics of the mosquito. The long colonized and therefore highly inbred laboratory RexD mosquito strain was used to phenotype 30P-NBX in this study and theoretically the genetics of this mosquito strain should be homogeneous from experiment to experiment (Miller and Mitchell, 1991). Therefore, extrinsic environmental factors apart from mosquito genetics may be influencing our artificial blood-feed experiments to a greater extent. Larval competition has been found to increase mosquito susceptibility to arboviruses (Alto *et al.*, 2005; Grimstad and Walker, 1991), including DENV2 in *Aedes albopictus* and to a lesser extent *Aedes aegypti* (Alto *et al.*, 2008). This may in turn affect the size of the mosquito, which has also been found to alter vector susceptibility to arboviruses (Alto, Reiskind, and Lounibos, 2008; Paulson and Hawley, 1991; Schneider *et al.*, 2007); a higher proportion of mosquitoes with small body size are infected compared to mosquitoes with large body size. However, this is contradicted by a separate study (Sumanochitrapon *et al.*, 1998), again showing that the

particulars of the mosquito system used help determine the outcome of the experiment. Also, decreasing the amount of endogenous bacteria in the mosquito midgut reduces basal levels of antimicrobial gene expression and therefore increases susceptibility to DENV infection (Xi, Ramirez, and Dimopoulos, 2008a), which could add to the variability in infection rates if the levels of endogenous bacteria are different between mosquito preparations. Although genetic factors cannot be definitively ruled out, the contribution of these extrinsic environmental factors may have contributed to the relatively high degree of variation in MIRs for 30P-NBX displayed in Figure 2.3 B, asserting the fact that *per os* challenge of *Aedes aegypti* mosquitoes with DENV2 strain 16681 requires a large sample size and many repetitions. Khin *et al.* (1994) did not specify how many (if any) repetitions were performed in their study and this may also be reflected in our differences in MIRs for this virus strain.

The high degree of variation made it difficult early on to determine the true MIR for 30P-NBX and therefore complicated efforts to statistically compare 30P-NBX derived mutant viruses to the parent (see remaining chapters). All mutant virus MIRs were statistically compared to the 30P-NBX data set presented in Figure 2.3B to determine significant differences. Each individual challenge experiment included a 30P-NBX internal control. In our system the virus titer in the blood-meal was not found to influence mosquito infection rates (Figure 2.1 B-D, and Figure 2.5). Table 2.1 shows MIRs decreased in a graded fashion after more than a 1:10 dilution and this is corroborated by other studies (Gubler *et al.*, 1985; Schoepp, Beaty, and Eckels, 1990; Schoepp, Beaty, and Eckels, 1991). However, even though high-titered virus increases the chance of obtaining higher infection rates, the quality of the virus at the time of

challenge seems to be a more important determinant. This is supported by Figure 2.5 (and Figure 2.1 B-D), in which each individual experimental data point from Figure 2.3 B is broken down into blood-meal titer versus MIR. Figure 2.5 shows that achieving a high virus titer does not always confer a high MIR (Figure 2.5; compare individual MIRs that have blood-meal virus titers 9 $\log_{10}$ $TCID_{50}$/ml). Since virus must be amplified in cell culture prior to each mosquito challenge experiment, the virus blood-meal titer is another extrinsic environmental factor that is difficult to control in our artificial bloodfeed system; including freeze-thawed virus in infectious blood-meals results in low mosquito infection rates (Miller, 1987; Miller *et al.*, 1982; Richards *et al.*, 2007). Despite the contribution of extrinsic and intrinsic factors that can influence mosquito midgut susceptibility to 30P-NBX in our system, these data suggest that 30P-NBX is not very efficient at infecting *Aedes aegypti* mosquito midguts.

A favorable phenotype for arbovirus vaccine formulations that use live-attenuated viruses is their inability to infect mosquitoes and this makes investigating viral determinants of mosquito infection a vital aspect of vaccine design. The infectious clone 30P-NBX, derived from the parent virus used to create a live-attenuated tetravalent vaccine candidate provides a powerful tool to elucidate E protein determinants that attenuate or enhance infectivity of mosquitoes and was used in further studies towards this end. Mutations that attenuate the virus could be included in vaccine formulations and mutations that enhance virus infectivity could be excluded and/or serve as a marker for identifying DENVs with enhanced vector pathogenesis.

CHAPTER 3

THE DENGUE VIRUS TYPE 2 ENVELOPE PROTEIN CONTAINS MOSQUITO INFECTION DETERMINANTS

Introduction

Dengue viruses (DENVs) (*Flaviviridae:Flavivirus*) are the most medically important arboviruses infecting humans today. There are four genetically and antigenically distinct subtypes of DENV (DENV1-4) that comprise the DENV serocomplex. These viruses have a single stranded positive sense RNA genome (~11 kb) encapsidated in a capsid protein core surrounded by a lipid envelope. In the envelope there are 180 copies of the envelope (E) structural protein arranged in an icosahedral scaffold of 90 homodimers that lie parallel to the virion surface (Kuhn *et al.*, 2002). The flavivirus E protein is a Class II fusion protein, the primary determinant of host cell tropism, and is responsible for host cell attachment, entry, and virus-mediated cell membrane fusion. The DENV2 E protein 2 Å crystal structure has been solved (Modis *et al.*, 2003) and revealed three distinct structural domains (DI, DII, and DIII), which correlate with three previously described antigenic domains defined by monoclonal antibody (MAb) mapping data (Heinz, 1986; Mandl *et al.*, 1989; Roehrig, Bolin, and Kelly, 1998; Roehrig *et al.*, 1990). Several important structures spanning each of the domains are vital to the virus replication cycle. Domains I and II are linearly discontinuous structures connected by a molecular hinge region that permits the

translocation and insertion of the flavivirus-conserved DII fusion peptide into the host cell endosomal membrane; a process required for virus-mediated cell membrane fusion. DIII is an immunoglobulin-like structure and is postulated to have host-cell receptor binding properties.

DENVs are maintained in nature via a human-to-mosquito transmission cycle and approximately one third of the world's population is at risk of becoming infected due to the widespread distribution of the primary vector, *Aedes aegypti* mosquitoes (Gubler, 1998). There are multiple steps integral to the transmission of mosquito-borne viruses to vertebrate hosts. Mosquitoes come into contact with virus when taking an infectious blood-meal from a viremic host. Virus must first infect and replicate in midgut epithelial cells and then disseminate into the hemocoel to amplify in secondary target tissues that include the salivary glands. The transmission cycle is continued when a salivary gland-infected mosquito inoculates a new host with virus during a subsequent blood-feed.

To date there has been limited research focusing on flavivirus genetic determinants that influence infection of mosquitoes. The introduction of the DENV2 Asian/American genotype in the Americas coincided with increases in DHF cases and the eventual displacement of the DENV2 American genotype with viruses from the Asian/American genotype (Rico-Hesse *et al.*, 1997). A similar phenomenon was recently observed with DENV2 in Vietnam and with DENV3s in Sri Lanka (Hanley *et al.*, 2008; Ty Hang *et al.*, 2010). The DENV2 Asian/American genotype was shown to not only produce higher infectious virus titers in dendritic cells than the American genotype but also to infect and disseminate more efficiently in field-caught *A. aegypti* mosquitoes (Armstrong and Rico-Hesse, 2003; Cologna and Rico-Hesse, 2003). Sequence

comparison of the two genotypes revealed several genome differences in the 5' and 3' non-coding regions and one mutation in the E protein (N/D390) (Leitmeyer *et al.*, 1999). Recombinant viruses containing combinations of these 5' and 3' non-coding regions and E genome differences showed viruses containing American genotype sequences (N390) had decreased virus output from mammalian cells (Cologna and Rico-Hesse, 2003). However, significant attenuation could not be attributed to any single sequence change and these recombinant viruses were not phenotyped in mosquitoes (Cologna and Rico-Hesse, 2003). Similarly, recombinant viruses utilizing yellow fever virus (YFV) virulent strain Asibi and vaccine strain 17D showed that DIII was a determinant for dissemination of virus from the midgut, albeit again not the sole determinant as it required synergism with the nonstructural genes (McElroy *et al.*, 2006).

While DENV2 strain 16681 infectious clone 30P-NBX (30P-NBX) infects and replicates efficiently in *A. aegypti* RexD strain mosquitoes after intrathoracic (IT) inoculation, infection of the midgut epithelium after introduction of virus by infectious blood-meal is less efficient (Erb et al., 2010). Given the E protein's importance to virus tropism, and that 30P-NBX has a relatively low midgut infection rate (MIR) after oral infectious challenge but not after IT inoculation, we hypothesized that mosquito infection determinants were located in the E protein. We serially passaged 30P-NBX four times in *A. aegypti* RexD strain mosquito midguts and identified an adaptive amino acid (AA) mutation in DII of the E protein that significantly enhanced midgut infectivity. Further analysis identified a second AA mutation in the same region of DII that conferred a similar phenotype. This study demonstrates for the first time that mosquito infection determinants are located in DII of the DENV2 E protein.

Methods and Materials

Cell culture

Vero cells were grown at 37^{o}C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). C6/36 cells used for virus phenotyping experiments were grown in YE-LAH medium (Huang *et al.*, 2000) and C6/36 cells used for virus propagation to infect mosquitoes were grown in Leibovitz L-15 infection medium (2% FBS, 1mM non-essential amino acids, 1mM penicillin-streptomycin, and 1 mM L-glutamine), both at 28^{o}C..

Aedes aegypti mosquito infection by blood-feeding and intrathoracic inoculation

Aedes aegypti RexD strain laboratory mosquitoes (RexD) originating from Rexville, Puerto Rico and *A. aegypti* Chetumal strain mosquitoes (Chet) originating from Chetumal, Mexico were reared from eggs and maintained as adults at 28^{o}C, 80% relative humidity with a photocycle of 12h light: 12h dark. Adult female mosquitoes were maintained in one-pint cartons with organdy covering, and given water and sugar until infection. To obtain DENV2 for infectious blood-feeds, C6/36 cells were infected at a multiplicity of infection (MOI) of 0.001 and maintained for 12-14 days with a medium change at 7 days. Infected cells were scraped into the medium mixed with an equal volume of defibrinated sheep blood and then supplemented with ATP to a final concentration of 1mM. Adult female mosquitoes 4-6 days post-emergence were starved for 24 hours, deprived of water for 4 hours, and then exposed to the infectious blood-meal for 45 minutes using a 37^{o}C water-jacketed glass feeding device with a hog gut membrane. Virus titers in the blood-meals for all experiments ranged from 6.2 to 9.2 $\log_{10}$ $TCID_{50}$/ml. In our previous experience with 30P-NBX and E protein mutant

viruses, there was no correlation between virus titer at this range and midgut infectivity (Chapter 2). Although the 30P-NBX MIR data set from Chapter 2 were used for all statistical comparisons between mutant and parent virus, a 30P-NBX internal control was included for every blood-feed experiment performed. Fully engorged mosquitoes were selected and maintained for 7 days, when midguts were dissected in PBS, fixed in 4% paraformaldehyde in PBS overnight, and analyzed for virus antigen via immunofluorescence (IFA) to determine midgut infection rates. Each blood-feed experiment was repeated at least three times with at least 19 mosquito midguts analyzed per experiment. To investigate mosquito infection rate kinetics, dissected mosquito midguts and head tissues assays were performed every two days for 14 days post bloodfeed (pbf). Virus antigen was detected in midguts and head squashes to determine infection rates as described below. Experiments were repeated three times and 17-30 mosquitoes were dissected on each day pbf.

Indirect immunofluorescence assay (IFA) for mosquito tissues

Midgut and head squash IFAs were performed as described previously (Brackney, Foy, and Olson, 2008). Virus antigen in midguts and head squashes was detected using flavivirus E protein DII group-reactive mouse MAb 4G2 (HB-112, ATCC, Manassas, VA) in wash buffer (PBS, 0.05% TritonX-100) or PBS, respectively. Secondary antibody was ImmunoPure biotin-labeled goat anti-mouse IgG (Thermo Scientific, Waltham, MA) with 0.005% Evan's Blue counter-stain, followed by streptavidin-fluorescein (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). MIRs and head tissue infectivity rates were determined by dividing the number of virus antigen-positive midguts or head squashes by the total number analyzed. The relative

infection intensity (RII) ratio is a quantitative measure of infection intensity in the midgut (Brackney, Foy, and Olson, 2008). Positive midguts were scored for infection intensity on a scale of 0.5 to 4, where 0.5 denotes less than 25% of the midgut surface area is positive for viral antigen, 1 denotes 25%, 2 denotes 50%, 3 denotes 75%, and 4 denotes 100% of the midgut surface area is positive for viral antigen. The RII ratio was determined by adding the infection intensity scores of positive midguts and dividing by the total number of positive midguts. Student's t tests (p-value 0.05) were performed using Excel 2007 and chi-square analysis (p-value 0.05) was done using SAS 9.1.

Envelope glycoprotein gene sequencing

DENV2 RNA was isolated from infected C6/36 cell cultures with the QIAamp Viral RNA Isolation Kit (Qiagen), and the E gene was amplified using Titan One-Step RT-PCR system (Roche, Indianapolis, IN) per the manufacturer's instructions. RT primers were used at 20 µM (Forward primer: D2 841 5'--atg atg gca gca atc ctg gca tac-- 3'; Reverse primer: cD2 2688 5'--cat gat tcc ttt gat gtc tcc tgt c-- 3'). The reaction parameters were one reverse transcription step (RT) at 50^{o}C for 30 min, followed by 96^{o}C for 2 min, and then 35 cycles at 96^{o}C for 20 sec, 55^{o}C for 30 sec, and 68^{o}C for 2 min. PCR products were gel extracted using QIAquick Gel Extraction kit (Qiagen) and sequencing reactions were performed using ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions with one notable exception. BigDye Termination Ready mix was diluted for one reaction as follows, 1 µl of BigDye Termination Ready Mix, 3.5 µl of 5x BigDye reaction buffer, and 3.5 µl of dH_2O. Reaction parameters were one exposure to 96^{o}C for 1 min, and then 25 cycles at 96^{o}C for 10 sec, 50^{o}C for 5 sec, and 60^{o}C for 4 min. Viral

genome sequencing was performed at the Centers for Disease Control and Prevention, Fort Collins, CO. Sequences were analyzed using Lasergene Seqman (DNASTAR, Madison, WI).

DENV2 viruses and their passage in Aedes aegypti mosquito midguts

Infectious virus derived from DENV2 strain 16681 infectious cDNA clone 30P-NBX, 30P-NBX derived FG loop mutants VEPGΔ and RGD (Chapter 4), and DENV2 strain Jamaica 1409 infectious cDNA clone (J1409-ic) (Chapter 2) were serially passaged in *A. aegypti* RexD midguts and C6/36 cells. To start the passage experiment, virus was amplified in C6/36 cells and the cell-virus suspension was included in an infectious blood-meal as described above. The remaining virus culture was stored at -80^{o}C for sequencing of the DENV E gene. RexD mosquitoes were challenged with the infectious blood-meal and fully engorged mosquitoes were maintained for 10 days. Mosquito midguts were dissected and placed into 4% paraformaldehyde for IFA analysis to determine MIRs (19-36 mosquitoes) or pooled on dry ice for trituration (at least 20 mosquitoes). Midguts were triturated in L15 infection medium and filtered through a 0.2 µm membrane syringe filter. The filtrate was placed directly onto naïve C6/36 cells to start the next passage. Four passages were completed for each virus.

Mutant virus construction

Construction of DENV2 strain 16681 infectious clone virus has been described previously (Huang *et al.*, 2010; Kinney *et al.*, 1997b). To introduce select amino acid mutations into the E gene of DENV2 strain 16681 infectious cDNA clone, pD2/IC-30P-A was modified to contain a BspE1 restriction site that results in silent mutations for introduction of unique cloning sites. The insertion of BspE1 between the E gene and

NS1 gene at genome nucleotide position 2425 splits pD2/IC-30P-A into two intermediate clones, pD2/I-5'NBAX and pD2/I-3'ABX, containing the structural genes and nonstructural genes, respectively. Ligation of these two intermediate clones yields the full length modified infectious clone pD2/IC-30P-NBX (Figure 3.1). The clone derived virus, 30P-NBX, exhibits all of the same phenotypes as the 16681 parent virus. Splitting up the full length infectious clone made it easier to introduce mutations into the E gene located in the smaller D2/I-5'NBAX plasmid via site-directed mutagenesis.

QuikChange® Lightning Site-directed Mutagenesis kit (Stratagene, Santa Clara, CA) was used to introduce mutations into the E gene of D2/I-5'NBAX per the manufacturer's instructions. Engineered mutations were targeted to DII of the E protein and included KK122/123EE and R120T. Primers used to make the KK122/123EE change included nucleotide substitutions a1300g and a1303g and the primers used to mutate R120T included g1295c (Forward primer: 5'--gtg acc tgt gct atg ttc aca tgc aaa aag aac atg gaa--3'; Reverse primer: 5'--ctt cca tgt tct ttt tgc atg tga aca tag cac agg tca c--3'). Amplified, mutated pD2/I-5'NBAX and its sister plasmid, pD2/I-3'ABX, were digested with BspI and XbaI restriction endonucleases, and gel purified via the QIAquick Gel Extraction kit (Qiagen, Valencia, CA). pD2/I-3'ABX insert (excluding vector) was ligated to pD2/I-5'NBAX via the BspI restriction site at DENV2 nucleotide position 2425 and inserted via the unique XbaI restriction site located at the 3' terminus of the DENV2 genomic cDNA (Fig 3.1) to create full length infectious clone pD2/IC-30P-NBX cDNA. Recombinant cDNA was amplified in electroporation-competent *Escherichia coli* XL1-Blue cells (Stratagene) and plasmid cDNA was isolated via HiSpeed Plasmid Midi kit (Qiagen) per the manufacturer's instructions with the exception that selected bacterial

colonies were amplified in 2xYT broth with ampicillin (0.1 mg/ml) prior to plasmid purification.

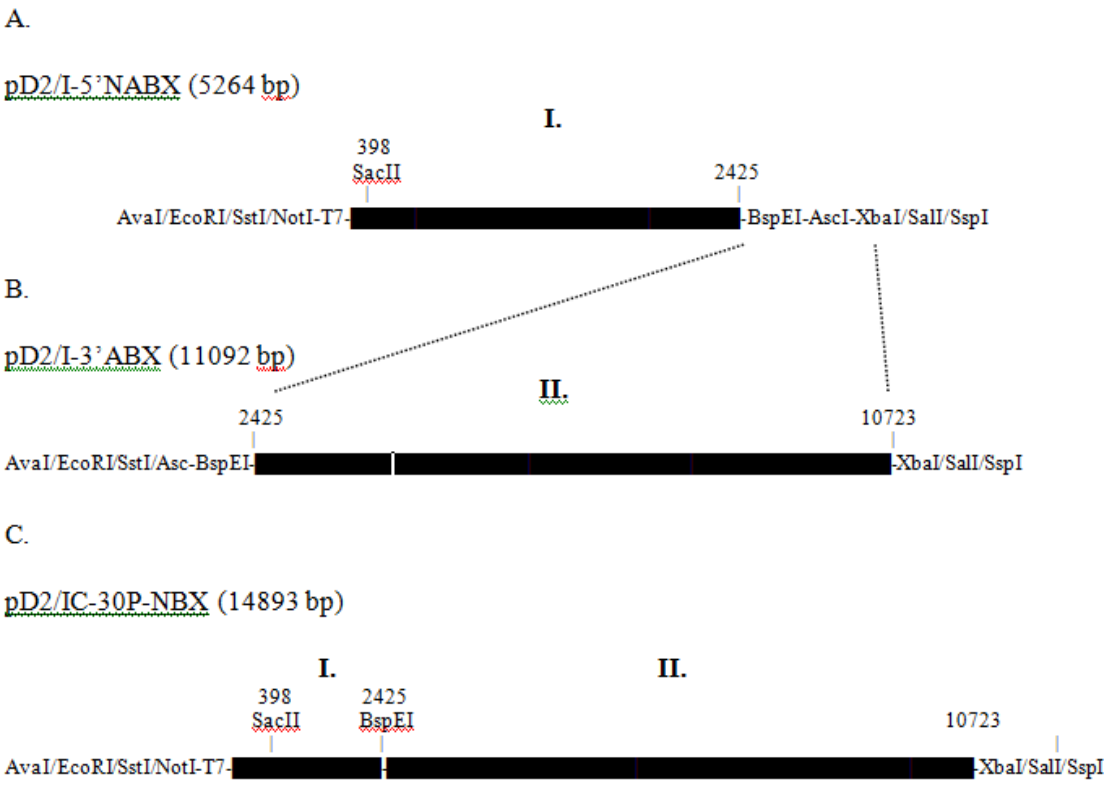

Figure 3.1. D2/IC-30P-NBX clone construction. Insertion of intermediate clone pD2/I-3'ABX (B) into pD2/I-5'NABX (A) at BspE1 and Xba1 restriction sites yields the full length infectious clone pD2/IC-30P-NBX (C); remaining vector sequence between AvaI and SspI not shown. Site-directed mutagenesis of pD2/I-5'NABX introduced desired nucleotide substitutions into the E gene prior to construction of the full length clone.

In vitro transcription of infectious RNA and transfection of cultured cells

In vitro transcription and transfection of Vero and C6/36 cells was performed as described previously (Huang *et al.*, 2010; Huang *et al.*, 2000; Kinney *et al.*, 1997b). Recombinant cDNA was linearized with XbaI and treated with 500 µg/ml proteinase K (GIBCO, Carlsbad, CA). Linearized cDNA was extracted once with phenol/chloroform/isoamyl alcohol, pH 8.0 (Ameresco, Framingham, MA), once with chloroform, and precipitated with ethanol. Approximately 80-100 ng of linearized cDNA

was used to transcribe genomic RNA via the AmpliScribe™ T7 kit (Epicentre Technologies, Madison, WI) according to the manufacturer's instructions with two notable changes; the transcription reaction (total volume 40 µl) contained 1.5 mM ATP, and 3 mM m^7-GpppA cap analog (New England Biolabs, Ipswich, MA) in order to increase the incorporation of A cap analog on the 5' end of the genome. The reaction was treated with DNaseI and the RNA was extracted once with phenol/chloroform/isoamyl alcohol, pH 6.8 (Ameresco, Framingham, MA), once with chloroform, and precipitated with ethanol. Since little RNA was recovered after the procedure, total resuspended RNA was split in half for transfection into Vero and C6/36 cells.

Positive-sense vRNA was transfected into Vero and C6/36 cells using a Bio-Rad Gene Pulser Xcell system (Bio-Rad, Hercules, CA) as described previously (Huang *et al.*, 2010). Vero and C6/36 cells were grown to 80-100% confluency, detached from flasks, washed twice with cold PBS, and resuspended to the desired concentration. Vero cells (4 x 10^6/0.4 ml) were mixed with RNA, transferred into a cold 0.4 cm gap cuvette, subjected to one square-wave pulse at 225 Volts for 25 milliseconds, and pipetted into 30 ml $10DMEM_{20}$ (DMEM, 10% FBS, 1X non-essential amino acids, 100 µg/ml penicillin-streptomycin, and 0.15% sodium bicarbonate) in a 75 cm^2 tissue culture flask. C6/36 cells (8 x 10^6/0.4 ml) were mixed with RNA, transferred into a cold 0.4 cm gap cuvette, subjected to two square-wave pulses at 225 Volts for 1 millisecond with a 5 second interval, and pipetted into 30 ml $10DMEM_{10}$ (DMEM, 10% FBS, 1X non-essential amino acids, 100 µg/ml penicillin-streptomycin, and 0.075% sodium bicarbonate) in a 75 cm^2

tissue culture flask. Transfected cells were maintained at 37°C and 28°C with 5% CO_2 for Vero and C6/36 cells, respectively.

Medium harvested from transfected Vero cells (day 12 post infection (pi), designated V-0) or C6/36 cells (day 14 pi, designated C-0) was centrifuged to remove cell debris, supplemented with 20% FBS, and stored at -80°C. An aliquot of V-0 and C-0 was used to infect naïve Vero and C6/36 cells to produce V-1 and C-1 seeds, respectively. Genome cDNA of V-1 and C-1 seeds were fully sequenced to evaluate their genomic stability as described previously (Huang *et al.*, 2010). Viral antigen was detected in acetone-fixed cells (day 8 post transfection/infection) by IFA using polyclonal anti-DENV2 New Guinea C hyperimmune mouse ascitic fluid (HMAF) and fluorescein isothiocynate-conjugated (FITC) goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA).

Assay of virus growth kinetics in cell culture

Twelve to fourteen day growth curves were performed to investigate mutant virus growth kinetics in various cell types. Cell cultures were infected in duplicate at a MOI of 0.001. Virus genomic equivalents were measured by quantitative (q)RT-PCR using 3'-NCR primers and probes via iScript™ One-Step RT-PCR Kit (Bio-Rad, Hercules, CA) as previously described (Butrapet, Kinney, and Huang, 2006), and samples were assayed for infectious virus by $TCID_{50}$ in C6/36 cells (Chapter 2).

Mosquito midgut-virus attachment assay

Aedes aegypti RexD mosquitoes were challenged orally with 30P-NBX or K122E as described above with the exception that virus was mixed 1:1 with heat-inactivated FBS instead of sheep's blood. Control mosquitoes were fed L15 infection medium mixed 1:1

with heat-inactivated FBS. Bloodfeeding was stopped after 30 minutes and mosquito cartons were placed on ice and put at 4°C. Two sets of midgut samples were dissected in three pools of five midguts for each virus. For the first set of samples, fully engorged mosquito midguts were dissected out, cut open, and the virus-meal was removed. Each midgut was then washed three times in ice-cold PBS to remove residual unattached virus and then placed on dry ice. For the second set of samples, fully engorged midguts were dissected in ice-cold PBS without removing the virus-meal. Total RNA was extracted from each pool using TRIzol® (Invitrogen, San Diego, CA) and viral RNA was quantified by qRT-PCR. Engorged mosquitoes not dissected to determine attachment efficiencies were maintained for 7 days post-bloodfeed to obtain MIRs.

Results

Serial passage of DENV2 in Aedes aegypti mosquito midguts

DENV2 strain 16681 infectious clone 30P-NBX, 30P-NBX-derived FG loop mutants VEPGΔ and RGD (Erb *et al.*, 2010), and infectious clone J1409-ic viruses were serially passaged in *A. aegypti* RexD strain mosquitoes to see if adaptive mutations would accumulate in the virus genome; the focus of our study was on the E gene because of its importance to virus tissue tropism. Pre-passaged virus MIRs show 30P-NBX and mutant virus RGD have similar MIRs while mutant virus VEPGΔ and J1409-ic have significantly lower and higher MIRs compared to 30P-NBX, respectively (Figure 3.2).

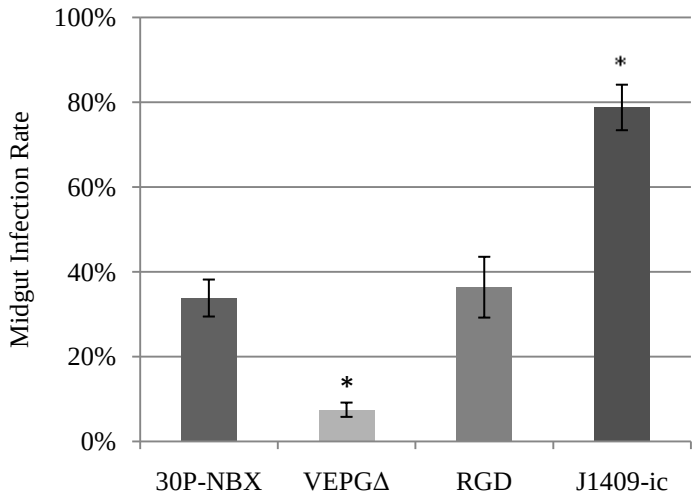

Figure 3.2. Virus MIRs in *A. aegypti* RexD mosquito midguts. RexD mosquitoes were orally challenged with each virus, maintained for seven days until midguts were dissected, and MIRs were determined by IFA. Data are the average of at least three experiments and significance was determined by comparison with 30P-NBX via student's t test (*p value < 0.05).

Each virus was serially passaged four times (SP0-4) in RexD mosquito midguts and C6/36 cells (Table 3.1). Comprehensive serial passage data for each virus is provided in Tables 3.2, 3.3, 3.4 and 3.5. Any mutations that occur in the E gene are suspected to be the result of adaptation to midgut epithelial cells because the E gene of 30P-NBX is genetically stable after successive passages in C6/36 cells (Huang *et al.*, 2010). SP0 MIRs are similar to the average MIRs for each of the viruses (Table 3.1 and Figure 3.2); 30P-NBX SP0 is lower than the average MIR for 30P-NBX but falls within the range of 30P-NBX MIRs in RexD mosquitoes found previously (Chapter 2). The MIRs of 30P-NBX and mutant virus RGD significantly increased after one passage in RexD midguts and in both cases this increase correlated with an amino acid change in the E protein (Table 3.1). Consensus sequencing of the E gene of SP1 virus showed there was a mixed nucleotide (nt) base population of adenine (wild type [wt]) and guanine (mutant) at nt position 1300 for both viruses. A change to guanine causes a non-

conservative AA change from lysine to glutamic acid in DII of the E protein at AA position 122 (Figure 3.3). As revealed by consensus sequencing, it took four passages of 30P-NBX in mosquito midguts before glutamic acid completely substituted lysine at position 122, while it took RGD only two passages before this complete AA change occurred. Two serial passage experiments were completed for 30P-NBX in RexD midguts and both experiments yielded the same results (data not shown). In contrast, VEPGΔ and J1409-ic MIRs did not demonstrate any discernable pattern after four serial passages in mosquito midguts and no nt changes were found in the E gene. VEPGΔ virus was lost after four passages and there was an insufficient amount of vRNA present in SP4 samples for sequencing. RGD-SP3 virus included in the infectious blood-meal was incubated in C6/36 cells for 17 days before it was harvested to challenge mosquitoes and this may account for why the MIR dropped from 93% to 57% between passages 2 and 3. It is unclear why the MIR for 30P-NBX-SP3 decreased to 44%, but it may be due to individual variation found in DENV blood-feed experiments, considering it increased again to 95% for SP4; the unpassaged 30P-NBX internal control for SP3 also had a below average MIR of 16% (data not shown). An adaptive AA mutation located in DII of the E protein that significantly enhances mosquito midgut infection was unexpected and is to our knowledge the first observation of its kind.

Table 3.1. Serial passage of 30P-NBX, VEPGΔ, RGD, and J1409-ic in *A. aegypti* RexD mosquitoes.

	30P-NBX			VEPGΔ			RGD			J1409-ic		
Passage	Midgut Infection Rate	Confidence Interval (%)	E protein AA at 122[a]	Midgut Infection Rate	Confidence Interval (%)	E protein AA at 122	Midgut Infection Rate	Confidence Interval (%)	E protein AA at 122	Midgut Infection Rate	Confidence Interval (%)	E protein AA at 122
0	18%	7-34	K	4%	1-20	K	33%	20-51	K	89%	68-97	K
1	68%	46-86	K/E	16%	7-33	K	83%	66-93	K/E	54%	36-71	K
2	61%	45-75	E/K	12%	5-27	K	93%	78-98	E	63%	46-77	K
3	44%	29-61	E/K	4%	0.9-18	K	57%	36-76	E	77%	60-89	K
4	95%	75-99	E	0%	0.07-10	n/a[b]	87%	70-95	E	86%	69-94	K

[a] AA present at DENV2 E protein position 122. Mixed consensus sequence verified by cDNA sequencing in both directions. The first AA labeled is present in greater amounts than the second AA; as determined by the sequence chromatogram.
[b] There was an insufficient amount of vRNA present for sequencing.

Table 3.2. 30P-NBX serial passage in *A. aegypti* RexD strain mosquito midguts.

30P-NBX	Passage Number				
	0	1	2	3	4
Log_{10} blood-meal titer	7.67	9.2	7.82	7.55	8.95
Positive Midguts	6	13	22	15	18
Total Midguts	34	19	36	34	19
MIR	18%	68%	61%	44%	95%
Confidence Interval (%)	8-34	46-86	45-75	29-61	75-99
E protein AA substitutions	none	K122K/E	K122E/K	K122E/K	K122E

Table 3.3. VEPGΔ serial passage in A. aegypti RexD strain mosquito midguts.

VEPGΔ	Passage Number				
	0	1	2	3	4
Log_{10} blood-meal titer	7.45	7.2	7.95	6.95	5.45
Positive Midguts	1	5	4	1	0
Total Midguts	24	31	34	27	35
MIR	4%	16%	12%	4%	0%
Confidence Interval (%)	1-20	7-33	5-27	1-18	0.1-10
E protein AA substitutions	none	none	none	none	none

Table 3.4. RGD serial passage in A. aegypti RexD strain mosquito midguts.

RGD	Passage Number				
	0	1	2	3	4
Log_{10} blood-meal titer	8.45	9.2	8.45	8.2	8.2
Positive Midguts	11	25	27	12	26
Total Midguts	33	30	29	21	30
MIR	33%	83%	93%	57%	87%
Confidence Interval (%)	20-51	66-93	78-98	36-76	70-95
E protein AA substitutions	none	K122K/E	K122E	K122E	K122E

Table 3.5. J1409-ic serial passage in A. aegypti RexD strain mosquito midguts.

J1409-ic	Passage Number				
	0	1	2	3	4
Log_{10} blood-meal titer	9.45	8.95	8.45	8.2	6.45
Positive Midguts	17	15	22	24	25
Total Midguts	19	28	35	31	29
MIR	89%	54%	63%	77%	86%
Confidence Interval (%)	68-97	36-71	46-77	60-89	69-94
E protein AA substitutions	none	none	none	none	none

Site-directed mutagenesis, transfection and mutant virus recovery in C6/36 and Vero cells

Serial passage of 30P-NBX and mutant virus RGD in RexD mosquito midguts selected for AA mutation K122E in DII of the E protein. AA sequence alignment of the E proteins shows there is high sequence variability in DII AA 120 to 130 between DENV serotypes and other arthropod-borne flaviviruses (Table 3.3). This region of the E protein was targeted previously for a different set of mutagenesis experiments where K122 and K123 were both mutated to glutamic acid (virus designated as K122/3E). This double mutation abolishes putative heparan sulfate binding sites and changes the AA charge from positive to negative. It was interesting that J1409-ic did not accrue any adaptive mutations after passage in mosquito midguts and DENV2 E protein AA sequence alignment shows that strain 16681 is the only DENV2 strain with arginine at position 120; all of the other DENV2 strains analyzed in this study have threonine (Table 3.3, Appendix Table 7.3). Comparison of E protein AA sequences shows there are nine differences between strains 16681 and J1409 and the difference at position 120 is the most interesting between the two strains because of its surface exposed location in the E protein and its difference in charge. Site-directed mutagenesis was used to introduce mutation R120T into the E protein of D2/IC-30P-NBX (virus designated as R120T).

Table 3.6. DENV2 and flavivirus E protein DII AA sequence alignment.

DENV2 Genotype[b]	Virus strain	DENV2 E glycoprotein AA numbering[a]																
		116	117	118	119	120	121	122	123			124	125	126	127	128	129	130
Asian 1	16681	C	A	M	F	R	C	K	K	--	--	N	M	E	G	K	V	V
	PUO-218	C	A	M	F	T	C	K	K	--	--	N	M	E	G	K	V	V
	M1	C	A	M	F	T	C	K	K	--	--	N	M	E	G	K	I	V
Asian 2	New Guinea C	C	A	M	F	T	C	K	K	--	--	N	M	K	G	K	V	V
	PL046	C	A	M	F	T	C	K	K	--	--	N	M	E	G	K	I	V
	CTD113	C	A	M	F	T	C	K	K	--	--	N	M	K	G	K	V	V
Asian/Am	Jamaica 1409	C	A	M	F	T	C	K	K	--	--	N	M	E	G	K	V	V
	13382-Tizimin	C	A	M	F	T	C	K	K	--	--	N	M	E	G	K	V	V
American	PR159	C	A	M	F	T	C	K	K	--	--	N	M	E	G	K	I	V
	Ven2	C	A	M	F	T	C	K	K	--	--	N	M	E	G	K	I	V
Cosmo	SL714	C	A	M	F	T	C	K	K	--	--	N	M	E	G	K	I	V
	CAMR5	C	A	M	F	T	C	K	K	--	--	N	M	E	G	K	I	V
Sylvatic	IC80-DAKAr578	C	A	M	F	T	C	L	K	--	--	K	M	E	G	K	V	V
	P8-1407	C	A	M	F	T	C	L	K	--	--	N	M	E	G	K	V	V
Flavivirus[c]																		
DENV1	16007	C	A	K	F	K	C	V	T	--	--	K	L	E	G	K	I	V
DENV3	PhMH-J1-97	C	A	K	F	Q	C	L	E	--	--	S	I	E	G	K	V	V
DENV4	Thailand/1985	C	A	K	F	S	C	S	G	--	--	K	I	T	G	N	L	V
YFV	Asibi	C	A	K	F	T	C	A	K	--	--	S	M	S	L	F	E	V
JEV	Nakayama	C	A	K	F	S	C	T	S	--	--	K	A	I	G	R	T	I
MVEV	NG156	C	A	K	F	T	C	S	S	--	--	S	A	A	G	R	L	I
WNV	NY99	C	A	K	F	A	C	S	T	--	--	K	A	I	G	R	T	I
SLV	Laderle	C	A	K	F	T	C	K	N	--	--	K	A	T	G	K	T	I
TBEV	Neudoerfl	C	V	K	A	A	C	E	A	K	K	K	A	T	G	H	V	Y
POWV	LB	C	A	K	F	E	C	E	E	A	K	K	A	V	G	H	V	Y

[a]AAs colored according to the ClustalX scheme in Jalview; cysteine [C, pink], glycine [G, orange], positively charged [K, R, red], negatively charged [E, D, purple], small and hydrophobic [A, W, L, V, I, M, F, blue], and hydroxyl and amine amino acids [T, S, N, Q, green]. AAs in black are significantly different from the consensus sequence at a specific residue position.
[b]AA alignment between dengue type 2 viruses.

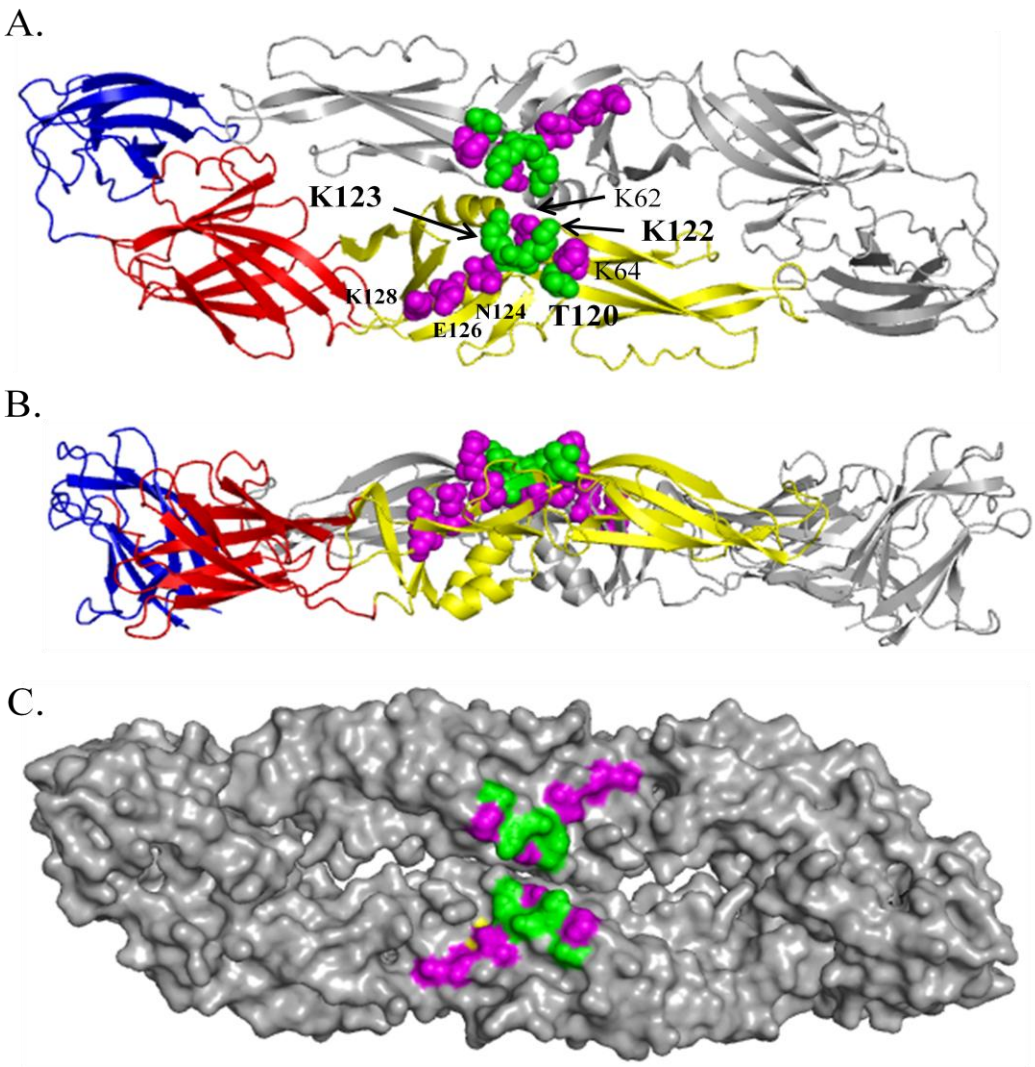

Figure 3.3. E protein structure and location of relevant AAs in DII. (A) Top-down view of the DENV2 E protein homodimer with D1 in red, DII in yellow, and DIII in blue. AAs identified in this study (T120, K122, and K123 in green) and in the literature (K63, K64, N124, E126, and K128 in magenta) are specified. Note, AA 120 is threonine in the published DENV2 E protein structure (Modis *et al.*, 2003). (B) Side-view of the DENV2 E protein homodimer. (C) Top-down view of the surface model of the DENV2 E protein homodimer. Specified AAs are colored the same as in A. Protein structures were obtained from the protein database bank (DENV2 E protein homodimer ID: 1oan) and were rendered in Polyview-3D (Porollo, Adamczak, and Meller, 2004).

After introduction of nucleotide substitutions into the E gene of the DENV2 infectious cDNA clone via site-directed mutagenesis, infectious RNA was transcribed *in*

vitro and transfected into C6/36 cells (C-0) and virus produced in the transfection was used to infect naïve C6/36 cells (C-1). Both K122/3E and R120T mutant viruses were able to replicate after transfection and one passage in C6/36 cells as evidenced by detectable viral antigen in cells. Consensus sequencing the genomes of C-1 viruses showed both mutant viruses had the expected full length genome sequence (Table 3.7), and therefore C-1 seeds were used for all subsequent phenotypic studies. Recovery of both mutant viruses from C-0 and C-1 samples demonstrated that these mutations have no effect on infectivity of or replication in C6/36 cells.

Table 3.7. Transfection and recovery of infectious virus in C6/36 or Vero cells.

Virus	C6/36 cells		Vero cells		
	C-0/C-1[a] virus recovery	C-0/C-1 E protein sequence	V-0/V-1[a] virus recovery	V-0[b] E protein sequence	V-1[b] E protein sequence
K122/3E	+/+	correct[b]	+/+	K122E[c]; K123K/E par. rev.[c]	K122E[c]; E123K full rev.[c]
R120T	+/+	correct[b]	+/+	correct[b]	correct[b]

[a] Transfection (C-0 or V-0) and passage (C-1 or V-1) was considered positive if virus antigen was detected by IFA.
[b] Sequencing verified all mutants contained the desired mutations and had no additional changes in the genome.
[c] Mutants with secondary mutations, partial reversions (par. rev.) or full reversions (full rev.) in the E protein are specified.

To investigate the ability of these mutant viruses to infect and replicate in mammalian cells, mutant virus RNA was transfected into (V-0) and resulting virus was passaged once in Vero cells (V-1). Full-length genome sequencing of K122/3E V-0 virus showed a partial reversion from glutamic acid at position 123 to lysine, while glutamic acid at position 122 remained unchanged (Table 3.7). V-1 consensus sequencing

revealed a complete reversion to lysine at position 123 while glutamic acid at position 122 continued to remain unchanged (Table 3.7). V-1 seed virus contains the desired mutation discovered in the serial passage experiments with no other differences in the virus genome compared to 30P-NBX, and was used in all subsequent phenotypic studies (virus designated as K122E). Virus recovery and genome sequencing showed that K123 is essential for virus replication in Vero cells, while threonine at position 120 has no effect on virus infectivity and replication in this cell type (Table 3.7).

Virus growth kinetics in C6/36 and Vero cells and temperature sensitivity

Mutant virus growth kinetics were analyzed by infecting cell cultures in duplicate with virus at a MOI of 0.001 and measuring virus genomic equivalents in medium by qRT-PCR (Figures 3.4 and 3.7) or infectious virus by $TCID_{50}$ (Figure 3.6) every two days. Growth kinetics for all three viruses was similar to 30P-NBX in C6/36 cells, corroborating the transfection data and the ability of these viruses to replicate efficiently in this cell type. Interestingly, 30P-NBX consistently presented more CPE in the form of syncytium formation, cell rounding, and cell detachment than K122E in our C6/36 cells used to amplify virus for blood-feed experiments (Figure 3.5). In Vero cells, K122E replicated similarly to 30P-NBX and had equivalent infectious virus titers in contrast to mutant K122/3E, which showed no virus replication and no infectious virus production (Figure 3.7). Sequencing virus RNA at the conclusion of the growth curves showed K122E was genetically stable, while there was not enough K122/3E viral RNA for sequencing at the conclusion of the experiment. R120T reached peak titers faster than 30P-NBX in Vero cells (Figure 3.6 B). However, these results do not mirror data

obtained previously, where the growth kinetics for 30P-NBX in Vero cells are more similar to those obtained for R120T (C.Y-H. Huang, personal communication).

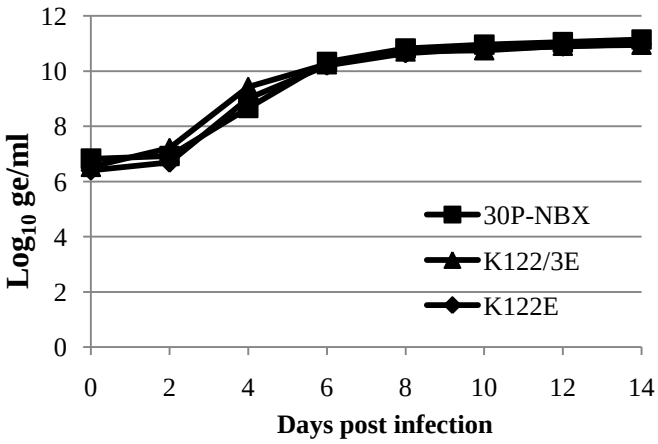

Figure 3.4. Virus growth kinetics in C6/36 cells. C6/36 cell cultures were infected in duplicate with virus at an MOI of 0.001 and sampled every two days for 14 days. Data presented are geometric mean titers of virus genome equivalents determined by qRT-PCR.

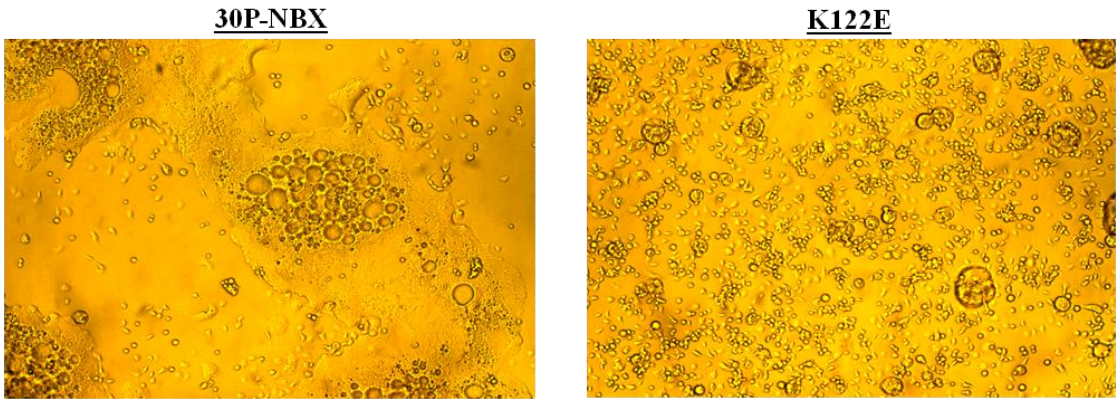

Figure 3.5. 30P-NBX and K122E CPE in C6/36 cells. C6/36 cells were inoculated at a MOI of 0.001 and maintained for 12 days with a medium change on day 7 pi. Pictures were taken on day 12 pi prior to harvesting for blood-feeding under bright light at 10x magnification. The medium for both infections had a pH of 6.5 to 7. MIRs for this experiment were 35.7% and 81.8% for 30P-NBX and K122E, respectively.

Temperature sensitivity was investigated for K122/3E by comparison of growth at 28°C and 37°C (Figure 3.7 B). Virus replication at 37°C, at first slower than virus grown at 28°C, increased at a more dramatic rate between days 4-8 pi when the rate of virus

replication became similar between the two temperatures. Virus genome sequencing revealed no addition nucleotide changes in mutant K122/3E at 28°C, while mutant K122/3E grown at 37°C partially or fully (results from duplicate cultures) reverted at position 123 from glutamic acid back to lysine. Infectious virus titers were similar at both temperatures. These results suggest that changing both lysines to glutamic acid is not tolerated for replication at 37°C and replication at this temperature requires only reversion at position 123; interestingly, reversion of AA 123 and not AA 122 occurred in the transfection, passage, and growth curve assays.

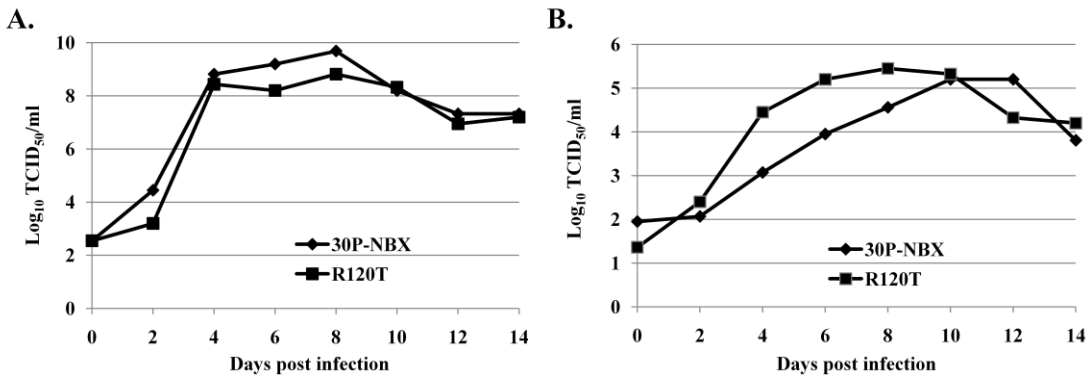

Figure 3.6. Virus growth kinetics in C6/36 and Vero cells. Cell cultures were infected with 30P-NBX and R120T at a MOI of 0.001 and virus replication was measured by $TCID_{50}$ for infectious virus in medium every two days for 14 days. The data presented are geometric means (in $\log_{10}$ $TCID_{50}$/ml) from duplicate flasks of C6/36 (A) and Vero (B) cell cultures.

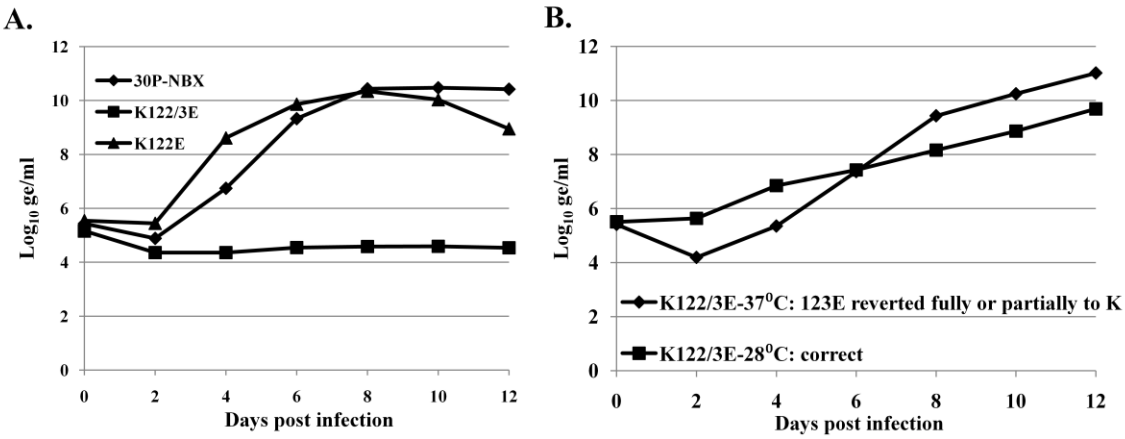

Figure 3.7. Virus growth kinetics in Vero cells at 37°C and 28°C. Vero cells grown at 37°C and 28°C were infected with 30P-NBX or mutant viruses at a MOI of 0.001 and replication was measured via qRT-PCR for virus genome RNA in medium every two days for twelve days. (A) The data presented are geometric mean titers (in $\log_{10}$ genome equivalents/ml) from duplicate flasks infected and maintained at 37°C. (B) Temperature sensitivity of K122/3E; data are geometric mean titers from duplicate Vero cell flasks infected and maintained at 37°C or 28°C. Sequencing virus RNA at the end of the growth curve verified the status of engineered mutations (specified where applicable).

Mutant virus infection rates in A. aegypti mosquitoes

Adult female *A. aegypti* mosquitoes were presented with an infectious blood-meal to determine if mutations in DII of the E protein enhance infection of the mosquito midgut compared to 30P-NBX. Mutant viruses K122/3E, K122E, and R120T each have

significantly higher MIRs than 30P-NBX in RexD mosquitoes (Figure 3.8 A). The only difference in AA sequence between K122E and 30P-NBX is glutamic acid at position 122, confirming this mutation alone is responsible for the enhanced infection rates found during the serial passage experiment. K122E also had a significantly higher MIR compared to 30P-NBX in Chetumal mosquitoes (compare 30P-NBXa to K122E, Figure 3.8 B and Table 3.8)), and these results are from 4 independent experiments, each completed at least one month apart. When R120T was assessed for its ability to infect Chetumal mosquitoes, the blood-feed experiments were completed concurrently in triplicate, with one additional experiment completed two weeks later (Table 3.8). 30P-NBX internal controls for these data showed high MIRs (30P-NBXb, Figure 3.8 B and Table 3.8), which is in contrast to the average MIR found for 30P-NBX internal controls in the previous Chetumal challenge experiments with K122E (30P-NBXa, Figure 3.8 B). Completing each of the R120T challenge experiments concurrently would have utilized mosquito eggs that were oviposited by the same parents. Also, all of the extrinsic environmental factors would have been similar for each of the repetitions, which would further reduce our previously observed general experimental variation. Despite 30P-NBX having a higher average MIR in the second set of experiments compared to the first, R120T still had a significantly higher MIR compared to 30P-NBX in those experiments (30P-NBXb compared to R120T, student's t test p value = 0.038). This shows that 30P-NBX is still limited in its capacity to infect mosquito midguts compared to R120T even when mosquito susceptibility is high, and also suggests that laboratory colonized mosquito strains can change their susceptibility for virus infection over time; however, more replicates are needed to verify that susceptibility has indeed changed.

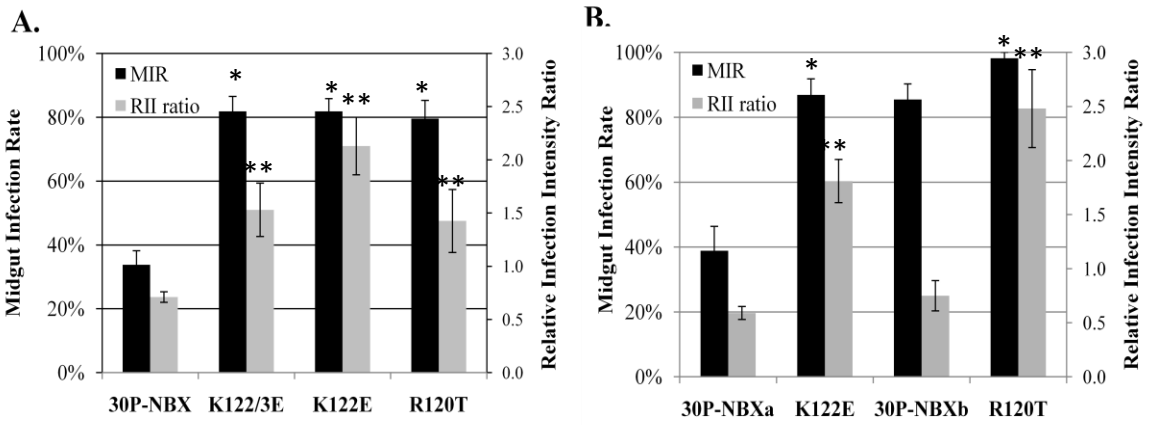

Figure 3.8. Virus MIRs and Relative Infection Intensity ratios in *A. aegypti* RexD and Chetumal mosquito midguts. RexD (A) and Chetumal (B) mosquitoes were orally challenged with virus, maintained for seven days until midguts were dissected, and MIRs and RII ratios were determined by IFA. 30P-NBXa and 30P-NBXb are data for the internal controls for K122E and R120T, respectively. Data are the average of at least three experiments and MIR (*) and RII ratio (**) significance differences were determined by comparison with 30P-NBX via student's t test (p value < 0.05).

Table 3.8. 30P-NBX MIR in *A. aegypti* Chetumal strain mosquitoes.

Date	Log $TCID_{50}$/ml	MG pos	MG total	MIR	RII ratio
1st Set[a]					
12/4/2009	8.45	18	34	52.94%	0.53
1/20/2010	8.45	11	24	45.83%	0.77
2/23/2010	8.2	6	33	18.18%	0.50
4/6/2010	8.2	10	26	38.46%	0.55
	Totals	45	117		
			Average	38.85%	0.59
			std dev	15.00%	0.12
			Std Error	7.50%	0.06
2nd Set[b]					
9/17/2010	8.45	13	18	72.22%	0.54
9/17/2010	8.95	18	19	94.74%	0.67
9/17/2010	8.95	16	18	88.89%	0.625
10/29/2010	8.45	31	36	86.11%	1.16
	Totals	78	91		
			Average	85.49%	0.75
			Std dev	9.55%	0.28
			Std Error	4.77%	0.14

[a]30P-NBX was the internal control for comparison of MIRs with K122E.
[b]30P-NBX was the internal control for comparison of MIRs with R120T.

IFA analysis of infected mosquito midguts showed that on average K122E, K122/3E, and R120T virus antigen encompassed the entire midgut more significantly than 30P-NBX at 7 days pbf as evidenced by their RII ratios (Figure 3.8) and even in spite of the elevated Chetumal mosquito susceptibility for the R120T comparisons, the 30P-NBX control virus still did not produce an infection that spread throughout the midgut (30P-NBXb, Figure 3.8 B). To further investigate K122E infection kinetics in the mosquito, RexD mosquitoes were presented an infectious blood-meal (titer ranging from 7 to 9 $\log_{10}$ pfu/ml) and midguts were dissected and head squashes were performed every two days for 14 days. The experiment was repeated 3 times with 17 to 31 mosquitoes being dissected per sampling day. MIRs for mutant K122E starting on day 2 pbf through day 14 are significantly higher than 30P-NBX, indicating that K122E infects a higher proportion of mosquitoes at an earlier time point than 30P-NBX (Figure 3.9). Similarly, the RII ratios for K122E are significantly higher than 30P-NBX starting on day 4 pbf and continuing to increase until day 14. In contrast, 30P-NBX maintained a low RII ratio throughout the time course (Figure 3.9 and 3.10). These results suggest that K122E generates a continuous and more productive infection in the mosquito midgut than 30P-NBX, which does not seem to spread throughout the midgut like K122E (Figure 3.9).

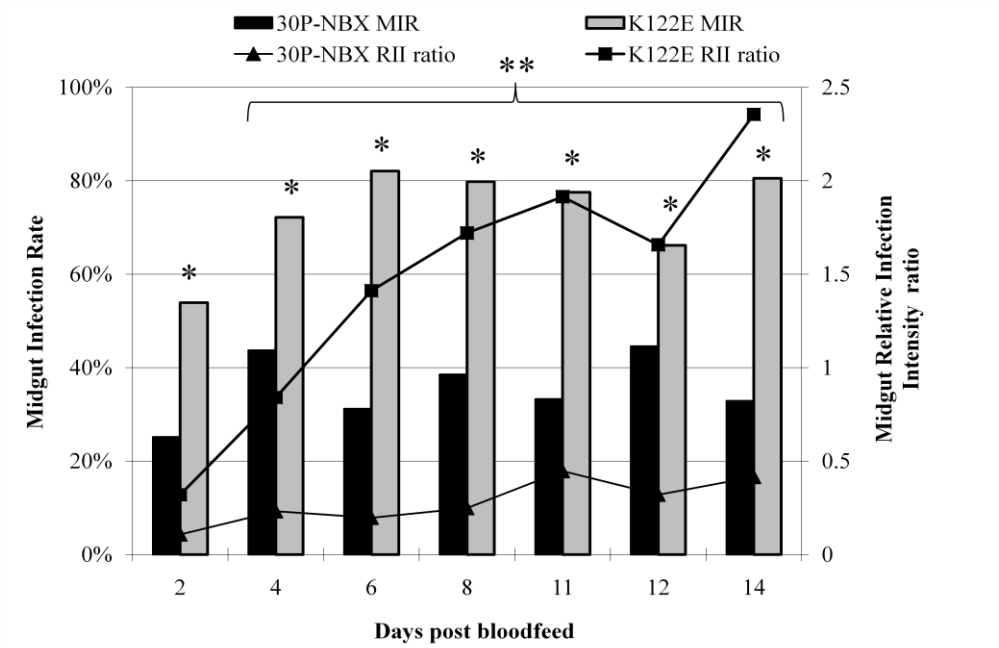

Figure 3.9. 30P-NBX and K122E MIR and RII ratio kinetics in *A. aegpyti* RexD mosquitoes. RexD mosqutioes were orally challenged with virus, midguts were dissected every other day for 14 days, and MIRs and RII ratios were determined by IFA. Challenge experiments were repeated three times and averaged 17-30 mosquitoes per day. MIRs were significantly different on days 2 to 14 (*) via chi-square (p value < 0.05) and RII ratios were significantly different on days 4 to 14 (**) via student's t test (p value < 0.05).

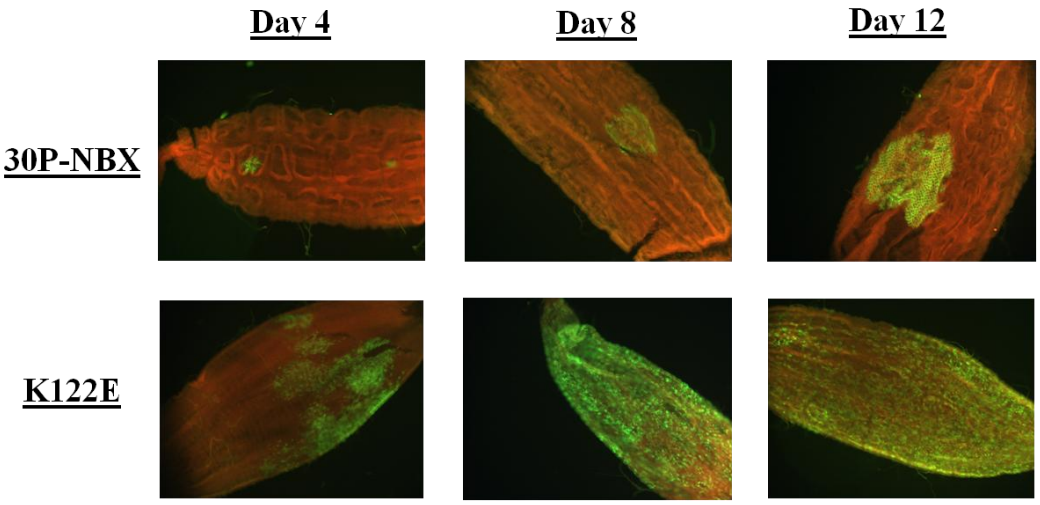

Figure 3.10. Infected midguts from the time course experiment. RexD mosqutioes were orally challenged with 30P-NBX or K122E, midguts were dissected every other day for 14 days, and virus antigen was detected by IFA. Midguts displaying virus antigen (green) are representative of the infection intensity seen for the days presented.

Virus dissemination from the midgut was also investigated. Head squash IFA analysis showed that both K122E and 30P-NBX escape the midgut after 4 days pbf; however, K122E has a significantly higher infection rate than 30P-NBX in tissues outside the midgut by day 6 pbf and until the end of the time course (Figure 3.11). In an independent experiment, dissemination rates determined by IFA analysis on paired midguts and heads showed that despite there being a distinct difference in dissemination rates between K122E (average 68.15%, standard deviation 28.66%) and 30P-NBX (average 31.75%, 16.89%), there is statistically no significant difference between the two viruses in RexD mosquitoes on day 14 pbf due to the high degree of variation between the three experiments (Table 3.9). Mosquito midguts and heads were not paired in the time course experiment shown in Figure 3.11, so it is difficult to determine whether the significantly different infection rates in tissues outside the midgut between K122E and 30P-NBX are the result of actual differences in dissemination or the result of K122E infecting a higher proportion of mosquitoes, although it is of note that K122E head tissue infection rates are close to the percent of mosquitoes with an infected midgut after day 12 pbf, while 30P-NBX are not (Figure 3.11). Either way, it is obvious that K122E infects and disseminates from a higher proportion of mosquito midguts than 30P-NBX.

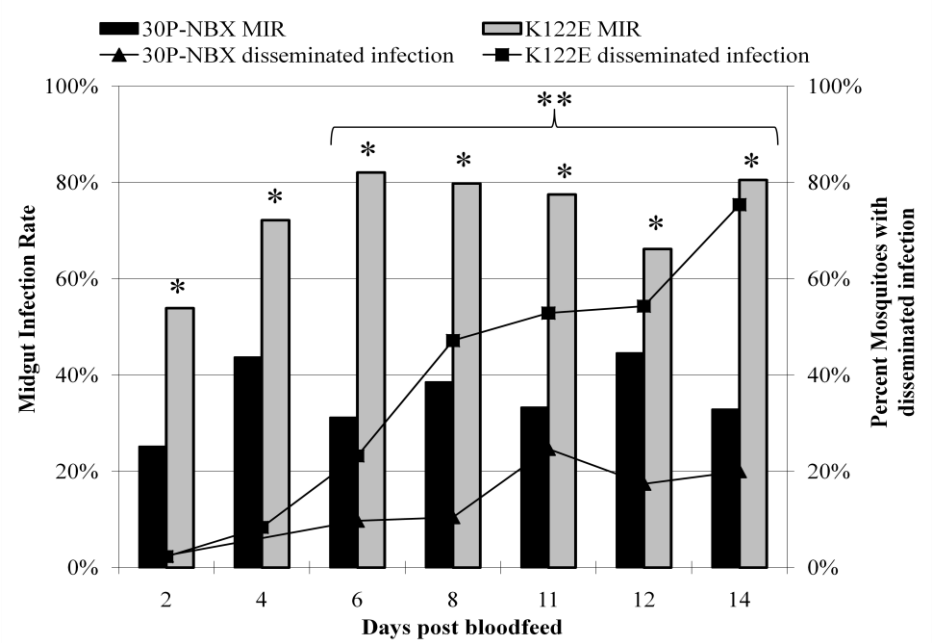

Figure 3.11. 30P-NBX and K122E MIR and dissemination kinetics in *A. aegpyti* RexD mosquitoes. RexD mosqutioes were orally challenged with virus, midguts were dissected and head squashes were performed every other day for 14 days, and MIRs and percent mosquitoes with a disseminated infection were determined by IFA. Challenge experiments were repeated three times and averaged 17-30 mosquitoes per day. MIRs were significantly different on days 2 to 14 (*) via chi-square (p value < 0.05) and percent mosqutioes with a disseminated infection were significantly different on days 4 to 14 (**) via student's t test (p value < 0.05).

Table 3.9. 30P-NBX and K122E dissemination rates in *A. aegypti* RexD strain mosquitoes.

	30P-NBX Dissemination				K122E Dissemination			
Exp.	Pos HS	Pos Midgut	Diss. Rate	HS pos MG neg	Pos HS	Pos Midgut	Diss. Rate	HS pos MG neg
A	1	6	16.67%	3	8	18	44.44%	1
B	1	2	50.00%	0	9	15	60.00%	0
C	2	7	28.57%	4	5	5	100.00%	1
		Average	31.75%			Average	68.15%	
		Std dev	16.89%			Std dev	28.66%	
		Std Error	9.75%			Std Error	16.55%	

Mosquito midgut-virus attachment assay

The first rate-limiting step in infection of any cell type with DENV is attachment to a cellular receptor and we hypothesized that the amino acid mutation from lysine to

glutamic acid might be increasing the efficiency of this step, considering that K122E infected a significantly higher proportion of midguts a short time after an infectious blood-meal. To test our hypothesis we developed a mosquito midgut-virus attachment assay. RexD mosquitoes were presented an infectious blood-meal for 30 minutes to ensure we obtained the desired number of fully engorged mosquitoes and were then placed on ice at 4°C to pause the replication cycle. After removal of the virus-containing blood-meal, dissected midguts were mechanically washed three times in ice-cold PBS by submersion and swishing to remove residual virus not attached to midgut epithelial cells. qRT-PCR for virus genome RNA was used to quantify the virus still attached to midgut cells. There was no significant difference between 30P-NBX and K122E in the number of virus genome copies associated with midguts (Figure 3.12). The MIRs for 30P-NBX (35.7%) and K122E (81.8%) were similar to their averages reported above. Infectious virus concentrations were equivalent for both viruses as were the number of virus genome copies associated with unwashed, fully engorged midguts (Figure 3.12). These results suggest that at least initial virus attachment to midgut cells is not affected by K122E mutation.

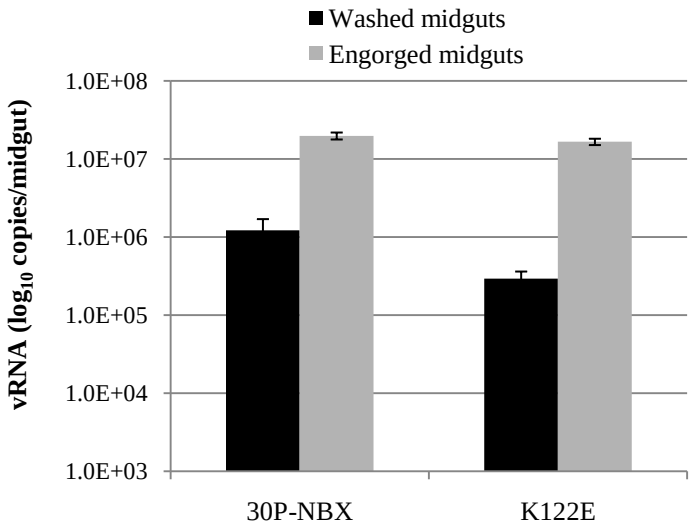

Figure 3.12. Virus attachment to *A. aegypti* RexD midgut cells. RexD mosquitoes in 3 pools of 5 were orally challenged with DENV2 for 30 mins, knocked down on ice at 4°C, and midguts were dissected and the virus-meal was removed with three washes in ice-cold PBS (washed midguts) or retained (engorged midguts). RNA was isolated via TRIzol and vRNA was measured via qRT-PCR. Data presented are the average of the three pools and there was no significant difference between 30P-NBX and K122E for either preparation. Mosquitoes that were not dissected after the oral challenge were maintained for seven days, when midguts were dissected and MIRs were determined by IFA. MIRs for each virus were equivalent to their reported averages (30P-NBX MIR 35.71%, virus-meal titer 8.2 $\log_{10}$ $TCID_{50}$/ml; K122E MIR 81.82%, virus-meal titer 8.2 $\log_{10}$ $TCID_{50}$/ml).

Discussion

In this study we demonstrate that single amino acid mutations in DII of the DENV2 E protein can result in significantly enhanced infection of *A. aegypti* mosquito midguts. Serial passage of 30P-NBX in RexD mosquitoes identified an adaptive mutation in DII of the E protein at position 122 from lysine to glutamic acid that correlated with increased infection rates in mosquito midguts. Incorporation of this mutation into the infectious clone recapitulated the results of the serial passage experiment, showing that this single amino acid mutation is responsible for the enhanced infectivity phenotype. In addition, a single mutation of neighboring amino acid R120 to threonine significantly enhanced mosquito midgut infection compared to the parent virus.

To our knowledge this is the first time mosquito infection determinants have been mapped to DII of the DENV E protein.

The identification of single AA mutations that enhance virus infectivity for mosquitoes is not unprecedented. Whereas few studies have investigated specific mosquito infection determinants in flaviviruses, mosquito infection determinants have been identified in the structural genes of zoonotic alphaviruses. Single AA mutations in the E2 protein of an enzootic VEEV subtype IE strain resulted in increased infectivity of the epizootic mosquito vector *Ochlerotatus taeniorhynchus*, which was responsible for outbreaks in Mexico in 1993 and 1996 (Brault *et al.*, 2002; Brault *et al.*, 2004; Weaver *et al.*, 2004). More recently, a large scale outbreak of Chikungunya virus (CHIKV) on Reunion island and additional Indian Ocean islands between 2004-2006 was instigated by a virus strain with a single AA mutation in the E1 class II fusion protein (Schuffenecker *et al.*, 2006; Tsetsarkin *et al.*, 2007; Vazeille *et al.*, 2007). The effects of this mutation were enhanced by mutations acquired previously in the receptor-binding E2 protein (Tsetsarkin *et al.*, 2009). These findings are consistent with studies conducted in our laboratories that have shown AA mutations in the E2 receptor binding protein of Sindbis virus can influence vector susceptibility (Myles, Pierro, and Olson, 2003; Pierro, Powers, and Olson, 2007; Pierro, Powers, and Olson, 2008). It is clear that point mutations in the structural genes of arboviruses can significantly enhance vector infectivity.

DIII of the flavivirus E protein is widely accepted to have receptor binding properties and the FG loop specifically was proposed to bind to mosquito cells (Hung *et al.*, 2004). This was further suggested by the absence of this loop structure in the tick-borne viruses. Previously we showed that deletion of the FG loop attenuated virus

infection in mosquito midguts, while mutation of the FG loop sequence from VEP to RGD did not significantly affect MIRs. These results showed that the FG loop structure itself and not the AA sequence is important for midgut infection (Erb *et al.*, 2010) (Chapter 4).

Mutant virus VEPGΔ was included in the serial passage experiments to see if deletion of this motif would place selective pressure on other regions of the E protein to compensate for its absence. Serial passage of VEPGΔ in mosquito midguts did not select for any enhancing adaptive mutations and the virus was lost between the third and fourth passages. Although this virus was able to infect mosquito midguts and secondary tissues (albeit at a significantly lower rate than 30P-NBX), our inability to continuously passage VEPGΔ in midguts indicates that the presence of the FG loop is vital to the overall life cycle of the virus *in vivo*. In contrast, the RGD mutant virus acquired the K122E mutation sooner than 30P-NBX, after only two passages in mosquito midguts. This may suggest that the RGD substitutions imposed greater selective pressure for the 122E mutation. We previously found that RGD virus (and VEPGΔ virus) derived from transfection and one passage in Vero cells acquired a K122I mutation (Erb *et al.*, 2010). Additionally, multiple passages of wild type DENV2 strain 16681 in Vero cells resulted in a mixed K122K/E population (C.Y-H Huang unpublished data). These findings at first suggested mutation of AA 122 was primarily a primate cell culture-adaptive mutation for DENV2, but the results of this study show the K122E mutation is also relevant to invertebrate systems. Whatever the selective pressure, it is clear that replacing positively charged AAs in this region may constitute a mutational hot spot in the E protein of DENV2 strain 16681.

The high MIR of J1409-ic may have precluded the necessity for this DENV2 strain to accumulate mutations after passage in mosquito midguts. DENV2 E protein AA sequence alignments show that strain 16681 has arginine at position 120 while all other DENV2 strains including J1409 have threonine at this position (Table 3.6). R120T had a significantly higher MIR than 30P-NBX showing that this AA mutation alone could also result in the increased midgut infectivity phenotype. Arginine at this position is therefore an attenuating determinant of midgut infection. Strain 16681 has been extensively passaged in different mammalian and invertebrate systems since isolation and the positively charged arginine could be the result of those passages. This is in congruence with findings that showed passage of DENV2 strain PUO-218 in BHK-21 cultured mammalian cells selected for a T120 to lysine change that resulted in higher binding capacity for glycosaminoglycans (GAGs) and reduced neurovirulence in mice (Lee *et al.*, 2006). The high MIR resulting from mutation of R120 to threonine may explain why J1409 did not accrue any adaptive mutations in the E gene and similarly, the absence of R120 in other DENV2 strains may explain why glutamic acid at position 122 is not present in any natural isolates. The ability of K122E to replicate as efficiently as 30P-NBX in mammalian cells suggests that this mutation could emerge and persist in DENV2 transmission cycles so it is intriguing that it has not been found before. Perhaps E protein AA sequence requirements for infection of mosquitoes are already satisfied with the threonine residue at position 120 and this could explain why 30P-NBX was not efficient at infecting highly susceptible Chetumal (Figure 3.8) and D2S3 strain mosquitoes (Chapter 2). It would be interesting to conduct mosquito and mammalian cell infection competition studies with K122E and R120T. DENV2s from the Asian/American

genotype were found to infect, disseminate, and out-compete viruses from the American genotype in *A. aegypti* mosquitoes as well as replicate to higher titers in isolated human dendritic cells (Anderson and Rico-Hesse, 2006; Armstrong, 2001; Armstrong and Rico-Hesse, 2003; Cologna, Armstrong, and Rico-Hesse, 2005), so it would be instructive to see if these single amino acid mutations can provide a replication advantage over other virus strains not included in this study.

Mutation of K122 to glutamic acid or R120 to threonine removes a positive charge at a surface exposed location on the E protein already crowded with positively charged amino acids (R120, K122, K123, K128, and K64) (Modis *et al.*, 2003) (Figure 3.3). AA mutations in this region are associated with increases and decreases in virus affinity for GAGs and neurovirulence in mice for TBEV, MVEV, JEV, and DENV2 (Bray *et al.*, 1998; Gualano *et al.*, 1998; Mandl *et al.*, 2001; McMinn *et al.*, 1995; Prestwood *et al.*, 2008; Shresta *et al.*, 2006; Tajima *et al.*, 2010). More interestingly, serial passage of DENV2 strain PL046 in AG129 mice and C6/36 cultured cells selected for virus with E protein K128E and N124D substitutions (in the same virus) that produced disease manifestations more akin to those produced in humans (Shresta *et al.*, 2006). These mutations reduce both the positive charge in this DII region and the affinity of the virus for GAGs. Reduced GAG binding resulted in increased systemic viral loads (Prestwood *et al.*, 2008). It would be fascinating to see if mutation of K122 to glutamic acid in the PL046 infectious clone could produce a similar disease phenotype to their K128E and N124D mutant virus. Additionally, it would be illuminating to see if their mutant virus can efficiently infect *A. aegypti* mosquitoes, which would provide a fruitful animal model system to analyze DENV transmission dynamics.

To date there has been no evidence to suggest that DENV binds to GAGs in mosquitoes or invertebrate cell cultures (Hung *et al.*, 2004) so it is intriguing that our mutations provide a fitness advantage in mosquito midguts. K122E and R120T substitutions remove positive charges on the E protein and may be increasing its availability for infection of midgut cells similar to how the N124D and K128E substitutions increase viremia in mice by reducing the virus affinity for heparan sulfate. Heparan sulfate has been identified in the midguts of *Anopheles stephensi* and is postulated to be present in *A. aegypti* as well (Sinnis *et al.*, 2007). If heparan sulfate plays no role in functional virus attachment and entry into midgut cells but is still non-specifically binding to virus it could possibly be sequestering virus on non-permissive midgut cells and/or preventing virus attachment to primary or secondary receptors. This could reduce the ability of 30P-NBX to infect a higher proportion of mosquitoes and spread throughout the midgut like K122E and R120T, and would also account for the equivalent attachment efficiencies shown in our attachment assay.

Time course experiments in mosquitoes showed that K122E infects a higher proportion of mosquitoes than 30P-NBX as soon as 2 days post blood-feeding (pbf) and suggested to us that early stage events including attachment and entry were enhanced by the K122E mutation. We tested this hypothesis by developing a mosquito midgut-virus attachment assay and showed that there was no significant difference in attachment to midgut cells using our assay. There are a few limitations to our experimental design. Artificial blood-feed experiments with DENV require fresh virus preparations (Miller, 1987; Richards *et al.*, 2007) and this precluded standardizing the virus concentration in the blood-meal, although the number of virus genomes in engorged midguts and the

infectious virus titers in the blood-meals were similar for both viruses (Figure 3.11). Midgut washing was completed mechanically, making it possible that unbound virus and/or vRNA non-specifically bound to midgut tissue was not washed away sufficiently before RNA isolation. Differences in attachment kinetics may have been masked by blood-feeding mosquitoes for 30 min and could have precluded our ability to see differences in attachment between the two viruses at an earlier time point. Additionally, challenging mosquitoes with such a high concentration of virus could have saturated the midgut with virus, which would make it difficult to discern differences in attachment between the two viruses. Several proteins of different molecular mass have been identified as DENV receptor proteins in the mosquito midgut (Mercado-Curiel, Black, and Munoz, 2008; Mercado-Curiel *et al.*, 2006) and it is possible that K122E and 30P-NBX could have different attachment affinities for a secondary receptor that we cannot detect using our assay. This could contribute to differences in infectivity rates. Furthermore, mosquitoes are challenged with virus that is maintained at 37°C and this higher temperature might have accelerated attachment and entry kinetics in the mosquito while they were allowed to feed for 30 min. Measuring the amount of virus negative strand RNA at short increments after oral challenge may help elucidate whether the two viruses are internalized at different rates.

Mosquito challenge studies using West Nile virus, VEEV, and Sindbis virus showed that only a small number of midgut cells are initially infected after oral challenge (Foy *et al.*, 2004; Scholle *et al.*, 2004; Smith *et al.*, 2008), which is consistent with our observations for day 2 infected midguts (data not shown). Scanning electron microscopy of the luminal side of *A. aegypti* showed that microvilli found on the majority of midgut

cells are covered by a network of fine strands called the microvilli-associated network (MN); some cells have less or no microvilli and are named bare-cells (Zieler *et al.*, 2000). This MN is suggested to protect midgut cells from phagocytes and other cells/proteins present in blood-meals. The high quantity of both viruses associated with midguts after washing could be the product of virus being trapped or nonspecifically bound to the MN. Additionally, bare-cells may account for the limited number of cells that become infected shortly after blood-feeding due to the accessibility of the cell surface to virus. Cells with an extensive MN on their surface may prevent virions from coming into contact with the cell surface and therefore preclude entry into the cell. *Culex tarsalis* mosquitoes have ca. $1x10^4$ cells per mosquito midgut (Houk *et al.*, 1990), and if this estimation is similar for *A. aegypti* mosquitoes, our results suggest that while high quantities of virus can attach to midgut cells, fewer cells are susceptible to infection.

It is possible that other steps in the virus replication cycle including maturation are affected by mutations in this DII region. DENV midgut infections generally start at a focus of infection and spread laterally from a single infected cell either by direct infection of neighboring cells at the edges of a focus or by budding out of cells and infecting local cells (Salazar *et al.*, 2007). K122E RII ratios in the midgut are significantly higher than 30P-NBX as early as day 4 pbf, showing that K122E produces an extremely productive infection that eventually encompasses the rest of the organ. 30P-NBX midgut infections stay relatively restricted by comparison. The midgut lumen of *A. aegypti* mosquitoes is basic (pH 8.5-9.5) (Corena *et al.*, 2005) and may increase the negative charge on the surface of the E protein of K122E, assuming that DENV is released from infected cells into the luminal space. This could contribute to attachment to a host cellular receptor or,

given the proximity of AA 122 to the dimer interface, could assist in E protein homodimer stability on mature virions. Likewise, mutation at position 122 may stabilize the E protein at various stages during protein maturation through the secretory pathway, helping to produce a higher infectious virus to particle ratio. Prestwood *et al.* (2008) suggest that their double K128E, N124D mutant virus produces a more equivalent particle to pfu ratio than the parent virus in BHK-21 cells; perhaps a similar phenomenon is occurring in mosquito midgut cells.

Two phenotypically distinct DENV2s with an AA difference at E protein position 62 were isolated from K562 and C6/36 cells inoculated with serum from the same DHF patient (Kinoshita *et al.*, 2009). Virus isolated from C6/36 cells had glutamic acid at this position and could not bind to and infect B cells while conversely, virus isolated from K562 cells had lysine at this position and was able to bind to and efficiently infect B cells. The K562 cell-derived virus was capable of infecting C6/36 cells but with low efficiency. AA 62 is located just below and slightly in between AAs 122 and 123 at the homodimer interface and Kinoshita *et al.* (2009) speculate that lysine (compared to glutamic acid) at this position causes high electrostatic repulsive effects with its sister AA on the opposite monomer, causing a loosening effect favorable to B cell binding. K122, K123, and R120T do not seem close enough in proximity to their equivalent AAs on the opposite monomer for contact (Figure 3.3 C) so it is unlikely that electrostatic repulsion is affecting these AAs. It is certain however, that engineering a double mutation at K122 and K123 to glutamic acid renders the virus unstable in Vero cells at 37°C (Figure 3.5 B). Interestingly, the double mutant was able to infect a significantly higher proportion of

mosquito midguts compared to 30P-NBX, suggesting this mutant is stable during replication in midgut cells.

Given the ability of the double mutant to infect a higher proportion of mosquito midguts than 30P-NBX and considering that the K122E mutation was the preferential mutation discovered in the serial passage experiments, it would be intriguing to see if a single mutation from K123 to glutamic acid provides the same phenotypes as K122E and R120T. This could again suggest that losing the positive charge is enough to change the phenotype rather then there being a specific mechanistic function for the K122E mutation. Additionally, it is speculated that E protein homodimers have varying dimerization affinities and can shift between monomeric and dimeric forms at physiologically relevant temperatures, thus facilitating the opening of otherwise cryptic neutralizing antibody sites (Nybakken *et al.*, 2006). If these DII mutations loosen the dimer interface, this phenomenon could be opening receptor binding sites for mosquito cell receptors or in contrast, could be increasing dimer affinity and stabilizing the homodimer during different stages in the virus life cycle.

A TBEV neutralizing antibody escape variant generated under selection with MAb IE3 had one AA change in the E protein (A123K) compared to the wild type (Holzmann *et al.*, 1997). TBEV has two additional AA in this region compared to other flaviviruses (Table 3.6). A later study investigating the quaternary structure of the TBEV E protein showed that individual AA mutations engineered into the TBEV E protein at positions 120, 122, 123, 124, and 126 were each found to disrupt binding of the MAb IE3 (Kiermayr, Stiasny, and Heinz, 2009). Interestingly, these mutations are too distant to be a part of the same Fab footprint, which suggests that mutations in this region disrupt the

overall secondary structure of E protein in this region. Changes in secondary structure created by mutations K122E and R120T may be influencing infectivity of mosquito midguts. The human neutralizing antibody response to DENV is not as exclusive for DIII as previously thought (Wahala *et al.*, 2009), and antibodies directed to epitopes in other domains, including DII, may be just as important. This AA region of DII is variable among the DENV serotypes and other flaviviruses and AA mutations in this region have been identified in flavivirus MAb neutralizing escape variants for DENV, JE, MVE, TBE, and YFV [summarized in Table 1, (Roehrig, 2003)]. Neutralizing escape variant mutations are located on surface accessible regions of the E protein and must be affecting the association of neutralizing antibody with its binding site suggesting that the AA variability in this region of the E protein and may be reflective of enhanced immune pressure.

Substitution of lysine and arginine residues could remove potential trypsin cleavage sites on the E protein. Brackney *et al.* (2008) showed suppression of late trypsin (5G1) expression (expressed ca. 5 to 6 hours pbf) resulted in increased RexD mosquito infection by J1409 virus but did not decrease RII ratios later on. This could explain the lower MIR of 30P-NBX compared to mutants K122E and R120T, but it does not explain why 30P-NBX does not productively infect the rest of the midgut. 5G1 is expressed approximately five hours after a mosquito ingests a blood-meal and unless 30P-NBX was unable to enter into cells to escape enzymatic digestion after an initial binding event, it is unlikely that detrimental cleavage of the E protein would occur. Additionally, introduction of a potential trypsin cleavage site by mutation of DIII surface exposed AA 304 from glycine to lysine did not reduce the ability of this mutant virus to

infect mosquito midguts compared to parent 30P-NBX (SM Erb, unpublished data, Chapter 4) suggesting that the differences in infection rates between 30P-NBX and the mutant viruses are not influenced by trypsin enzyme activity.

Although arboviruses generally produce very little cytopathic effects (CPE) in C6/36 cells (Knipe, 2006), CPE presented as syncytium formation, cell rounding, and cell detachment is commonly observed in our C6/36 cells infected with 30P-NBX after 10 days pi. We observed that C6/36 cells infected with K122E consistently present less CPE than 30P-NBX infected cells (Figure 3.5). The pH of the medium at the time of mosquito challenge (12 to 14 days pi) for both viruses is usually 6.5 to 7.0 in our system and the fusion threshold for 30P-NBX is pH 6.3 to 6.5. This implies that E protein in mature virus would not be prematurely transitioning to the fusion conformation prior to infecting new cells. 30P-NBX and K122E have comparable fusion indexes in C6/36 cells via the fusion from within assay (data not shown) so it is unlikely that differences in CPE are due to differences in fusion efficiency; considering both viruses have similar fusion indexes, we would expect K122E to show similar levels of syncytium formation to 30P-NBX. Apoptosis in midgut epithelial cells was found to limit infection and transmission of WNV in a refractory strain of *Culex pipiens* mosquitoes (Vaidyanathan and Scott, 2006). If the elevated CPE exhibited by 30P-NBX infected C6/36 cells compared to K122E is occurring in midgut epithelial cells, this virus may be inducing midgut cell death, thus preventing a productive infection that spreads throughout the midgut epithelium as observed with K122E. However, J1409-ic (and mutant virus R120T) exhibited CPE similar to 30P-NBX starting around day 10 pi. Unless J1409-ic is able to produce and release more infectious virus than 30P-NBX prior to inducing cell death, it is unlikely

that apoptosis is causing low 30P-NBX infection rates and limited virus spread throughout the midgut. It is puzzling how mutation of these AAs are enhancing DENV infection of midguts, but it is obvious that this region of the E protein contains important infection determinants.

DENV2 are suggested to have evolved only recently from sylvatic-DENV2 viruses sustained in nature between non-human primates and canopy-dwelling *Aedes spp.* mosquitoes (Holmes and Twiddy, 2003; Wang *et al.*, 2000). Phylogenetic analysis of DENV2 E proteins suggested that a mutation from leucine to lysine at E protein position 122 (Table 3.6) was predicted to accompany DENV2 E protein evolution, possibly under immune selection (Leclerc *et al.*, 1993; Twiddy, Woelk, and Holmes, 2002; Vasilakis *et al.*, 2008; Wang *et al.*, 2000). Most of the AA mutations proposed to correlate with emergence of endemic/epidemic DENV1-4 from sylvatic progenitors were located in DIII, so it is unclear from the phylogenetic analysis whether the AA 122 change occurred independently or in combination with changes in DIII. If this mutation was necessary for the transition from the sylvatic to the human cycle, it could explain why no natural DENV2 endemic/epidemic isolates have mutations at position 122.

The epidemiological implications of our findings are highlighted by the dissemination data from the time course experiment. Head squash IFA analysis, used as a surrogate for transmission potential, showed that K122E disseminated from the mosquito midgut in a higher proportion of mosquitoes compared to 30P-NBX. Even though this may correlate more with the higher proportion of mosquitoes exhibiting a midgut infection than with increased dissemination capacity over 30P-NBX virus, the ability of this mutant virus to disseminate from the midgut and infect secondary tissues is

clearly greater than 30P-NBX. It will be interesting to see if K122E can infect field-caught or genetically diverse laboratory strain *A. aegypti* mosquitoes as efficiently as the laboratory colonized strains used in this study. Gradual increases in viral fitness that increase transmission rates in mosquitoes and produce higher viremias in humans can lead to genotype and strain displacements (Armstrong and Rico-Hesse, 2003; Cologna, Armstrong, and Rico-Hesse, 2005; Hanley *et al.*, 2008; Ty Hang *et al.*, 2010). Given that the K122E mutation does not result in fitness costs to replication in mammalian cells, the epidemic potential of a virus that accumulates this point mutation would likely be high.

We have shown for the first time that single AA mutations in DII of the DENV2 E protein can significantly enhance infection of *A. aegypti* mosquitoes. Natural mosquito isolates of DENV2 should be monitored for variations in gene sequence at this surface exposed region of DII and could provide biological markers for virus emergence in the future. Inclusion of arginine at position 120 in DII of live-attenuated DENV2 vaccine viruses in conjunction with other attenuating mutations could reduce the transmission potential of vaccine viruses from vaccinees. Further research investigating the contribution of this AA region to protective immunity, DENV transmission, and viral pathogenesis in mammals should not be overlooked and merits further study.

CHAPTER 4

DOMAIN III FG LOOP OF THE DENGUE VIRUS TYPE 2 ENVELOPE PROTEIN IS IMPORTANT FOR INFECTION OF MAMMALIAN CELLS AND *AEDES AEGYPTI* MOSQUITOES

Introduction

This Chapter has been published:

Steven M. Erb, Siritorn Butrapet, Kelly J. Moss, Betty Luy, Thomas Childers, Amanda E. Calvert, Shawn J. Silengo, John T. Roehrig, Claire Y.-H. Huang, and Carol D. Blair. (2010). Domain-III FG loop of the dengue virus type 2 envelope protein is important for infection of mammalian cells and *Aedes aegypti* mosquitoes. Virology, 406(2): 328-35.

Siritorn Butrapet is responsible for making the engineered mutant viruses.
Thomas Childers performed fusion from within assays.
Amanda Calvert completed antibody mapping experiments.
Kelly J. Moss, Betty Luy, and Shawn J. Silengo contributed equally to virus titrations and sequencing.

Dengue viruses (DENVs) are the most medically important arthropod-borne viruses infecting humans today. The DENVs comprise a serocomplex in the family *Flaviviridae*, genus *Flavivirus* that includes DENV serotypes 1-4 (DENV1-4) and are the etiological agents of dengue fever and dengue hemorrhagic fever/dengue shock syndrome. Approximately one third of the world's population is at risk of becoming infected by DENV due to the distribution of their primary vector, *A. aegypti* (Gubler, 1998). While there are licensed vaccines for other medically important flaviviruses such as Japanese encephalitis virus (JEV), yellow fever virus (YFV), and tick-borne

encephalitis virus (TBEV), no currently licensed vaccines or antiviral drugs are available for dengue.

Flaviviruses are composed of a capsid protein core complexed with a positive sense RNA genome (~11 kb), surrounded by a lipid envelope. The envelope contains 180 copies of the envelope (E) protein, which are arranged in an icosohedral scaffold of 90 homodimers that lie parallel to the virion surface (Kuhn *et al.*, 2002). The DENV E protein is a class II fusion protein responsible for host cell attachment, entry, and virus-mediated cell membrane fusion. The 2 angstrom crystal structure has been solved for the ectodomain of the TBEV (Rey *et al.*, 1995), DENV2 (Modis *et al.*, 2003), DENV3 (Modis *et al.*, 2005), and West Nile virus (Kanai *et al.*, 2006) E proteins revealing three important structural domains (DI, DII, and DIII), which correlate with earlier monoclonal antibody (MAb) mapping data that defined three antigenic domains (C, A, and B) in the E protein (Heinz, 1986; Mandl *et al.*, 1989; Roehrig, Bolin, and Kelly, 1998; Roehrig *et al.*, 1990). Domains I and II are linearly discontinuous structures separated by four peptide strands that comprise a molecular hinge region. The flavivirus-conserved fusion peptide (CD loop, amino acids [AAs] 98-111), located at the distal end of DII is responsible for E protein insertion into the endosomal membrane and subsequent fusion of the host membrane and viral envelope (Modis *et al.*, 2004). DIII is an immunoglobulin-like structure connected to DI by a single linker peptide and is postulated to have host-cell receptor binding properties. Antibodies with high neutralizing activity have been mapped to DIII and soluble DIII has been used to block infection of cells with whole virus, both suggesting DIII contains receptor-ligand epitopes

(Abd-Jamil, Cheah, and AbuBakar, 2008; Chin, Chu, and Ng, 2007; Chu *et al.*, 2005; Crill and Roehrig, 2001; Huerta *et al.*, 2008; Roehrig, Bolin, and Kelly, 1998).

The DENV2 and TBEV E protein structures only differ noticeably at surface exposed loops. One major difference in surface structure is located in DIII, where DENV2 has an extended loop motif between the F and G beta strands (FG loop) while TBEV does not (Figure 1) (Rey *et al.*, 1995; Zhang *et al.*, 2004). The extended portion of this loop is composed of four AAs that are present in all mosquito-borne flaviviruses but absent in tick-borne flaviviruses (Table 4.1, Figure 4.1). The absence of the extended loop in the tick-borne viruses has implicated the FG loop as a mosquito cell-specific binding motif. In addition, the four central AAs comprising this loop vary between each DENV serotype and are suggested to be involved with binding to mosquito cells in a serotype-specific manner, while binding of DIII to mammalian cell lines is suspected to be independent of the FG loop (Hung *et al.*, 2004).

In this study we investigated the importance of the DENV2 FG loop for infection of mammalian and mosquito cell cultures and live mosquitoes. Site-directed mutagenesis was used to delete or substitute AAs in the FG loop of DIII in an infectious cDNA clone of DENV2 strain 16681 to mimic the tick-borne viruses, JEV, and YFV17D. The results of this study show that the FG loop is critical for virus infectivity of cultured mammalian cells and *A. aegypti* midguts.

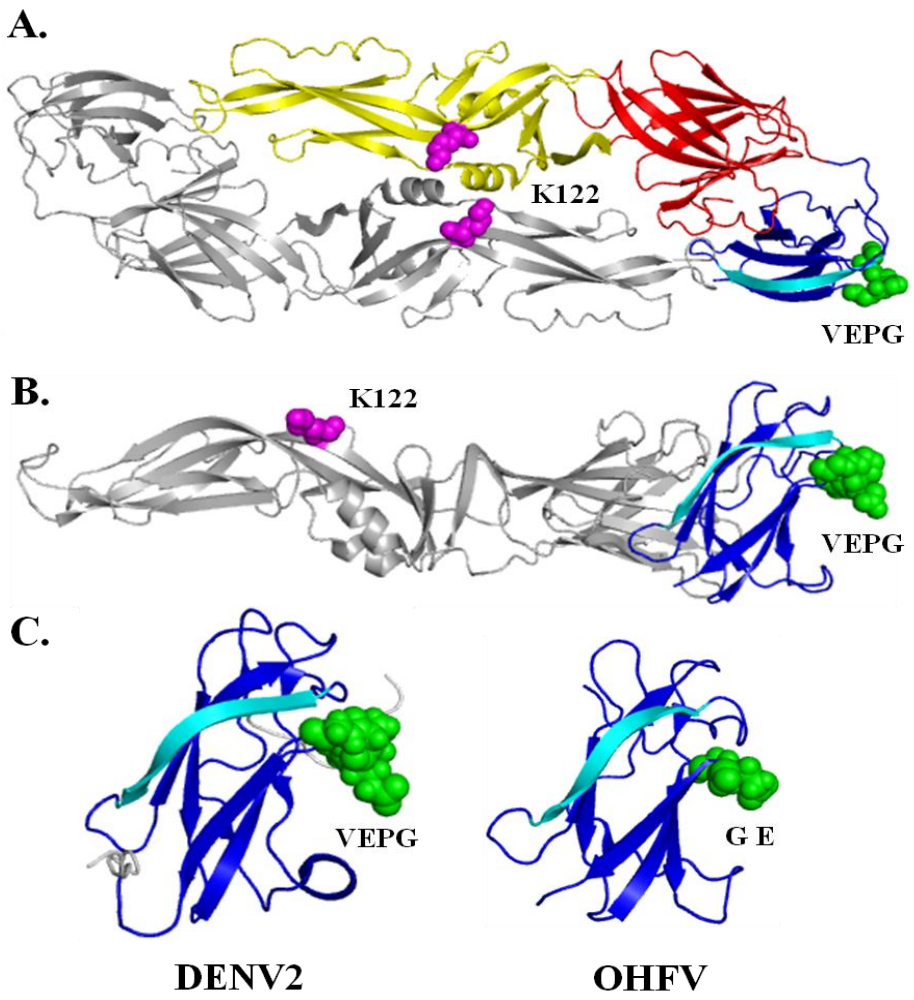

Figure 4.1. E protein structure and location of AA on the lateral ridge of DIII. (A) Top-down view of the DENV2 E protein dimer with DI in red, DII in yellow, and DIII in blue on one monomer. (B) Side-view of the DENV2 E protein monomer. (C) Side-view of DENV2 E protein DIII and Omsk hemorrhagic fever virus (OHFV) E protein DIII. The A beta strand (cyan), the FG loop (green), and K122 (magenta) are highlighted. Protein structures were obtained from the protein database bank (DENV2 E protein homodimer ID: 1oan, DENV2 DIII ID: 2jsf, and OHFV DIII ID: 1z3r) and were rendered using Polyview-3D (Porollo, Adamczak, and Meller, 2004).

Methods and Materials

Cell Culture

Vero, HepG2, and K562 cells were grown at 37^{o}C in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS). C6/36 cells used for virus phenotyping experiments were grown in YE-LAH medium with 10% FBS (Huang *et al.*,

2000) and C6/36 cells used for virus propagation to infect mosquitoes were grown in Leibovitz L-15 medium, with 2% FBS, both at 28^{o}C.

Mutagenesis, transfection, and virus recovery

DENV2 strain 16681 infectious cDNA plasmid, pD2/IC 30P-NBX (Huang *et al.*, 2010) was modified from pD2/IC-30P-A (Kinney *et al.*, 1997a) and used to construct all mutant viruses. QuikChange site-directed Mutagenesis kit (Stratagene) was utilized to engineer desired mutations into the envelope (E) gene of the cDNA plasmid. Mutations engineered into the E gene were a VEPG382 motif deletion, VEP382RGD, VEPG382RGDK, VEPG382RGDS, K305/307/310E, and G304K. Virus derived from the parental plasmid (30P-NBX) has a similar phenotype to DENV2 strain 16681 and was used as the positive control for all experiments.

Transfection of Vero and C6/36 cells with infectious vRNA has been described previously (Huang *et al.*, 2010; Huang *et al.*, 2003). Briefly, 200 ng of linearized infectious clone cDNA was transcribed *in vitro* using the Ampliscribe T7 High-yield transcription kit (EPICENTRE Biotechnologies) and the recovered RNA was electroporated into 4 x 10^6 Vero cells or 8 x10^6 C6/36 cells using the BioRad Gene Pulser Xcell system. Electroporated cells were seeded into a 75 cm^2 flask and maintained for 10-14 days until virus was harvested. Medium harvested from transfected Vero cells (V-0) or C6/36 cells (C-0) was centrifuged to remove cell debris, supplemented with 20% FBS, and stored at -80^{o}C. An aliquot of V-0 and C-0 was used to infect naïve Vero and C6/36 cells to produce V-1 and C-1 seeds, respectively. Genome cDNA of V-1 and C-1 seeds were fully sequenced to evaluate their genomic stability as described before (Huang *et al.*, 2010). Medium from transfected and infected cells was subjected to RT-PCR to

detect the presence of viral genomes. Viral antigen was detected in acetone-fixed cells by IFA using polyclonal anti-DENV2 New Guinea C hyperimmune mouse ascitic fluid (HMAF) and fluorescein isothiocynate-conjugated (FITC) goat anti-mouse IgG (Jackson Immunoresearch Laboratories).

Assay of DENV2 growth kinetics in cell cultures

Twelve day growth curves were performed to investigate mutant virus growth kinetics in different cell types. Cell cultures were infected in duplicate at a MOI of 0.001 and virus genomic equivalents were measured by qRT-PCR (Butrapet, Kinney, and Huang, 2006). Samples with peak qRT-PCR titers were assayed for infectious virus by $TCID_{50}$ in C6/36 cells (Bryant *et al.*, 2007; Huang *et al.*, 2010).

Aedes aegypti mosquito infection by blood-feeding and intrathoracic inoculation

A. aegypti RexD strain laboratory mosquitoes (RexD) originating from Rexville, Puerto Rico were reared from eggs and maintained as adults at 28^{o}C, 80% relative humidity with a photocycle of 12h light: 12h dark. Adult female mosquitoes were maintained in one-pint cartons with organdy covering, and given water and sugar until infection. To obtain DENV2 for infectious blood-feeds, C6/36 cells were infected at a MOI of 0.001 and maintained for 12-14 days with a medium change at 7 days. Infected cells were scraped into the medium, mixed with an equal volume of defibrinated sheep blood and then supplemented with ATP to a final concentration of 1mM. Adult female mosquitoes 4-6 days post-emergence were starved for 24 hours, deprived of water for 4 hours, and then exposed to the infectious blood-meal for 45 minutes using a 37^{o}C water-jacketed glass feeding device with a hog gut membrane. Virus titers in the blood-meals ranged from 7.2 to 9.45 $\log_{10}$ $TCID_{50}$/ml. In our experience with 30P-NBX and E protein

mutant viruses, there was no correlation between virus titer at this range and midgut infectivity (Chapter 2). Fully engorged mosquitoes were selected and maintained for 7 days, when midguts were dissected in PBS, fixed in 4% paraformaldehyde in PBS overnight, and analyzed for virus antigen via IFA to determine midgut infection rates. Each blood-feed experiment was repeated three times with at least 23 mosquito midguts analyzed per experiment.

IT inoculations were performed as described previously (Huang *et al.*, 2010). C-1 virus seeds were diluted to $1x10^5$ $TCID_{50}$/ml and adult female mosquitoes were IT inoculated with 0.3-0.5 μl of inoculum. Injected mosquitoes were maintained for 7 days until head squashes were performed. Virus antigen was detected in head tissues by IFA to determine infection rates. Each IT injection experiment was repeated three times for a total of 76-100 mosquitoes for each virus.

Indirect immunofluorescence assay for mosquito tissues

Midgut and head squash IFAs were performed as described previously (Brackney, Foy, and Olson, 2008). Virus antigen in midguts and heads was detected using flavivirus E protein DII group-reactive mouse MAb 4G2 (ATCC, HB-112) in wash buffer (PBS, 0.05% TritonX-100) or PBS, respectively. Secondary antibody was ImmunoPure biotin-labeled goat anti-mouse IgG (Thermo Scientific) with 0.005% Evan's Blue counter-stain, followed by streptavidin-fluorescein (GE Healthcare). MIRs and head infectivity rates were determined by dividing the number of virus antigen-positive midguts or heads by the total number analyzed. The relative infection intensity (RII) ratio was created by Dr. Doug Brackney and is a quantitative measure of infection intensity in the midgut. Positive midguts were scored for infection intensity on a scale of 0.5 to 4, where 0.5

denotes that less than 25% of the midgut surface area is positive for viral antigen, 1 denotes 25%, 2 denotes 50%, 3 denotes 75%, and 4 denotes 100% of the midgut surface area is positive for viral antigen. The RII ratio was determined by adding the infection intensity scores of positive midguts and dividing by the total number of positive midguts. Student's t tests (p-value 0.05) were performed using Excel 2002 and chi-square analysis (p-value < 0.05) was done using SAS 9.1.

Epitope mapping of the E protein

The antigenic structure of the DENV E protein was analyzed using a well defined panel of DENV Mabs (Roehrig, Bolin, and Kelly, 1998), which included MAbs reactive with the E protein DI (1B4C-2), DII (6B6C-1 and 2H3), DIII (3H5, 9A3D-8, and 1A1D-2), and prM (2H2). Antibody reactivity was assessed by IFA on acetone-fixed C6/36 cells infected with each mutant virus. Antibody endpoint concentrations with a greater than 4-fold difference from that of parent virus 30P-NBX were considered significant.

Fusion from within assay

The capacity of the mutant viruses to fuse with host cell lipid membranes was assessed using infected C6/36 cells exposed to pH 5.5 in the FFWI assay (Guirakhoo *et al.*, 1993). Briefly, C6/36 cells in 24-well plates were infected with virus at a MOI of 0.1 and maintained for 7 days in culture medium buffered to pH 7.7. Medium was removed and the infected cells were exposed to fusion medium buffered with MES to pH 5.5 for 2 hours. After the incubation period, cells were re-exposed to the pH 7.7 culture medium for an additional 24 hours. Cells were stained with Hema-3 quick stain (Fisher) and the number of cells and nuclei were counted for calculation of the fusion index and percent fusion (Huang *et al.*, 2010).

Results

Rationale for mutagenesis of the FG extended loop

DENV type-specific antibodies have been mapped to a surface exposed amino acid motif in the E protein located on the lateral ridge of DIII that forms the FG extended loop (Gentry *et al.*, 1982; Gromowski, Barrett, and Barrett, 2008; Hiramatsu *et al.*, 1996; Sukupolvi-Petty *et al.*, 2007). To investigate the involvement of the DENV2 FG loop (specifically AAs 382-385; VEPG) in viral replication, we used site-directed mutagenesis of the E protein gene of DENV2 strain 16681 infectious clone 30P-NBX to alter the VEPG sequence (Huang *et al.*, 2010). The flaviviruses in the JEV serocomplex and YFV have a semi-conserved RGD integrin binding motif as part of the FG loop, and changes to this motif have been shown to affect the ability of viruses to infect vertebrate and invertebrate cells (Hahn *et al.*, 1987; Lee and Lobigs, 2000; Lobigs *et al.*, 1990; van der Most, Corver, and Strauss, 1999). The mutations we introduced into the E gene of the full length DENV2 infectious clone were VEPGΔ (deletion), VEP382RGD (RGD), VEPG382RGDK (RGDK), VEPG382RGDS (RGDS), KKK305/307/310EEE (triple substitution, K305/7/10/E), and G304K (substitutions). Changing VEP to RGD inserts a known integrin-binding motif and changes the DENV2 type-specificity of the FG loop. More specifically, mutating VEPG to RGDK or RGDS imitates the FG loops found in JEV and YFV17D, respectively (Table 1). Furthermore, we deleted completely the FG loop, mimicking the tick-borne flaviviral E protein. The triple mutant removes putative heparan sulfate and receptor binding sites, and conversely the G304K mutation adds a heparan sulfate binding site to the A strand of DIII.

Table 4.1. Domain III FG extended loop amino acid sequences for vector-borne flaviviruses.

Virus	Strain	FG loop Sequence (AA 381-386)*	Accession Number
DENV-2	16681	G **VEPG** Q	AAB58782
DENV-1	16007	G **AGEK** A	AAF59976
DENV-3	PhMH-J1-97	G **IGDK** A	AAS49486
DENV-4	Thailand/1985	G **VGDS** A	AAV49746
YFV	Asibi	G **TGDS** R	AAT58050
YFV	17D	G **RGDS** R	AAX47570
JEV	Nakayama	G **RGDK** Q	AAB40688
MVE	NG156	G **RGDK** Q	ABM65594
WNV	NY99	G **RGEQ** Q	AAF20092
SLEV	Laderle	G **RGTT** Q	ACA28960
TBEV	Neudoerfl	G **----** E	AAA86870
OHFV	S-4-7/9867	G **----** E	AAO65829

*E protein AA position in DENV-2 16681

Transfection and recovery of infectious virus from C6/36 cells

After site-directed mutagenesis to introduce nucleotide substitutions into the E gene of the DENV2 infectious cDNA clone, infectious RNA was transcribed *in vitro* and transfected into C6/36 cells (C-0). All mutant viruses were able to replicate in C6/36 cells after transfection as evidenced by detectable virus antigen in cells and virus genome in medium. After passage of resulting C-0 virus to naive C6/36 cells (C-1), virus antigen and virus genome were again detected, confirming the ability of all mutant viruses to infect this cell type. Sequencing the genomes of C-1 viruses showed each mutant had the expected full length genome sequence (Table 4.2), and therefore C-1 seeds were used for all subsequent phenotypic studies. The recovery of all mutant viruses from C-0 and C-1 samples demonstrated that DENV2 infectivity of C6/36 cells is not solely dependent on the presence or sequence of the FG loop or the A strand.

Table 4.2. Transfection and recovery of infectious virus in C6/36 or Vero cells.

Virus	C6/36 cells		Vero cells		
	C-0/C-1[a] virus recovery	C-0/C-1[b] E protein sequence	V-0/V-1[a] virus recovery	V-0[b] E protein sequence	V-1[b] E protein sequence
VEPGΔ	+/+	correct	+/+	+K122I/K +K388K/E	+K122I/K +K388E/K
RGD	+/+	correct	+/+	correct	+K122K/I
RGDK	+/+	correct	+/+	+K122K/I	+K122I/K
RGDS	+/+	correct	+/+	correct	+K122I/K
K305/7/10E	+/+	correct	-/-	lethal	lethal
G304K	+/+	correct	+/+	reversion	reversion

[a] Transfection (C-0 or V-0) and passage (C-1 or V-1) was considered positive if virus antigen and virus genome was detected by IFA and RT-PCR, respectively.
[b] Sequencing verified all mutants contained the desired mutations. Mutants that could not be recovered from transfection or passage (lethal), with no additional changes in the genome (correct), a reversion of the engineered mutation (reversion), and secondary mutations in the E protein are specified.

Transfection and recovery of virus from Vero cells

To investigate the ability of these mutant viruses to infect and replicate in mammalian cells, mutant virus RNA was transfected into Vero cells (V-0) and resulting virus was passaged once in Vero cells (V-1). Full-genome sequencing of V-1 samples revealed that all of the FG loop mutant viruses retained the desired mutation and gained a partial mutation in the envelope gene, K122I/K (mixed populations, consensus sequence verified by cDNA sequencing in both directions). V-0 genome sequencing showed that mutants VEPGΔ and RGDK acquired this additional mutation following transfection while mutants RGD and RGDS did not have this change until the subsequent passage. Mutant VEPGΔ had a partial mutation in the envelope protein, K388E/K (mixed populations), which appeared in V-0 and was retained in the V-1 seed. Mutant K305/7/10E virus could not be recovered after transfection of vRNA into Vero cells and therefore no passage could be completed in this cell type, demonstrating that this set of

mutations is lethal for infection of Vero cells. Mutant G304K virus was recovered after transfection of vRNA in Vero cells but consensus sequencing revealed a full reversion back to wild type sequence. Consequently, passage in Vero cells also replicated virus with the wild type sequence. Due to the instability of these mutants in Vero cells, all the mutant seeds used for further phenotype studies were derived from C6/36 cells (C-1).

Epitope mapping of mutant viruses

A well-defined panel of murine MAbs that recognize all three distinct antigenic domains of the E protein was used to investigate whether epitopes in the E protein are affected by mutation of AAs in DIII (Roehrig, Bolin, and Kelly, 1998). Antibody reactivity was assessed by immunofluorescent assays (IFA) on acetone-fixed C6/36 cells infected with each virus. MAb 3H5 was the only antibody to show a decrease in binding greater than 4-fold for all FG loop mutants compared to wild type 30P-NBX (Table 4.3). In addition, reduction in 3H5 binding to the triple mutant was also found, which corroborates findings from researchers who have shown that the FG loop and the A strand contribute to the binding site for this type-specific antibody (Gromowski, Barrett, and Barrett, 2008; Hiramatsu *et al.*, 1996; Sukupolvi-Petty *et al.*, 2007; Trirawatanapong *et al.*, 1992). Binding of subcomplex-specific MAb 1A1D-2 was affected by mutation of residues K305/7/10 (Table 4.3), which is consistent with reports that show these are critical residues for binding of this MAb, specifically residues K307 and K310. (Gromowski *et al.*, 2010; Lok *et al.*, 2008; Roehrig, Bolin, and Kelly, 1998; Sukupolvi-Petty *et al.*, 2007). Type-specific MAb 9A3D-8 binding to the K305/7/10E mutant was also reduced and is consistent with a study that showed reduced binding when residue K307 was mutated to glutamic acid (Sukupolvi-Petty *et al.*, 2007). Sukupolvi-Petty *et al.*

(2007) also showed that mutation of G304 to tyrosine resulted in reduced binding to 9A3D-8. However, considering that this antibody was able to bind to our G304K mutant suggests this residue may not be absolutely critical for binding of 9A3D-8.

Table 4.3. Epitope mapping of DIII virus mutants.

Virus	Monoclonal antibody reactivity[a]						
	2H2 prM	6B6C-1 A1[b]	1B4C-2 C1	2H3 A4	9A3D-8 B2	3H5 B1	1A1D-2 B4
30P-NBX	0.16	0.12	0.12	0.94	0.94	0.47	0.31
VEPGΔ	0.24	0.2	0.12	1.25	1.25	**>10**	0.31
RGD	0.16	0.12	0.12	0.94	1.25	**>10**	0.47
RGDK	0.12	0.12	0.12	1.25	1.25	**>10**	0.31
RGDS	0.16	0.12	0.08	1.25	0.94	**>10**	0.47
K305/7/10/E	0.12	0.12	0.12	0.94	**>10**	**>10**	**7.5**
G304K	0.12	0.12	0.24	0.47	1.88	0.63	0.12

[a]MAb reactivity was assessed by IFA on acetone-fixed C6/36 cells infected with each virus. Numbers indicate endpoint concentration of purified antibody and numbers equal to or greater than 4-fold endpoint differences compared to 30P-NBX are specified (bold).
[b]MAbs were described previously (Roehrig, Bolin, and Kelly, 1998).

Virus growth kinetics in C6/36, Vero, and HepG2 cells

Mutant virus growth kinetics were determined by infecting cell cultures in duplicate with each virus at a multiplicity of infection (MOI) of 0.001 and measuring virus genomic equivalents in medium by quantitative (q)RT-PCR every two days. Given the genetic stability of these mutant viruses in C6/36 cells and their genetic instability in Vero cells, infectious virus titers of selected samples were determined using a C6/36 cell-based 50% tissue culture infectious dose ($TCID_{50}$) assay. Genome equivalents achieved by day 8 and peak infectious titers are reported in Table 4.4.

Table 4.4. Genome equivalents and peak infectious virus titers in invertebrate and vertebrate cell lines.

Virus	C6/36		Vero		HepG2	
	ge/ml[a]	$TCID_{50}$/ml[b]	ge/ml	$TCID_{50}$/ml	ge/ml	$TCID_{50}$/ml
30P-NBX	10.81	8.75	10.16	6.88	9.06	6.25
VEPGΔ	10.85	8.50	5.07[c]	4.50[c]	6.38	Lethal
RGD	10.93	9.00	9.21	7.25	9.14	5.75
RGDK	10.68	8.63	8.60[c]	5.75[c]	7.46	3.25
RGDS	10.56	9.13	9.19	6.63	8.01	4.75

[a]Geometric mean Log_{10} ge/ml on day 8 post infection
[b]Peak Log_{10} $TCID_{50}$/ml achieved during growth curves
[c]Virus acquired a K122I mutation in the E protein

All four mutant viruses had similar replication kinetics and peak infectious titers in invertebrate C6/36 cells compared to 30P-NBX, corroborating the C6/36 cell transfection data and the ability of the FG loop mutant viruses to infect and replicate efficiently in these cells. This includes both K305/7/10E and G304K viruses (data not shown in Table 4.4). In contrast, mutant virus growth kinetics in mammalian cells was more variable. By day 10 pi in Vero cells, mutants RGD, RGDK, and RGDS reached similar genome equivalent (ge) levels to 30P-NBX, although replication before this day was delayed compared to 30P-NBX (Figure 4.2A). Sequencing genomes of virus harvested at the completion of each growth curve showed that mutants RGD and RGDS were genetically stable, while RGDK acquired an additional mixed K122K/I mutation during growth. Mutant VEPGΔ replication kinetics differed greatly from the other mutants in Vero cells. Duplicate growth curves for VEPGΔ yielded two different results. Both infections showed no genome replication until day 6 pi, then only one of two cultures demonstrated increased replication kinetics (Figure 4.2A) and attained a low peak infectious virus titer of 4.50 log_{10} $TCID_{50}$/ml (Table 4.4). Virus genome sequences

from the culture exhibiting genome replication contained a K122I mutation, similar to mutant RGDK and the V-0 virus resulting from transfection. The temperature sensitivity of mutant VEPGΔ in Vero cells was investigated by comparison of growth at 28^{o}C and 37^{o}C. Replication of mutant VEPGΔ from day 2-6 pi was slower at 37^{o}C than at 28^{o}C, but the replication rate was more rapid at 37^{o}C than at 28^{o}C after day 8 pi (Fig 4.2B). Viral genome sequencing revealed no additional genome sequence changes in mutant VEPGΔ at 28^{o}C, while additional E protein mutations occurred in both samples cultured at 37^{o}C. One culture acquired K122K/I and N390H mutations, and the other had additional K122K/I, N390N/H, and Q400H/Q mutations. These data suggest that the VEPGΔ deletion may have rendered the virus thermally unstable at 37^{o}C until extra mutations were evolved.

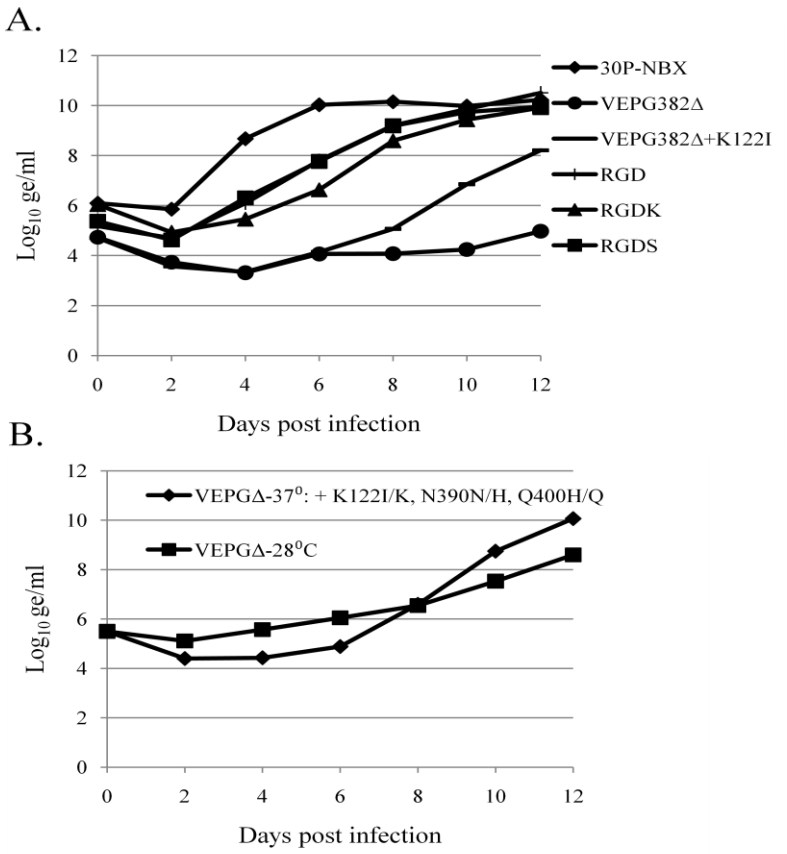

Figure 4.2. Virus growth kinetics in Vero cells at 37°C and 28°C. Vero cells grown at 37°C and 28°C were infected at a MOI of 0.001 and virus replication was measured via qRT-PCR every two days for twelve days. (A) The data presented are geometric mean titers from duplicate flasks infected and maintained at 37°C except for VEPGΔ and VEPGΔ+K122I, which represent results from individual flasks. (B) Temperature sensitivity of VEPGΔ data are geometric mean titers from duplicate flasks infected and maintained at 37°C or 28°C.

Mutant virus phenotypes also were analyzed in HepG2 cells. Replication kinetics and peak infectious titer of mutant RGD were similar to 30P-NBX after infection of HepG2 cells, in stark contrast to mutant VEPGΔ, which was unable to produce infectious virus in this cell type. Although there was no detectable infectious virus, low level increases (12-fold) of viral genome equivalents were detected by qRT-PCR between day 0 and day 8 pi, suggesting that virus could enter these cells but could not complete a productive infectious cycle. All other mutants had 10^2 to 10^4-fold viral RNA increases,

while 30P-NBX viral RNA increased more than 10^5-fold between day 0 and day 8. Genome replication of mutants RGDK and RGDS was reduced by 32- and 10-fold compared to 30P-NBX, respectively. Also, their peak infectious titers were lower than 30P-NBX by 50- to 1000-fold (Table 4.3). Replication kinetics were additionally analyzed for all mutant viruses in K-562 cells. All mutants replicated in K-562 cells, but at significantly lower levels (2-4 logs lower titers) than the 30P-NBX (data not shown).

Mutant virus phenotypes in A. aegypti mosquitoes

Adult female *A. aegypti* RexD mosquitoes were presented with an infectious blood-meal to determine if mutations in DIII affected mosquito midgut infectivity. RGD, RGDK, RGDS, and G304K mutant viruses did not have statistically different midgut infection rates (MIRs) from 30P-NBX. However, mutants VEPGΔ and K305/7/10E had significantly lower MIRs than 30P-NBX, in contrast to the ability of these viruses to efficiently infect C6/36 cells (Figure 4.3 A). The intensity of infection for each mutant virus was similar to 30P-NBX (Figure 4.3 A).

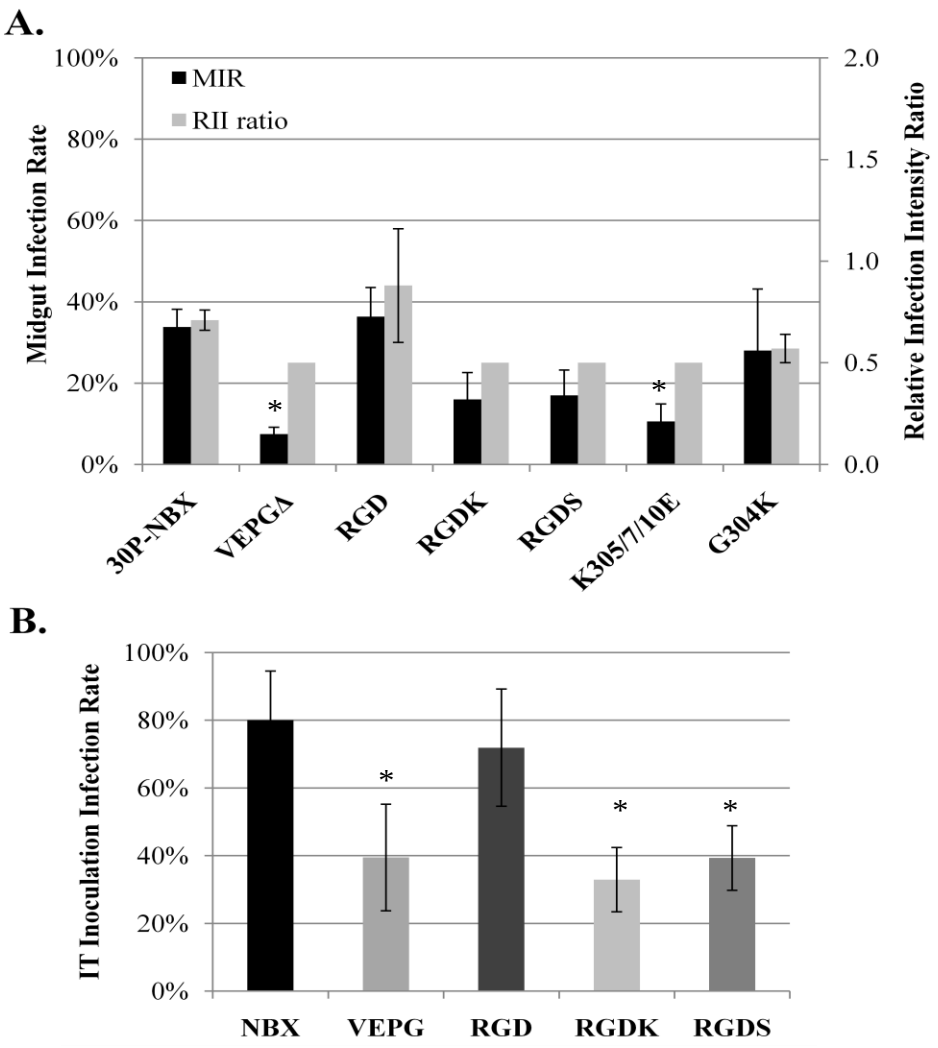

Figure 4.3. Mosquito MIR post-oral infection and mosquito head infection rates post IT inoculation. *Aedes aegypti* RexD strain mosquitoes were presented an infectious bloodmeal (A) or IT inoculated with virus (B), and maintained for seven days until midguts or heads were dissected. IFA analysis was used to detect virus antigen and infection rates were calculated. Experiments were repeated at least three times. Student's t test was used to calculate significance (* p-value < 0.05) for MIR and RII ratio and chi-square analysis was used to calculate significance for head infection rates (* p-value < 0.05) as compared to 30P-NBX, respectively.

The low MIR of the parent virus 30P-NBX made it difficult to analyze differences in dissemination rates between 30P-NBX and mutant viruses and we wanted to investigate whether these mutants could infect tissues outside the midgut. Therefore, midgut infection was bypassed by intrathoracic (IT) inoculation of mosquitoes with 30-

50 $TCID_{50}$ of virus, a concentration previously used to show significant differences in mutant virus infectivity of tissues outside the midgut (Huang *et al.*, 2010). Virus will first infect and amplify in tissues in the mosquito hemocoel and then spread to the head by 7 days pi, where there do not seem to be any barriers to infection that would require the accumulation of adaptive mutations. Mutants VEPGΔ, RGDK, and RGDS were less efficient at infecting mosquito tissues outside the midgut compared to 30P-NBX, as indicated by their significantly different head infection rates. Mutant RGD infection rate for tissues in the head was similar to 30P-NBX (Figure 4.3 B). No IT inoculations were completed for the triple mutant or G304K because the available virus stocks had titers below $1x10^5$ $TCID_{50}$/ml. These data clearly show a disparity between mutant virus infection of C6/36 cells and infection of mosquito tissues.

Fusion competence of FG extended loop mutant viruses

The ability of the dengue virion to escape the host cell endosome before degradation in lysosomes is an important step in the life cycle of the virus and is mediated by the CD loop located at the distal end of DII of the E protein monomer. Our fusion from within (FFWI) assay results using infected C6/36 cells showed no significant difference in fusion activity at a pH range of 5.5 to 7.5 for any of the FG loop mutant viruses compared to 30P-NBX (data not shown), confirming that these FG loop mutations in DIII did not affect the CD loop fusion function.

Discussion

Previous studies have implied that DIII of the flaviviral E protein is required for attachment to and infection of susceptible cells. Soluble DIII of the DENV E protein can block binding of multiple cultured cell types by homologous and heterologous DENV

serotypes (Chin, Chu, and Ng, 2007; Hung *et al.*, 2004). Hung *et al.* (2004) found that binding of DENV2 soluble DIII to C6/36 cells can be blocked by a peptide (AA380-389) containing the homologous FG loop sequence, but not by heterologous peptides containing FG loop sequences of other DENV serotypes. Additionally, these peptides were unable to block either homologous or heterologous DIII binding to BHK-21 cells. These results and the absence of the FG loop in the tick-borne viruses (Table 1), which typically are not able to infect C6/36 cells, led to speculation that the FG loop plays a specific role in binding invertebrate cells but not mammalian cells. However, the results of our study suggest that the DIII FG loop structure is important for infection and replication in mammalian cells as well as mosquito tissues but a specific amino acid sequence is not required.

Other studies have indicated that the receptors and/or entry pathways for DENV infection of C6/36 cells might be atypical. A previous report utilizing a chimera containing Langat virus (tick-borne flavivirus) prM/E genes on a DENV4 nonstructural gene backbone showed that this virus was able to infect and replicate efficiently in C6/36 cells, while the wild type Langat virus could not, implying that the C6/36 cell infection restriction for Langat virus is due to the nonstructural genes and not the E glycoprotein (Pletnev and Men, 1998). Both our results and those of Pletnev and Men (1998) suggest that other structures or sequence motifs in the E protein are responsible for binding to and entry of C6/36 cells. Additionally, although there is evidence that flaviviruses enter C6/36 cells by receptor mediated endocytosis (Acosta, Castilla, and Damonte, 2008; Chu and Ng, 2004a; Chu and Ng, 2004b; Mosso *et al.*, 2008), it has also been suggested that

flaviviruses can infect these cells by direct fusion with the plasma membrane (Hase, Summers, and Cohen, 1989; Hase, Summers, and Eckels, 1989; Nawa *et al.*, 2007).

It is widely assumed that DENV replication in C6/36 cells is a useful model for predicting viral replication in live adult mosquitoes; however, our studies and others have shown that this is not necessarily the case. Previous studies showed that E protein glycosylation was critical for growth in Vero and C6/36 cells but not for growth in *A. aegypti* mosquitoes (Bryant *et al.*, 2007). Additionally, we found that DENV2 E protein fusion peptide mutants could replicate to high titer in C6/36 cells but had significantly lower infection rates in mosquitoes after IT inoculation (Huang *et al.*, 2010).

We showed that the AA sequence of the FG loop is unimportant compared to the presence of the structure itself for mosquito midgut infection. Similarly, engineering multiple mutations into the A strand also reduces infection of mosquito midguts. The low MIR resulting from deletion of the FG loop and mutation of the A strand may be due to one of the following: (i) Absence or mutation of these motifs may reduce viral attachment/entry to midgut cells, suggesting either the FG loop and/or the A strand is directly involved in interactions with midgut cell receptors or manipulation of these two motifs results in changes in other DIII structures necessary for efficient receptor binding/cell entry. (ii) Absence of the FG loop and mutation of the A strand may affect proper E protein folding/maturation in midgut cells, resulting in reduced assembly of infectious progeny virions.

IT inoculation of virus into mosquitoes has been shown to be a sensitive method for growing DENVs that cannot be cultured readily in other systems (Rosen and Gubler, 1974) and it was employed in this study to analyze the infection rates of the FG loop

mutant viruses in tissues outside the midgut. In addition to mutant VEPGΔ, mutants RGDS and RGDK also had significantly lower infection rates than 30P-NBX in mosquito head tissues. Mutant RGDK and RGDS FG loop motifs mimic those found in DIII of JEV and YFV17D, respectively, and both of these viruses are capable of replicating in *A. aegypti* mosquitoes after IT inoculation (Bhatt *et al.*, 2000; McElroy *et al.*, 2006). Since the RGD virus had similar infection rates to 30P-NBX virus in head tissues, it appears that in the context of DENV2 DIII, substitution of glycine at position 385 is most detrimental to virus replication in these tissues.

A surprising finding in this study was that the FG loop affects DENV2 infection of mammalian cells. All of our FG loop mutants replicated more slowly than the 30P-NBX virus in Vero, HepG2, and K562 cells during the first 8 days pi. Productive replication of VEPGΔ in Vero cells was only observed after the acquisition of a K122I mutation in DII of the E protein (Figures 4.1 and 4.2). Our results indicated that deletion of AA 382-385 significantly impaired virus replication in mammalian cells, although VEPGΔ was able to enter cells to initiate virus replication. This finding is consonant with the previous report that the FG loop is not important for DENV2 binding to mammalian cells (Hung *et al.*, 2004). VEPGΔ and the other FG loop mutants acquired the K122I mutation after transfection (RGDK) or passage (RGD and RGDS) in Vero cells implying that this mutation is an adaptation for replication in Vero cells at 37^{o}C. Interestingly, viruses with engineered mutations in the molecular hinge region of the E protein also acquired this K122I mutation following transfection of Vero cells, suggesting that this mutation may not specifically compensate for alterations in the FG loop (C.Y-H Huang, unpublished data). We have also previously found a K122E mutation in wild

type DENV2 strain 16681 after multiple Vero cell passages (C.Y-H Huang personal communication) as well as after passage of 30P-NBX in *Aedes aegypti* mosquito midguts (Chapter 3). The K122E mutation identified in the serial passage experiments was an adaptive mutation for enhanced infectivity of mosquito midguts. It is unclear why this mutation was selected for after passage of wild type virus in Vero cells considering both K122E mutant virus and 30P-NBX virus replicate with equivalent efficiencies in this cell type. In a separate study, mutations introduced into the FG loop of YFV17D were found to affect protein stability at 37^{o}C, but not mammalian host cell attachment (van der Most, Corver, and Strauss, 1999).

Our study also identified possible compensatory mutations to VEPG deletion at DIII K388E (Table 1), N390H, and Q400H (Fig. 2B). AAs K388 and N390 sit below the FG loop and mutations to these residues may serve to stabilize the lateral ridge of DIII itself or could cause changes to the tertiary structure of DIII that can compensate for the space left behind after deletion of the FG loop. Since the amino acids seem to project from the lateral ridge, they may also provide stability for the 5-fold axis of symmetry at 37^{o}C. DENV2 strains in the Asian/American genotype have asparagine at position 390 and replicate to higher titers in human dendritic cells compared to strains in the American genotype, which have aspartic acid at this position (Cologna and Rico-Hesse, 2003; Leitmeyer *et al.*, 1999). A mechanism has yet to be elucidated for the increased replication capacity of viruses with asparagines at position 390. Mutation at AA Q400 to H is more puzzling considering its position at the start of helix 1 in the conserved membrane proximal "stem" region located underneath the E protein (Schmidt, Yang, and Harrison, 2010).

Substitution of VEP by the integrin-binding motif RGD did not affect DENV2 infection of mosquitoes and had minimal effects on growth in cultured cells, suggesting that the integrin binding motif could also be utilized by DENV2 in host infection. However, additional substitutions at AA 385 significantly reduced infection of mosquito head tissues, suggesting that RGDK or RGDS motifs are not optimal for DENV infection in *A. aegypti*. Also, when single AAs in the RGD motif of YFV-17D and Murray Valley encephalitis virus were mutated, mutant viruses were still able to infect cultured vertebrate and invertebrate cells (Hurrelbrink and McMinn, 2001; van der Most, Corver, and Strauss, 1999). These data taken together with the fact that none of the DENV serotypes has the RGD motif, and that other flaviviruses have variations of the RGD motif (Table 4.1), suggest that integrin-dependent binding may not play a major role in *Aedes* spp. mosquito infection. It is possible that the AA motif in the FG loop is a determinant of vector selection/preference considering that none of the DENV serotypes has the RGD motif, whereas most of the flaviviruses with the RGD motif in the FG loop are transmitted primarily by C*ulex* spp. mosquitoes.

If the FG loop contributes to receptor binding, it may be either in concert with or independently from another ligand on the E protein. Several groups have identified putative cell receptors capable of binding DENV E protein on mammalian and invertebrate host cells that include glycosaminoglycans (Vero and BHK21) (Chen *et al.*, 1997; Hung *et al.*, 2004; Hung *et al.*, 1999), heat shock proteins (U937 and C6/36) (Salas-Benito *et al.*, 2007; Valle *et al.*, 2005), stress response protein Grp78 (HepG2) (Jindadamrongwech, Thepparit, and Smith, 2004), DC-SIGN (monocyte-derived dendritic cells) (Navarro-Sanchez *et al.*, 2003; Tassaneetrithep *et al.*, 2003), mannose

receptor (macrophages) (Miller *et al.*, 2008), prohibitin (C6/36, CCL-125, and *A. aegypti* whole mosquitoes) (Kuadkitkan *et al.*, 2010), and other as yet unidentified proteins of various sizes (Vero, C6/36, and mosquito midgut cells) (Martinez-Barragan and Del Angel, 2001; Mercado-Curiel *et al.*, 2006) indicating that the E protein may be capable of attaching to different cellular receptors via several E protein motifs.

In this study we showed that binding of type-specific, strongly neutralizing MAb 3H5 is abolished by deletion or substitution of AA in the FG loop. MAb 3H5 was found to bind to the DENV2 conserved FG loop AAs E383 and P384 as well as semi-conserved G304 and K305 on the neighboring A beta strand (Gromowski and Barrett, 2007; Gromowski, Barrett, and Barrett, 2008; Roehrig, Bolin, and Kelly, 1998; Sukupolvi-Petty *et al.*, 2007). Interestingly, 3H5 binding was not affected by mutation of G304 to lysine suggesting that K305 is more critical than G304 for binding of this MAb. Binding of subcomplex-specific MAb 1A1D-2, which targets AAs on the A strand (more specifically 307 and 310) was not affected by mutation of the FG loop in this study, demonstrating that the antigenic structure of the A strand was still intact (Lok *et al.*, 2008; Roehrig, Bolin, and Kelly, 1998; Sukupolvi-Petty *et al.*, 2007). However, this was not the case for the triple mutant, which was not bound by either 1A1D-2 or 3H5 as expected.

Evidence that a peptide containing the DENV2 FG loop sequence did not reduce binding of DENV2 DIII to mammalian cells (Hung *et al.*, 2004) suggests that a common attachment site apart from the FG loop in DIII has a role in binding to vertebrate cells. In addition to 1A1D-2, two other MAbs (9F12 and 4E11) have also been mapped to the A strand and can bind to and neutralize all four DENV serotypes (Lisova *et al.*, 2007; Rajamanonmani *et al.*, 2009; Thullier *et al.*, 2001). Furthermore, a group using phage

display technology showed evidence that phages expressing different truncated portions of DENV2 DIII (specifically AAs 297-423 and AAs 380-423) both bound to C6/36 cells with high affinity and found that there was no competitive binding between the phages (Abd-Jamil, Cheah, and AbuBakar, 2008). They suggest that DENV2 DIII binding to C6/36 cells could be a multistep process that includes binding to the A strand in addition to the FG loop and this may explain how the VEPGΔ mutant can successfully bind to and enter this cell type. However, this multistep binding suggestion may be more relevant for infection of Vero cells, considering the K305/7/10E mutations were lethal for replication in this cell type but not C6/36 cells. Even the G304K mutation in the A strand was not tolerated in Vero cells as evidenced by the reversion of this mutation after transfection of vRNA in this cell type. This would suggest that Vero cell infection may be dependent on the integrity of both the A strand and the FG loop while infection of C6/36 cells seems dependent on the integrity of at least one of these motifs. In a separate study analyzing virus attachment to host cells, the triple mutant was capable of attaching to Vero cells at 4°C but unable to enter cells when the temperature was returned to 37°C (C.Y-H Huang, unpublished data). This suggests that there may be a secondary binding event necessary for internalization of the virus that is disrupted by mutation of the A strand or the protein structure is rendered unstable by the elevated temperature. In fact, it should be highlighted that engineering three non-conservative amino acid mutations to opposite charge changes within close proximity may have profound effects on all aspects of the biology of the virus, which could very well include temperature sensitivity at 37°C. Nevertheless, evidence for the A strand being important for mammalian cell receptor binding is also supported by groups who have mapped neutralization escape mutants to

this region of DIII (Lin *et al.*, 1994; Lok, Ng, and Aaskov, 2001). Additionally, as depicted in Figure 4 by Zhang *et al.* (2004), the FG loop may also be important for forming a hydrogen bond network with the A strand around the 5-fold axis in the mature virion that could help maintain the structural integrity of the icosohedral protein scaffold, aiding in thermal stability and providing a concentration of multiple attachment sites within close proximity.

If the FG loop were responsible for binding to one particular receptor, it is possible that other structures in DIII such as the A strand, or other sites in DII could bind to co-receptors and partially compensate for the absence of the FG loop in the VEPGΔ mutant. However, deletion of VEPG may decrease the overall efficiency of virus entry, resulting in suboptimal virus infection in vertebrate cells. The presence of other receptor binding sites outside DIII could have implications for vaccine design and may explain why DIII-specific antibodies seem to play a minor role in neutralization of DENV by human sera (Wahala *et al.*, 2009). Binding assays using mutant VEPGΔ may be successful in discerning what other E protein structures contribute to receptor binding and could help determine if DENV E protein attachment to host cells is indeed a multistep process.

The findings of this study emphasize the importance of the FG loop and A strand in DENV infection of mosquitoes, however they do not determine if the presence of the FG loop alone is sufficient for mosquito infection, nor why tick-borne flaviviruses lack this structure. The results provide additional evidence that C6/36 cells are not a complete surrogate for DENV replication in mosquitoes and also point to the importance of using whole virus particles for analyzing the effect of mutations on the biology of the E protein.

CHAPTER 5

DENGUE VIRUSES WITH MUTATIONS IN THE FUSION PEPTIDE AND HINGE REGION OF THE ENVELOPE PROTEIN VARY IN THEIR ABILITY TO INFECT *AEDES AEGYPTI* AFTER INTRATHORACIC INOCULATION

Fusion mutant virus data has been published:
Claire Y.-H Huang, Sirritorn Butrapet, Kelly J. Moss, Thomas Childers, Steven M. Erb, Amanda E. Calvert, Shawn J. Silengo, Richard M. Kinney, Carol D. Blair, John T. Roehrig. (2010). The dengue virus type 2 envelope protein fusion peptide is essential for membrane fusion. Virology, 396(2): 305-15.

Hinge mutant virus data has been published:
Siritorn Butrapet, Thomas Childers, Kelley J. Moss, Steven M. Erb, Betty E. Luy, Amanda E. Calvert, Carol D. Blair, John T. Roehrig, and Claire Y.-H. Huang. (2011). Amino acid changes within the E protein hinge region that affect Dengue virus type 2 infectivity and fusion. Accepted to Virology, January 2011.

Introduction

Aedes aegypti is a highly domesticated mosquito that is distributed throughout the tropical and subtropical regions of the world. This mosquito prefers to breed in artificial containers and feeds almost exclusively on humans, often more than once during a single reproductive cycle (Scott *et al.*, 2000; Scott *et al.*, 1993). The four serotypes of dengue virus (DENV1-4) are the most medically important arboviruses infecting humans today and they are transmitted to humans by *Aedes aegypti* mosquitoes. Due to combined global human population growth, uncontrolled urbanization, air travel, and poor public health infrastructure in developing countries that results in ineffective mosquito control programs, DENV is endemic and epidemic in many countries throughout the world (Gubler, 1989; Gubler, 1998).

DENV is cycled in nature between humans and mosquitoes. There has been little published information regarding the contribution of DENV genetics to mosquito infection. DENV have a single stranded positive sense RNA genome (~11 kb) encapsidated in a capsid protein core surrounded by a lipid envelope. In the envelope there are 180 copies of the envelope (E) structural protein arranged in an icosahedral scaffold of 90 homodimers that lie parallel to the virion surface (Kuhn *et al.*, 2002) as well as 180 copies of the M protein. The soluble DENV2 E protein 2 Å crystal structure has been solved (Modis *et al.*, 2003) and revealed three distinct structural domains (DI, DII, and DIII) (Figure 5.1 A).

The E protein is a class II fusion protein that is responsible for host cell attachment, entry, and virus-mediated cell membrane fusion. Following internalization of a virion into a host cell endosome, the E protein will experience a pH-catalyzed molecular reorganization where E protein homodimers disassociate and DII moves away from the virion surface. The fusion peptide at the distal end of DII (CD loop, amino acids [AA] 98-111) (Figure 5.1 C) and the hinge region (composed of four peptide strands that connect DI and DII) (Figure 5.1 B) are vital to the function of the E protein during low pH-catalyzed molecular rearrangement. The fusion peptide is a highly conserved AA motif among flaviviruses and mediates fusion of virus and host cell endosomal membranes when it is inserted into a target membrane (Modis *et al.*, 2004). The hinge region allows the movement of the fusion peptide towards the endosomal membrane by flexing DII ca. 37° during the molecular conformational shifts (Modis *et al.*, 2004). Mutations engineered into the fusion peptide affect the fusion pH threshold of the protein and can result in a lethal phenotype (Huang *et al.*, 2010). Mutations

introduced into the molecular hinge region have variable effects on virus phenotypes that include adaptation to cell culture, escape from neutralizing antibody, and neurovirulence in mice (Butrapet et al., 2011; Hurrelbrink and McMinn, 2001; Lee, Weir, and Dalgarno, 1997; McMinn, Weir, and Dalgarno, 1996). The hinge region is also a target for antiviral compounds (Li *et al.*, 2008b; Modis *et al.*, 2003). The contribution of these two structures to infection of mosquitoes has not yet been investigated.

Invertebrate C6/36 cells are used in the laboratory as a surrogate for live mosquitoes and results obtained for virus infectivity of this cell type do not always translate to infection of live mosquitoes (Erb *et al.*, 2010; Huang *et al.*, 2010). Intrathoracic (IT) inoculation of virus into mosquitoes is a sensitive method of growing DENV that cannot be cultured in other systems (Rosen and Gubler, 1974) and was employed in this study to analyze the effect that fusion peptide and hinge region AA mutations have on virus infectivity of live mosquitoes. The results of this study show that infection of C6/36 cells with DENV is not an accurate model for predicting virus infectivity of live mosquitoes.

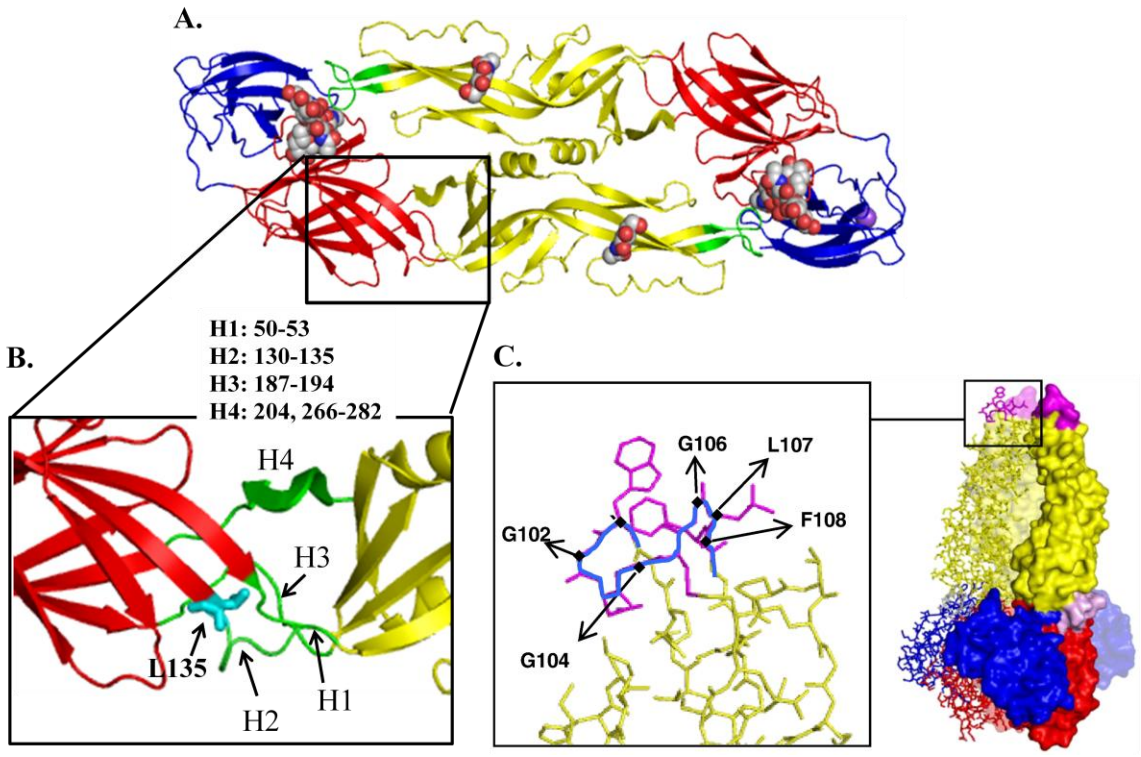

Figure 5.1. DENV2 E protein structure in mature virus and after membrane fusion. (A) Top down view of the DENV2 E protein homodimer (protein database bank ID: 1oan) with D1 in red, DII in yellow, and DIII in blue. (B) Magnified view of the four hinge peptide strands in green with L135 in cyan. AA that comprise H1 to H4 are specified. (C) DENV2 E protein homotrimer after membrane fusion and magnified view of the fusion peptide (protein database bank ID: 1ok8) with the same coloring as in A, except for the fusion peptide, which is magenta (picture is borrowed from Huang *et al.*, 2010). Protein structures were obtained from the protein database bank and were rendered in Polyview-3D (Porollo, Adamczak, and Meller, 2004).

Methods and Materials

Mutagenesis, transfection, and virus recovery

DENV2 strain 16681 infectious cDNA plasmid pD2/IC 30P-NBX (Huang *et al.*, 2010) was modified from pD2/IC-30P-A (Kinney *et al.*, 1997a) and used to construct all mutant viruses. QuikChange site-directed Mutagenesis kit (Stratagene) was utilized to engineer desired mutations into the envelope (E) gene of the cDNA plasmid. Two sets of mutations were engineered into the E gene. The first set was targeted at the CD fusion loop in DII and includes G102S, G104S, G106A, G106L, L107F, L107M, and F108W

(Huang *et al.*, 2010), and the second set was targeted to hinge region 2 between DI and DII and includes L135W, L135G, L135K, and L135M (Butrapet et al., 2011). Virus derived from the parental plasmid (30P-NBX) has a similar phenotype to DENV2 strain 16681, and was used as the positive control for all experiments. Viruses used in all experiments were obtained after transfection and one passage in C6/36 cells.

Mosquito maintenance and intrathoracic inoculation

Aedes aegypti RexD strain laboratory mosquitoes (RexD) originating from Rexville, Puerto Rico, were reared from eggs and maintained as adults at 28^{o}C, 80% relative humidity with a photocycle of 12h light: 12h dark. Adult female mosquitoes were maintained in one-pint cartons with organdy covering, and given water and sugar until infection.

IT inoculations were performed as described previously (Huang *et al.*, 2010). C-1 virus stocks with measured infectious virus concentrations were diluted to 1x10^{5} $TCID_{50}$/ml and adult female mosquitoes (5-7 days post emergence) were IT inoculated with 0.3-0.5 µl of diluted virus. Injected mosquitoes were maintained for 7 days until head squashes were performed. Virus antigen was detected in head tissues by IFA to determine infection rates. Each IT injection experiment was repeated three times for a total of 76-100 mosquitoes for each virus. To verify that mutation L135G was stable after replication in live mosquitoes, virus RNA was isolated via TRIzol® from the body of a mosquito that was positive for virus antigen in its head tissue, and then sequenced according to procedures described previously (Huang *et al.*, 2010).

Indirect immunofluorescence assay

Head squash IFAs were performed as described previously (Brackney, Foy, and Olson, 2008). Virus antigen in head squashes was detected using flavivirus E protein DII group-reactive mouse MAb 4G2 (HB-112, ATCC, Manassas, VA) or DENV2 type specific mouse MAb 3H5 (Henchal *et al.*, 1985) in PBS. Secondary antibody was ImmunoPure biotin-labeled goat anti-mouse IgG (Thermo Scientific, Waltham, MA) with 0.005% Evan's Blue counter-stain, followed by streptavidin-fluorescein (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Infectivity rates were determined by dividing the number of virus antigen-positive head tissues by the total number analyzed. Chi-square analysis (p-value 0.05) was done using SAS 9.1.

Results

30P-NBX has a low midgut infection rate in *A. aegypti* RexD strain mosquitoes and this made it difficult to analyze differences in mosquito infectivity between the 30P-NBX and mutant viruses in oral challenge experiments. For that reason we bypassed midgut infection by IT inoculation of virus in order to assess the ability of mutant viruses to infect live mosquitoes. In Chapter 4 we demonstrated that after IT inoculation of mosquitoes with 30 to 50 $TCID_{50}$, FG loop mutant viruses showed significant differences in infectivity of mosquito tissues compared to 30P-NBX. When these viruses were inoculated into mosquitoes at 3000 to 5000 $TCID_{50}$, an alphavirus concentration used in a previous study with *Anopheles gambiae* (Keene *et al.*, 2004), we found that mutant virus infectivity rates were equivalent to 30P-NBX ($\geq$ 96% for each mutant virus) (data not shown). The fact that 30P-NBX consistently exhibited high infection rates at both virus inoculation doses and that mutant viruses showed reduced infectivity rates at lower doses

suggested that inoculating high concentrations of mutant virus can preclude the ability to see differences in virus infectivity rates. Therefore, we proceeded to check mutant virus mosquito infectivity rates by IT inoculation with 30 to 50 $TCID_{50}$ of virus.

The results of the IT inoculation experiments for fusion peptide mutant viruses are shown in Table 5.1 and graphically in Figure 5.2 (Huang *et al.*, 2010). Each of the fusion mutant viruses included in this study replicates with similar growth kinetics and peak infectious titers compared to 30P-NBX in C6/36 cells. With the exception of mutant L107F, each of the mutant viruses had a significantly lower infection rate than 30P-NBX after IT inoculation into mosquitoes, contrary to their ability to efficiently infect invertebrate C6/36 cells.

Table 5.1. Infectivity of fusion peptide mutant viruses after IT inoculation in *A. aegypti* mosquitoes.

Virus	Exps.[a]	Negative	Positive	Total	% infected	p value[b]	Significance[c]
30P-NBX	7	6	161	167	96.41%	n/a	n/a
G102S	5	46	94	140	67.14%	<0.0001	*
G104S	5	63	58	121	47.93%	<0.0001	*
G106A	4	43	65	108	60.19%	<0.0001	*
G106L	4	49	62	111	55.86%	0.013	*
L107F	4	3	82	85	96.47%	>0.05	none
L107M	3	19	93	112	83.04%	<0.0001	*
F108W	4	29	78	107	72.90%	<0.0001	*

[a] Number of experimental repetitions.
[b] Significance was determined by chi-square analysis (p value ≤ 0.05) and is marked by asterisks.
[c] Significant p value ≤ 0.05 is marked by *.

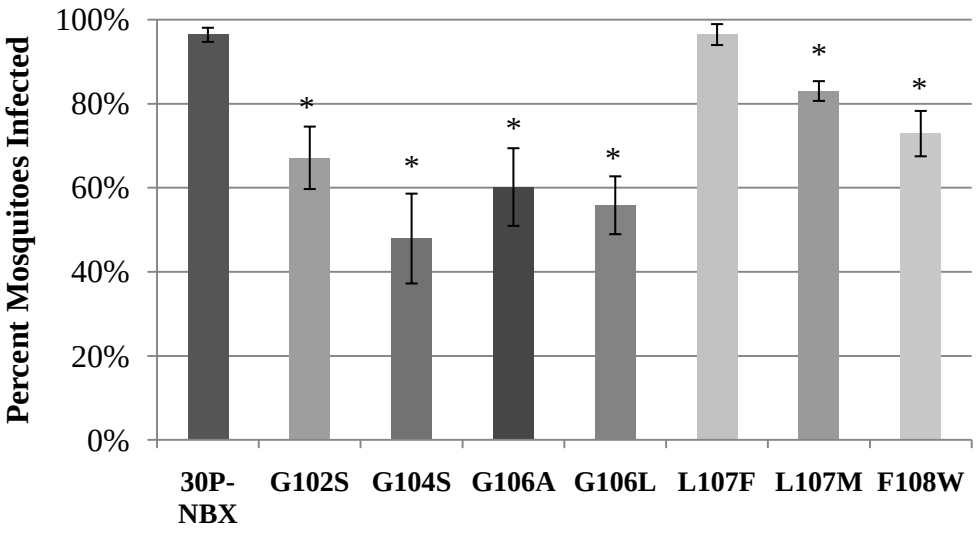

Figure 5.2. Infectivity rates of fusion peptide mutant viruses after IT inoculation in *A. aegypti* mosquitoes. Infection of adult mosquitoes by IT inoculation was evaluated via IFA assay for DENV2 E protein in head tissues 7 days post inoculation. Percent infectivity rates are the same numbers presented in Table 5.1. Chi-square analysis was used to compare each mutant virus to 30P-NBX (* p value $\leq$ 0.05).

The results for hinge region mutant viruses are shown in Table 5.2 and graphically in Figure 5.3 (Butrapet et al., 2011). Hinge mutant viruses with mutations at AA 135 were chosen for IT inoculation experiments because they showed variable replication kinetics in C6/36 and Vero cultured cells compared to 30P-NBX (Butrapet et al., 2011). With the exception of L135M, each of the mutant viruses had significantly lower mosquito infection rates than 30P-NBX. Additionally, sequencing the E protein of L135G virus after replication in mosquito tissues revealed that the engineered mutation was unchanged and no compensatory mutations were acquired.

Table 5.2. Infectivity of hinge region mutant viruses after IT inoculation in *A. aegypti* mosquitoes.

Virus	Exps.[a]	Negative	Positive	Total	% infected	p value[b]	Significance[c]
30P-NBX	6	8	149	157	94.90%	n/a	n/a
L135W	5	141	21	164	12.80%	<0.0001	*
L135G	4	25	88	113	77.88%	<0.001	*
L135K	3	16	73	89	82.02%	0.0228	*
L135M	3	8	88	94	93.62%	>0.05	none

[a] Number of experimental repetitions.
[b] Significance was determined by chi-square analysis (p value ≤ 0.05) and is marked by asterisks.
[c] P value ≤ 0.05 is marked by *.

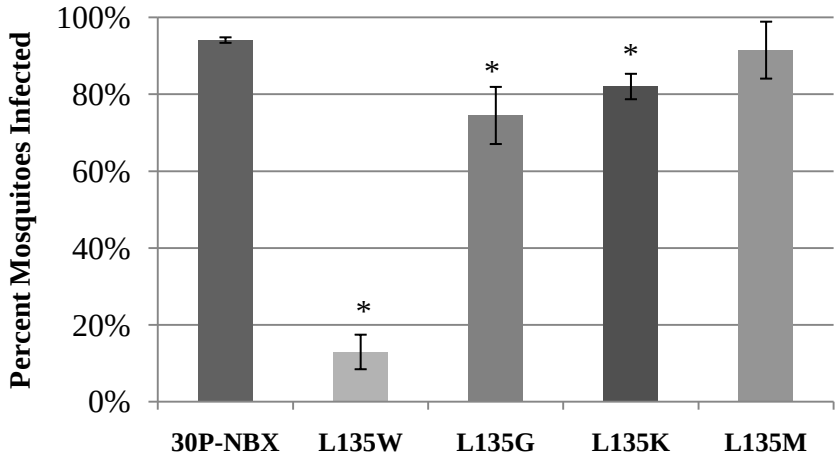

Figure 5.3. Infectivity rates of hinge region mutant viruses after IT inoculation in *A. aegypti* mosquitoes. Infection of adult mosquitoes by IT inoculation was evaluated via IFA assay for DENV2 E protein in head tissues 7 days post. Percent infectivity rates are the same numbers presented in Table 5.2. Chi-square analysis was used to compare each mutant virus to 30P-NBX (* p value ≤ 0.05).

IT inoculation of viruses with mutations in the E protein produces results with a relatively high degree of variation as evidenced by the standard deviations presented in Table 5.3; the data shown in this table are the same as in Tables 5.1 and 5.2. 30P-NBX infection rates were relatively stable after each IT inoculation experiment and this low variance made it difficult to statistically compare infectivity rates via student's t test with mutant viruses that have high variance. The student's t test, unequal variance was used to

analyze the data in Butrapet *et al.* (2011) and showed that mutant L135G is not significantly different from 30P-NBX (p value of 0.052). This value is technically not considered significant although the raw data show there is a difference between infectivity rates. Therefore, in this dissertation, chi-square analysis for proportions was utilized because it takes into account the raw data shown in Tables 5.1 and 5.2. Our null hypothesis states that mosquito infectivity rates are independent of mutations in the E protein. This improved the significance level between 30P-NBX and mutant viruses without considerably changing the conclusions.

Table 5.3. Infectivity rates of fusion peptide and hinge region mutant viruses after IT inoculation in *A. aegypti* mosquitoes.

Virus	Exps.[a]	% Infected[b]	Std Dev.[b]
30P-NBX	13	95.70%	4.05%
G102S	5	67.14%	16.61%
G104S	5	47.93%	21.38%
G106A	4	72.90%	18.49%
G106L	4	60.19%	13.77%
L107F	4	55.86%	4.98%
L107M	4	96.47%	4.07%
F108W	3	83.04%	10.80%
L135W	5	12.80%	10.05%
L135G	4	77.88%	12.87%
L135K	3	82.02%	5.73%
L135M	3	93.62%	12.83%

[a] Number of experimental repetitions.
[b] Standard deviation of all experimental repetitions.

Discussion

IT inoculation of virus into mosquitoes has been shown to be a sensitive method for growing DENVs that cannot be cultured readily in other systems (Rosen and Gubler, 1974). Bypassing the midgut infection barrier will result in a systemic infection in the mosquito where virus will infect and amplify in tissues in contact with the hemocoel and

then spread to the head by 7 days pi. In contrast to the mosquito midgut, the types of cells and tissues outside the midgut are varied and provide a myriad of cellular environments for virus replication; including tissues in the head. Cell types outside of the midgut will likely have different degrees of susceptibility to viruses and similarly, the mutant viruses included in this study will most likely have different replication kinetics in varying cell types. Therefore, IT inoculation of mosquitoes with 30 to 50 $TCID_{50}$ of virus would be able to show differences in mutant virus infectivity of tissues outside the midgut.

Comprehensively, the results of this study show that the ability of viruses to replicate in C6/36 cells does not always correlate with the ability to infect and replicate in live adult mosquitoes. All of the fusion peptide mutants and hinge mutant L135W replicated with similar efficiency to 30P-NBX in C6/36 cells and in contrast, each of these viruses (except hinge mutant L107F) had significantly lower infection rates in live adult mosquitoes compared to 30P-NBX (Huang *et al.*, 2010). C6/36 cultured cells were isolated from Singh's *A. albopictus* larval line (Singh, 1967) for their ability to replicate DENV and Chikungunya viruses to high titers (Igarashi, 1978). The tissue origin of these cells is unknown so it is not entirely surprising that *A. aegypti* mosquito infection rates may not reflect the same susceptibility as C6/36 cells. Researchers investigating DENV infectivity phenotypes in C6/36 cells should take note.

Mutations engineered into the fusion peptide were designed to change the biochemistry of the structure and a detailed analysis and description of their phenotypic properties has been published (Huang *et al.*, 2010). Each of the fusion peptide mutants included in this study was less efficient by at least 50% at inducing cell-cell fusion in

C6/36 cells compared to 30P-NBX via the fusion from within (FFWI) assay (Huang *et al.*, 2010). This defect in fusion efficiency may have reduced the ability of these viruses to infect mosquito tissues (Table 5.1 and Figure 5.2). However, L107F infected mosquitoes at an equivalent rate to 30P-NBX (Table 5.1 and Figure 5.2). There may be different microenvironments and pH threshold requirements for virus-mediated cell membrane fusion in different mosquito tissues and this could be reflected by the different infection rates. Similarly, differences in virus replication kinetics caused by mutating the fusion peptide may account for the lower infection rates recorded at day 7 post inoculation compared to 30P-NBX. Additionally, antigen presentation in head tissues at day 7 post inoculation was variable between mutant viruses and usually less than 30P-NBX (data not shown), suggesting that these viruses are replicating at different rates. Performing a virus growth curve analysis by measuring virus RNA quantities or infectious virus titers after IT inoculation might discern answers to some of these questions. However, experiments using live mosquitoes are subject to high variability and demand the use of high numbers and multiple repetitions. This would make growth curve assays labor intensive and not very cost-effective if the replication kinetics for each one of these viruses were going to be analyzed. For this reason, mosquito infectivity was analyzed by the detection of virus antigen by IFA at a standard time post inoculation. Although reading head squash IFA samples can be subjective, simple analysis of positive and negative control slides greatly diminishes false positive/negative readings.

Mutations engineered into the hinge region were designed to evaluate which AA are determinants for DENV replication and a detailed phenotypic analysis for these viruses has been submitted for publication (Butrapet et al., 2011). L135W had

comparable replication kinetics and peak infectious virus titers to 30P-NBX in C6/36 cells (Butrapet et al., 2011), but this particular mutation produced the most significant attenuation in mosquitoes (Table 5.2 and Figure 5.3). L135G and L135K produced significantly lower peak infectious virus titers than 30P-NBX in C6/36 cells (Butrapet et al., 2011) and also had lower infection rates compared to 30P-NBX in mosquitoes, although not as drastic as L135W. L135G was the most attenuated of the hinge mutant viruses in invertebrate and mammalian cultured cells and was completely fusion defective at all pH's tested in the FFWI assay (Butrapet et al., 2011). It is interesting that this virus was able to infect mosquitoes relatively well considering its attenuation in C6/36 cells and mammalian cells and for this reason, we sequenced the E gene of L135G from an infected mosquito 7 days post IT inoculation. The L135G mutation was present without the addition of secondary mutations in the E gene showing that this virus is capable of infecting and replicating in mosquito tissues. When we tried to recover L135K after transfection in Vero cells, the lysine substitution evolved into methionine during replication in this cell type. L135M mutant virus was recovered and included in IT inoculation experiments to see how this mammalian cell adaption would influence virus fitness in live mosquitoes. Interesting, L135M had a similar mosquito infection rate compared to 30P-NBX (Table 5.2 and Figure 5.3). The results of these hinge mutant IT inoculations reveal that there are different AA requirements at position 135 for replication in invertebrate and mammalian systems.

IT inoculation of virus into mosquitoes has identified AA mutations G102S, G104S, G106A, G106L, L107M, F108W, L135W, L135G, and L135K as determinants for reduced infection of mosquito tissues outside the midgut. It should be noted that each

of these viruses were still able to infect mosquito tissues after IT inoculation and no single AA mutation completely abrogated infection. Mutations of G104 to serine and L135 to tryptophan caused the most dramatic reductions in mosquito infectivity, while mutations of L107 to phenylalanine and L135 to methionine had no effect on virus infectivity of live mosquitoes. These four mutations should be evaluated for their ability to infect mosquito midguts after oral infectious challenge.

CHAPTER 6

EXTENDED PROJECT DISCUSSION AND PROSPECTS FOR FUTURE RESEARCH

The DENV E protein is the primary determinant for initiation of host cell infection. To date, studies investigating mosquito infection determinants for DENV2 are limited. The results of the studies herein analyze the effects that AA mutations in the E protein have on infectivity of mosquitoes and cultured cells. We show that (1) DENV2 strain 16681 has a low MIR, (2) single AAs in DII of the E protein are mosquito infection determinants, (3) the FG loop in DIII of the E protein is important for mammalian cell and mosquito infection and (4) virus phenotypes in insect cell culture do not necessarily translate to live mosquitoes. These findings significantly enhance our understanding of DENV biology.

Our experiments investigating DENV2 strain 16681 infectious clone 30P-NBX infectivity for *A. aegypti* RexD strain mosquitoes reveal that this virus has a low infectivity phenotype for mosquitoes. This was in contrast to one existing report (Khin *et al.*, 1994) that suggested strain 16681 had an infection rate more similar to another virus strain (J1409) consistently used in our laboratory. Repeated mosquito challenge experiments confirm that in our system 30-NBX has a mean MIR of 33.79%. Continuous repetition of these challenge experiments highlighted the variability that exists in the laboratory when performing artificial blood-feed experiments and groups investigating mosquito infection rates with DENV should take note. Challenge experiments with 30P-

NBX require at least three repetitions, each consisting of ≥ 20 mosquitoes to obtain data with statistical relevance and even then more repetitions may be necessary to see significant differences when comparing infectivity of two DENV strains. Minimum infectious virus titers required for successful artificial challenge of mosquitoes with 30P-NBX were shown to be 6 $\log_{10}$ pfu/ml or 7 $\log_{10}$ $TCID_{50}$/ml. Infecting virus titers higher than those did not correlate with increased mosquito infection rates.

The results from Chetumal mosquito strain challenge experiments show that colonized mosquitoes can change in their susceptibility to 30P-NBX infection over time (Appendix, Table 7.8). As discussed in Chapter 3, Chetumal mosquito challenge experiments comparing 30P-NBX and K122E infectivity showed 30P-NBX had an average MIR of 38.85% after 4 experiments, each completed ca. 1 month apart from December to April of 2009-2010. Approximately 6 months later, challenge experiments comparing 30P-NBX and R120T infectivity showed 30P-NBX had an average MIR of 85.49% in 4 experiments, this time completed in the span of 1 month. Maintenance of colonized mosquitoes may need to include periodic challenge experiments with the same virus strain to verify that their susceptibility is not changing. Despite 30P-NBX having a higher average MIR in the second set of experiments compared to the first, R120T still had a significantly higher MIR compared to 30P-NBX (student's t test p value = 0.038), showing that 30P-NBX is still limited in its capacity to infect mosquito midguts compared to R120T even when mosquito susceptibility is higher.

It should be noted that the *in vitro* transcription protocol employed to make infectious virus RNA reduces the concentration of ATP (by 1/5) included in the assay in order to increase the likelihood of A-cap analog binding to the 5' end of the RNA for

translation (Contreras *et al.*, 1982). The reduced amount of ATP could have instigated the introduction of nucleotide substitutions into areas of the genome that have stretches of adenines, such as the virus genome region at nts 1300 to 1305. This may have increased the propensity of producing virus populations with mutations at E protein AA position 122, including mutations to isoleucine (codon aua) and glutamic acid (codon gaa). Transfection and passage of several mutant viruses (with mutations in different locations throughout the E protein) in Vero cells resulted in a K122I mutation. Any change to one of the three nucleotides in the codon encoding for lysine at 122 (aaa) can result in 6 potential non-synonymous substitutions (isoleucine, threonine, asparagine, glutamine, arginine, and glutamic acid) and K122I (nt substitution a1301u) and K122E (nt substitution a1300g) were the only AA mutations selected for at this position. It is unclear how K122I stabilizes E protein mutant viruses *in vitro* at 37°C (see VEPGΔ temperature sensitivity, Chapter 4). Even more interesting is that a mixed K122K/E mutation was identified in 30P-NBX strain 16681 after passage in Vero cells (C.Y-H. Huang personal communication). This mutation was acquired during passage in cultured cells and not by manipulation via molecular biology techniques, indicating that this region of DII is structurally important to replication in mammalian cells. Regardless of whether the cloning process produced virus populations with mutations at position 122, results from the serial passage experiments show that the K122E mutation was specific for adaptation to replication in mosquito midgut cells.

Identifying the specific mechanism by which mutations to 122 and 120 are enhancing midgut infection may be complicated. Cell culture assays used to investigate the virus life cycle are difficult to translate to the mosquito midgut. The limitations to

our mosquito midgut-virus attachment assay were discussed in Chapter 4. Experiments can build on this assay. Viruses with extremely low MIRs like VEPGΔ or K305/7/10E might show more significant differences in attachment compared to 30P-NBX and K122E, and would suggest that our assay can in fact show differences in virus attachment to midgut cells. A more robust method of washing midguts after removal of the blood-meal may also help resolve differences in this assay. Since the only difference between K122E and 30P-NBX is a single nucleotide and AA change in the E gene, it can be assumed that there will be no differences in genome replication between the two viruses. Therefore, measuring the amount of negative strand viral RNA at short time intervals after blood-feeding could lead to inferences about the amount of virus entering midgut cells. Additionally, using radiolabeled virus in a midgut attachment assay might enable us to show differences in midgut cell binding between viruses, although this might be hampered by the necessity of using fresh virus for mosquito challenge experiments. Radiolabeling virus would involve purification steps and these could negatively affect virus binding. It is unclear by what mechanism freeze-thawing virus reduces midgut infection but it is most likely influencing virus attachment and entry, which would preclude being able to store radiolabeled virus after its production and purification.

There has been no published evidence to suggest that DENV is binding to glycosaminoglycans (GAGs) in mosquitoes. Heparan sulfate has been shown to be a determinant of mammalian cell infection (Chen *et al.*, 1997; Germi *et al.*, 2002; Hung *et al.*, 1999), but not important for attachment and infection of invertebrate C6/36 cells (Hung *et al.*, 2004). Heparan sulfate and chondroitin sulfate can bind circumsporozoite protein, the major surface protein of *Plasmodium*, and both molecules have been

identified in the midguts and salivary glands of *Anopheles stephensi* mosquitoes (Dinglasan *et al.*, 2007; Sinnis *et al.*, 2007). It is postulated that heparan sulfate is also present in *Aedes aegypti* mosquitoes considering that the genes necessary to produce heparan sulfate in *Anopheles stephensi* have homologs present in the *A. aegypti* genome (Sinnis *et al.*, 2007). Therefore, it is conceivable that DENV can also bind to negatively charged heparan sulfate on mosquito midgut cells via positively charged surfaced exposed AA. K122E and R120T mutations remove a positive charge on a surface exposed region in the E protein and could potentially reduce virion binding to GAGs in the mosquito midgut. If heparan sulfate plays no role in attachment and entry of DENV into midgut cells, non-specific binding to heparan sulfate could sequester virus and prohibit it from entering cells. This could happen by either preventing virus from attaching to a primary or secondary receptor or by sequestering virus on non-permissive cells in the midgut. Similar to the mutant virus engineered by Prestwood and colleagues (2008), which had reduced heparan sulfate binding affinity and therefore increased serum half-life, K122E and R120T mutant viruses may have reduced affinity for heparan sulfate in the mosquito midgut, which could in turn increase midgut infectivity rates and enhance virus spread throughout the midgut (two phenotypes not characteristic of 30P-NBX). DENV binding to heparan sulfate and midgut cell receptors are most likely not differentiated by our midgut-virus attachment assay. Potentially, RNAi can be used to knock down heparan sulfate in adult mosquitoes. By identifying genes that are necessary for the biosynthetic processing of heparan sulfate in *A. aegypti* mosquitoes and targeting them via RNAi, mosquitoes with low levels of heparan sulfate can be challenged orally with our viruses (Brackney, Foy, and Olson, 2008; Dinglasan *et al.*, 2007; Sinnis *et al.*,

2007). If heparan sulfate is negatively affecting 30P-NBX infection of midguts, 30P-NBX MIRs should increase in these mosquitoes and be more comparable to mutants K122E and R120T. Similarly, infection rates for K122E and R120T would be unaffected by RNAi knockdown of heparan sulfate.

Engineering more AA mutations into the DII region of the E protein could provide information about this region. It was interesting that reversion of AA E123 and not AA 122 occurred in the transfection, passage, and growth curve assays for double mutant K122/3E, and that serial passage of 30P-NBX in mosquito midguts selected for virus with a mutation at K122. Engineering a single K123E mutation could show if the loss of a positively charged residue in this region is sufficient to enhance mosquito infectivity similar to K122E. Introduction of K122/123/128E mutations into the infectious clone was lethal for virus replication in C6/36 and Vero cells, indicating that too many AA mutations with charge changes in this region is lethal (C.Y-H. Huang unpublished data). It would also be interesting to see if engineering both R120T and K122E into the infectious clone would cause temperature sensitivity at 37°C, similar to K122/3E, or lethality similar to the triple mutant. The identification of mosquito infection determinants in this AA 120 to 130 region in DII is new to the field and is a fruitful area for future exploration.

Shresta et al (2006) passaged non-mouse adapted DENV2 strain PL046 in AG129 mice and C6/36 cells and identified two AA mutations (N124D and K128E) in the E protein that mediated viscerotropic disease and high viremia in mice. DENV2 strain 16681 is not adapted to mice so it could be valuable to see what type of disease K122E will produce in AG129 mice. If the virus pathology is similar to that of the N123/K128E

mutant, this system could provide a unique and much desired model to study dengue virus transmission. Since an infectious clone is also available for strain PL046, a K122E mutation could be engineered into this virus and analyzed in the model created by Shresta and colleagues (2006). Conversely, it might be instructive to introduce the N124D/K128E mutations into our infectious clone and assess the ability of that mutant virus to infect mosquitoes.

The structural protein prM prevents premature fusion of the E protein with intracellular host cell membranes during E protein maturation from the ER through the secretory pathway. Host cell furin cleaves pr from M after virions exit the trans-Golgi network and instead of dissociating immediately after cleavage, the pr protein stays bound to the E protein to prevent membrane fusion until the particles are released from the infected cell (Yu *et al.*, 2008). Pr protein residues E46 and D47 are suggested to contact E protein residue K52 via charge interactions due to their close proximity (atoms less than 4.5 Å apart) (Li *et al.*, 2008a). Published E protein structures show that K122 does not seem to interfere with prM/E contacts at low or neutral pH (Figure 6.1 B and C). However, K64 neighbors both K122 and R120 forming a patch of positively charged AA in DII (3.12 B and C). It is difficult to say how these AA are influencing this part of the virus life cycle, if at all. It is interesting to speculate that if 122E were to interact with K64 via charge interactions, this could potentially reduce the affinity of pr for E at this protein-protein contact point, thus increasing the amount of mature K122E virus production compared to 30P-NBX. This does not give any insight as to how mutation of R120 can influence pr binding and it is difficult to speculate on how this AA change would influence maturation in relation to pr. It might be beneficial to measure how much

pr protein is still associated with K122E and 30P-NBX after amplification in C6/36 cells (taking into consideration these cells are used to produce fresh virus for blood-feed experiments). Also, K122 does not seem to interfere with contact points in the trimer necessary for membrane fusion; it is located on the outside of DII in the fusion conformation (Modis *et al.*, 2004) (Figure 6.1 A), suggesting it could potentially be bound by a host cell receptor without obstructing the fusion process. Currently, it is unclear how receptor binding influences the conformational changes necessary for fusion. Obviously, these points are based entirely on conjecture and are pointed out in this dissertation solely for discussion about how K122E and R120T mutations are influencing the virus life cycle. Just as it cannot be denied that K122 may be involved with important contacts during the molecular conformational transitions necessary to generate the snapshot structures in the published literature, it also cannot be denied that K122 may have no influence on any of the molecular conformational changes necessary for fusion or maturation via prM.

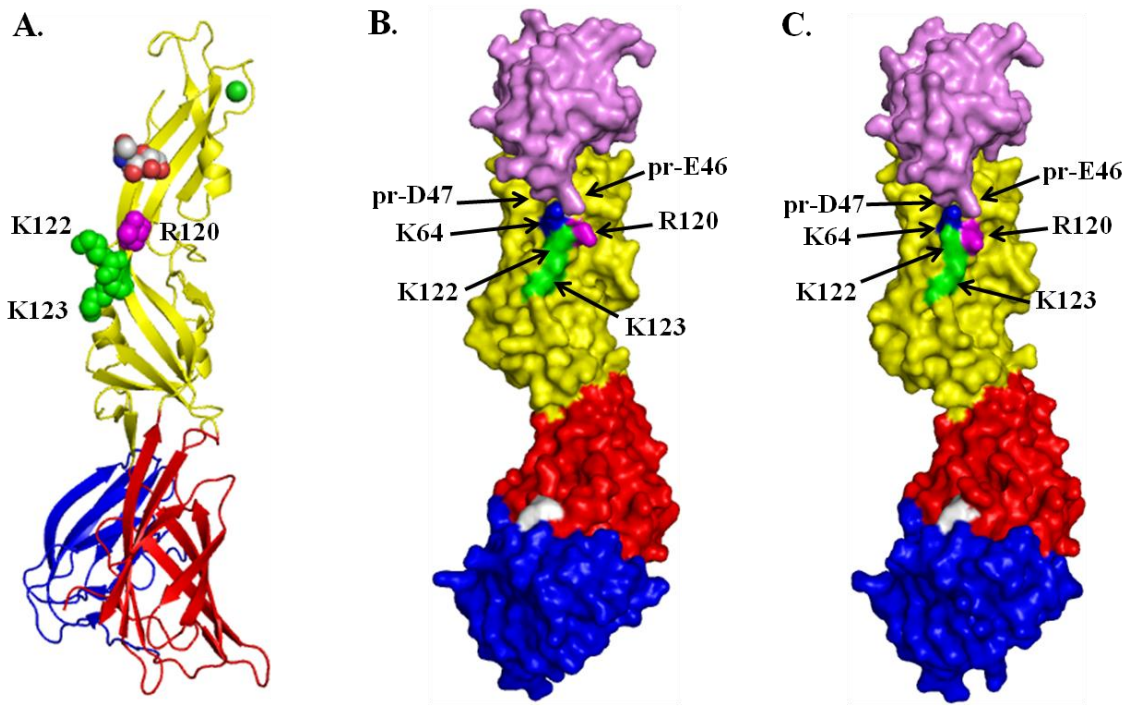

Figure 6.1. DENV2 E protein structures and location of relevent AAs. (A) Side view of the DENV2 E protein monomer in post fusion intermediate conformation (protein database bank ID: 1ok8) with D1 in red, DII in yellow, and DIII in blue. K122 and K123 are colored green, and R120 is colored magenta. (B) Side-view of pymol surface rendition of the E monomer complexed with pr at low pH post furin cleavage (protein database bank ID: 3c5x) with the same coloring as in A with two exceptions: K64 is colored blue and the pr protein is colored purple. (C) Side-view of pymol surface rendition of the E monomer complexed with pr at neutral pH post furin cleavage (protein database bank ID: 3c5x) with the same coloring as in B. Protein structures were obtained from the protein database bank and were rendered in Polyview-3D (Porollo, Adamczak, and Meller, 2004).

There are nine AA differences in the E protein between DENV2 strains 16681 and J1409 (Appendix, Figure 6.3) and one of them has been investigated previously. Pierro *et al.* (2006) showed that continuous passage of J1409 in C6/36 cells resulted in an AA mutation from isoleucine to methionine at E protein position 6 (16681 has methionine at this position). This mutation was introduced into the J1409 infectious clone and, while it was observed to form reduced syncytia in C6/36 cells, it had no effect on mosquito infectivity via oral infectious blood-meal (Pierro *et al.*, 2006). It is interesting that J1409-ic (has I6) and 30P-NBX (has M6) caused similar CPE in C6/36

cells despite I6 being suggested as a determinant for syncytium formation in this cell type (Chapter 3 discussion). In addition, VEPGΔ produced hardly any CPE in C6/36 cells compared to 30P-NBX (data not shown). Infectious virus titers produced in this cell type were similar for both viruses so it is unclear how mutations in areas like the FG loop and in DII of the E protein cause attenuation or increase of CPE in C6/36 cells.

As discussed in the Chapter 3, R120T was the most interesting AA difference between DENV2 strains 16681 and J1409-ic given its surface exposed location and difference in charge. This mutation was introduced to make the 30P-NBX E protein "like" J1409, so future studies comparing mutant R120T and strain J1409 in parallel should be pursued. This mutation is also relevant to other DENV2 strains, since threonine at position 120 is present in E protein AA sequence alignments of 53 other DENV2 strains (data not shown). The 16681 cDNA infectious clone used in this study is much easier to manipulate than the one created by Pierro *et al.* (2006) and, combined with the R120T mutation introduced, provides a useful tool to analyze how virus genetics contribute to mosquito infectivity. Additionally, both K122E and R120T viruses can be used in studies requiring a DENV2 strain that consistently infects a high proportion of mosquitoes (i.e. challenging transgenic mosquitoes that have engineered resistance to DENV).

The FG loop has widely been assumed to have receptor binding properties, and while this still may be the case, the ability of the VEPGΔ virus to infect mammalian and insect cell cultures and live mosquitoes suggests it is not the only structure required for attachment and entry into cells. In contrast to the findings of Hung *et al.* (2004), we showed that the FG loop was important for infection of mammalian cells but dispensible

for infection of C6/36 cells. A common theme suggested by the results of this study is that the FG loop is more important for general structure than for a sequence specific function. Furthermore, although VEPGΔ was significantly less efficient at infecting mosquito midguts, consensus sequencing of this virus during the serial passage experiment verified that it is capable of infecting mosquito midguts without the acquisition of compensatory mutations. All of the mosquito-borne flaviviruses have an extended FG loop while the tick-borne flaviviruses do not so it is interesting that deletion of this loop does not completely abrogate mosquito infection. These results point to other structures in the E protein being involved with attachment and entry into host cells and further illustrates the fact that the E protein is extremely dynamic in its ability to compensate for mutations to its sequence and structure.

These studies show that virus phenotypes in cell culture do not necessarily translate *in vivo*. A large number of viruses in this study were engineered to have mutations in the E protein and most of them were able to infect and replicate in C6/36 cells similar to the 30P-NBX. However, after IT inoculation into adult mosquitoes, many of these viruses were shown to have reduced infectivity rates compared to 30P-NBX. Researchers investigating DENV phenotypes should not assume that there is any connection between virus infectivity of C6/36 cells and live mosquitoes. Without directly comparing the two in the laboratory, assumptions in the published literature can only be regarded as conjecture.

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APPENDIX

Table 7.1. 30P-NBX MIR data in *A. aegypti* RexD strain mosquitoes.

Infection Medium	YE-LAH	L15	L15
Titer method	Plaque	Plaque	$TCID_{50}$
Titer range/ml[a]	6.3-7.52	6.67-8.27	7.45-9.45
Positive midguts	40	166	172
Total midguts	553	607	517
MIR	7.23%[b]	27.35%[c]	33.27%[c]

[a] Log_{10} pfu or $TCID_{50}$/ml
[b] MIR is significantly different from L15 plaque by student's t test
[c] No statistical difference between L15 plaque and L15 $TCID_{50}$ MIRs

Table 7.2. 30P-NBX MIR in *A. aegypti* Thailand strain mosquitoes.

Exp. #	Log_{10} pfu/ml	Midgut Positive	Total Midguts	MIR
1	8.03	14	17	82.35%
2	8	1	14	7.14%
3	7.82	5	35	14.29%
4	7.6	29	41	70.73%
			Average	43.63%
			Std Dev	38.41%
			Std Error	19.21%

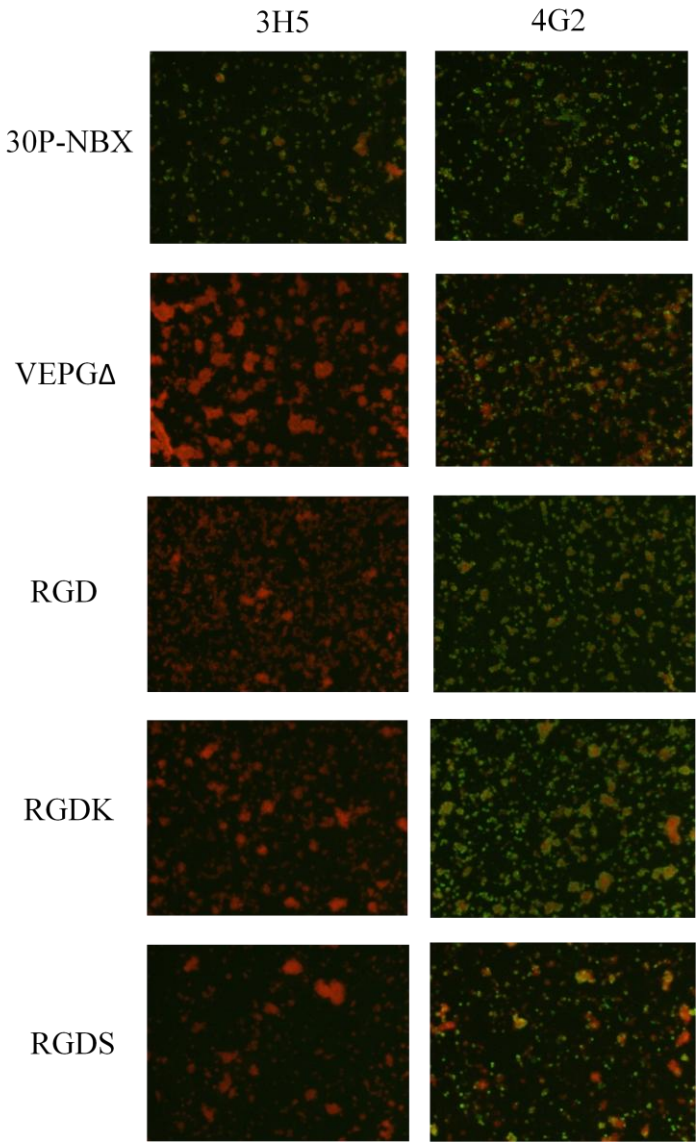

Figure 7.1. DENV2 FG loop mutant virus infected C6/36 cells stained with MAb 3H5 or 4G2. C6/36 cells were infected with each virus (specified on left) at a MOI of 0.001, fixed in acetone on day 7 pi, and stained with MAb 3H5 (left panel) or 4G2 (right panel).

Table 7.3. Viruses included in the DENV2 and flavivirus E protein AA sequence alignment.

DENV2[a] Genotype	Virus Strain	Country of Origin	GenBank accession no.
Asian 1	16681	Thailand	U87411
	PUO-218	Thailand	D00345
	M1	Malaysia	X15434
Asian 2	New Guinea C	New Guinea	AF038403
	PL046	Taiwan	ABQ18242
	K0005	Thailand	AY158336
Asian/American	Jamaica 1409	Jamaica	M20558
	13382-Tizimin	Mexico	AY449684
	China-04	China	AF119661
American	PR159	Puerto Rico	L10046
	Ven2	Venezuela	AF100465
	IQT2913	Peru	AF100468
Cosmopolitan	SL714	Sri Lanka	L10055
	CAMR5	Australia	AF410370
	CAMR16	Saudi Arabia	AF410378
Sylvatic	Guinea-81-ON33974	Guinea	AF231719
	IC80-DAKAr578	Ivory Coast	AF231718
	Mal70-P8-1407	Malaysia	AF231717
Flavivirus[b]			
DENV2	16681		U87411
DENV1	16007		AF180818
DENV3	PhMH-J1-97		AA549486
DENV4	Thailand-1985		AAV49746
YFV	Asibi		AAT58050
	17D		AAX47570
JEV	Nakayama		AAB40688
MVEV	NG156		ABM65594
WNV	NY99		AAF20092
SLEV	Laderle		ACA28960
TBEV	Neudoerfl		AAA02739
POWV	LB		AAA86870

[a] Viruses designated in the alignment below are in the order of this Table and begin with a virus abbreviation followed by the virus strain (*e.g.* DENV2 [D2-], DENV1 [D1-], DENV3 [D3-], DENV4 [D4-], YFV-, JEV-, MVEV-, WNV-, SLEV-, TBEV-, and POWV-). E protein AAs were aligned and colored using ClustalX. One blank row was inserted in between DENV2 Sylvatic strain Mal70-P8-1407 and DENV2 Asian 1 strain 16681. Virus strain 16681 was included a second time above DENV1 for ease of comparison.

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D2-16681          MRCIGMSNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-PUO-218        MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-M1             MRCIGISNRDLVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-New Guinea C   MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-PL046          MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQLATLRKYC 60
D2-K0005          MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-Jamaica 1409   MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-13382/Tizimin  MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-China-04       MRCIGVSNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPVTLRKYC 60
D2-PR159          MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-Ven2           MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-IQT2913        MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-SL714          MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-CAMR5          MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-CAMR16         MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D2-DAKAr578       MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQLATLRKFC 60
D2-PM33974        MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKFC 60
D2-P8-1407        MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKFC 60

D2-16681          MRCIGMSNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
D1-16007          MRCVGIGNRDFVEGLSGATWVDVVLEHGSCVTTMAKNKPTLDIELLKTEVTNPAVLRKLC 60
D3-PhMH-J1-97     MRCVGVGNRDFVEGLSGATWVDVVLEHGGCVTTMAKNKPTLDIELQKTEATQLATLRKLC 60
D4-Thailand/1985  MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELIKTTAKEVALLRTYC 60
YFV-Asibi         AHCIGITDRDFIEGVHGGTWVSATLEQDKCVTVMAPDKPSLDISLETVAIDGPAEARKVC 60
YFV-17D           AHCIGITDRDFIEGVHGGTWVSATLEQDKCVTVMAPDKPSLDISLETVAIDRPAEVRKVC 60
JEV-Nakayama      FNCLGMGNRDFIEGASGATWVDLVLEGDSCLTIMANDKPTLDVRMINIEAVQLAEVRSYC 60
MVEV-NG156        FNCLGMSSRDFIEGASGATWVDLVLEGDSCITIMAADKPTLDIRMMNIEATNLALVRNYC 60
WNV-NY99          FNCLGMSNRDFLEGVSGATWVDLVLEGDSCVTIMSKDKPTIDVKMMNMEAANLAEVRSYC 60
SLEV-Laderle      FNCLGTSNRDFVEGASGATWIDLVLEGGSCVTVMAPEKPTLDFKVMKMEATELATVREYC 60
TBEV-Neudoerfl    SRCTHLENRDFVTGTQGTTRVTLVLELGGCVTITAEGKPSMDVWLDAIYQENPAKTREYC 60
POWV-LB           TRCTHLENRDFVTGTQGTTRVSLVLELGGCVTITAEGKPSIDVWLEDIFQESPAETREYC 60
                   .*    .**:: *  * : :  .** . *:*  :  **::*. :        .  *  *

D2-16681          IEAKLTNTTTESRCPTQGEPSLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFR 120
D2-PUO-218        IEAKLTNTTTESRCPTQGEPSLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-M1             IEAKLTNTTTESRCPTLGEPSLNEEQDKRLVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-New Guinea C   IEAKLTNTTTDSRCPTQGEPSLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-PL046          IEAKLTNTTTESRCPTQGEPSLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-K0005          IEAKLTNTTTESRCPTQGEPSLKEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-Jamaica 1409   IEAKLTNTTTESRCPTQGEPSLNEEQDKRFLCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-13382/Tizimin  IEAKLTNTTTESRCPTQGEPSLNEEQDKRFICKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-China-04       IKAKLTNTTTESRCPTQGEPSLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCATFT 120
D2-PR159          IEAKLTNTTTDSRCPTQGEPTLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-Ven2           IEAKLTNTTTDSRCPTQGEPTLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-IQT2913        IEAKLTNTTTDSRCPTQGEPTLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-SL714          IEAKLTNTTTASRCPTQGEPSLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-CAMR5          IEAKLTNTTTASRCPTQGEPSLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-CAMR16         IEAKLTNTTTASRCPTQGEPSLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-DAKAr578       IEAKLTNTTTESRCPTQGEPSLVEEQDKRFVCRHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-PM33974        IEAKLTNTTTESRCPTQGEPSLVEEQDKRFVCRHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
D2-P8-1407        IEAKLTNTTTESRCPTQGEPSLVEEQDKRFVCKHSMVDRGWGNGCGLFGKGGVVTCAMFT 120

D2-16681          IEAKLTNTTTESRCPTQGEPSLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFR 120
D1-16007          IEAKISNTTTDSRCPTQGEATLVEEQDANFVCRRTFVDRGWGNGCGLFGKGSLITCAKFK 120
D3-PhMH-J1-97     IEGKITNVTTDSRCPTQGEAILPEEQDQNYVCKHTYVDRGWGNGCGLFGKGSLVTCAKFQ 120
D4-Thailand/1985  IEASISNITTATRCPTQGEPYLKEEQDQQYICRRDVVDRGWGNGCGLFGKGGVVTCAKFS 120
YFV-Asibi         YNAVLTHVKINDKCPSTGEAHLAEENEGDNACKRTYSDRGWGNGCGLFGKGSIVACAKFT 120
YFV-17D           YNAVLTHVKINDKCPSTGEAHLAEENEGDNACKRTYSDRGWGNGCGLFGKGSIVACAKFT 120
JEV-Nakayama      YHASVTDISTVARCPTTGEAHNEKRADSSYVCKQGFTDRGWGNGCGLFGKGSIDTCAKFS 120
MVEV-NG156        YAATVSDVSTVSNCPTTGESHNTKRADHNYLCKRGVTDRGWGNGCGLFGKGSIDTCAKFT 120
WNV-NY99          YLATVSDLSTKAACPTMGEAHNDKRADPAFVCRQGVVDRGWGNGCGLFGKGSIDTCAKFA 120
SLEV-Laderle      YEATLDTLSTVARCPTTGEAHNTKRSDPTFVCKRDVVDRGWGNGCGLFGKGSIDTCAKFT 120
TBEV-Neudoerfl    LHAKLSDTKVAARCPTMGPATLAEEHQGGTVCKRDQSDRGWGNHCGLFGKGSIVACVKAA 120
POWV-LB           LHAKLTNTKVEARCPTTGPATLPEEHQANMVCKRDQSDRGWGNHCGFFGKGSIVACAKFE 120
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D2-16681           CKKN--MEGKVVQPENLEYTIVITPH-SGEEHAVGNDTGKHGKEIK----ITPQSSTTEA 173
D2-PUO-218         CKKN--MEGKVVQPENLEYTIVVTPH-SGEEHAVGNDTGKHGKEIK----VTPQSSITEA 173
D2-M1              CKKN--MEGKIVQPENLEYTIVVTPH-SGEEHAVGNDTGKHGKEIK----ITPQSSITEA 173
D2-New Guinea C    CKKN--MKGKVVQPENLEYTIVITPH-SGEEHAVGNDTGKHGKEIK----ITPQSSITEA 173
D2-PL046           CKKN--MEGKIVQPENLEYTIVITPH-SGEEHAVGNDTGKHGKEIK----ITPQSSITEA 173
D2-K0005           CKKN--MEGKIVQPENLEYTIVVTPH-SGEEHAVGNDTGKHGKEIK----VTPQSSITEA 173
D2-Jamaica 1409    CKKN--MEGKVVLPENLEYTIVITPH-SGEEHAVGNDTGKHGKEIK----ITPQSSITEA 173
D2-13382/Tizimin   CKKN--MEGKVVQPENLEYTIVITPH-SGEEHAVGNDTGKHGKEIK----ITPQSSITEA 173
D2-China-04        CKKN--MEGKIVQPENLEYTIVITPH-SGEEHAVGNDTGKHGKEIK----ITPQSSITEA 173
D2-PR159           CKKN--MEGKIVQPENLEYTVVITPH-SGEEHAVGNDTGKHGKEVK----ITPQSSITEA 173
D2-Ven2            CKKN--MEGKIVQPENLEYTVVITPH-SGEEHAVGNDTGKHGKEVK----ITPQSSITEA 173
D2-IQT2913         CKKN--MEGKIVQPENLEYTVVITPH-SGEEHAVGNDTGKHGKEVK----ITPQSSITEA 173
D2-SL714           CKKN--MEGKIVQPENLEYTIVITPH-SGEENAVGNDTGKHGKEIK----VTPQSSITEA 173
D2-CAMR5           CKKN--MEGKIVQPENLEYTIVVTPH-SGEENAVGNDTGKHGKEIK----VTPQSSITEA 173
D2-CAMR16          CKKN--MEGKIVQPENLEYTIVITPH-SGEENAVGNDTGKHGKEIK----VTPQSSITEA 173
D2-DAKAr578        CLKK--MEGKVVQPENLEYTIVITPH-SGEEHAVGNDTGKHGKEVK----ISPQSSIAEA 173
D2-PM33974         CLKK--MEGKVVQPENLEYTIVITPH-SGEEHAVGNDTGKHGKEVK----ITPQSSIAEA 173
D2-P8-1407         CLKN--MEGKVVQPENLEYTIVITPH-SGEEHAVGNDTGKHGKEVK----ITPQSSITEA 173

D2-16681           CKKN--MEGKVVQPENLEYTIVITPH-SGEEHAVGNDTGKHGKEIK----ITPQSSTTEA 173
D1-16007           CVTK--LEGKIVQYENLKYSVIVTVH-TGDQHQVGNETTEHGTTAT----ITPQAPTSEI 173
D3-PhMH-J1-97      CLES--IEGKVVQHENLKYTVIITVH-TGDQHQVGNET--QGVTAE----ITPQASTVEA 171
D4-Thailand/1985   CSGK--ITGNLVQIENLEYTVVVTVH-NGDTHAVGNDTSNHGVTAT----ITPRSPSVEV 173
YFV-Asibi          CAKS--MSLFEVDQTKIQYVIRAQLHVGAKQENWNTDIKTLKFDAL--------SGSQEA 170
YFV-17D            CAKS--MSLFEVDQTKIQYVIRAQLHVGAKQENWNTDIKTLKFDAL--------SGSQEV 170
JEV-Nakayama       CTSK--AIGRTIQPENIKYEVGIFVHGTTTSENHGNYSAQVGASQAAKFTVTPNAPSITL 178
MVEV-NG156         CSSS--AAGRLILPENIKYEVGIFVHGSTDSTSHGNYSTQIGANQAARFTISPNAPAITA 178
WNV-NY99           CSTK--AIGRTILKENIKYEVAIFVHGPTTVESHGNYSTQVGATQAGRFSITPAAPSYTL 178
SLEV-Laderle       CKNK--ATGKTILRENIKYEVAIFVHGSTDSTSHGNYFEQIGKNQAARFTISPQAPSFTA 178
TBEV-Neudoerfl     CEAKKKATGHVYDANKIVYTVKVEPH--TGDYVAANETHSGRKTAS----FTISSEKTIL 174
POWV-LB            CEEAKKAVGHVYDSTKITYVVKVEPH--TGDYLAANETNSNRKSAQ----FTVASEKVIL 174
                   *              :: * :    *         .                 :

D2-16681           ELTGYGTVTMECSPRTGLDFNEMVLLQMENK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-PUO-218         ELTGYGTVTMECSPRTGLDFNEMVLLQMENK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-M1              ELTGYGTVTMECSPRTGLDFNEMVLLQMENK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-New Guinea C    ELTGYGTVTMECSPRTGLDFNEMVLLQMENK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-PL046           ELTGYGTVTMECSPRTGLDFNEMVLLQMENK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-K0005           ELTGYGTVTMECSPRTGLDFNEMVLLQMENK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-Jamaica 1409    ELTGYGTVTMECSPRTGLDFNEMVLLQMEDK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-13382/Tizimin   ELTGYGTVTMECSPRTGLDFNEMVLLQMEDK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-China-04        ELTGYGTVTMECSPRTGLDFNEMVLLQMEDK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-PR159           ELTGYGTVTMECSPRTGLDFNEMVLLQMKDK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-Ven2            ELTGYGTVTMECSPRTGLDFNEMVLLQMEDK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-IQT2913         ELTGYGTVTMECSPRTGLDFNEMVLLQMEDK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-SL714           ELTGYGTVTMECSPRTGLDFNEMVLLQMENK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-CAMR5           ELTGYGTVTMECSPRTGLDFNEMVLLQMENK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-CAMR16          ELTGYGTVTMECSPRTGLDFNEMVLLQMENK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-DAKAr578        ELTDYGTITMECSPRTGLDFNEMVLLQMESK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-PM33974         ELTGYGTITMECSPRTGLDFNEMVLLQMESK------AWLVHRQWFLDLPLPWLPGADTQ 227
D2-P8-1407         ELTGYGTITMECSPRTGLDFNEMVLLQMEKK------AWLVHRQWFLDLPLPWLPGADTQ 227

D2-16681           ELTGYGTVTMECSPRTGLDFNEMVLLQMENK------AWLVHRQWFLDLPLPWLPGADTQ 227
D1-16007           QLTDYGTLTLDCSPRTGLDFNEMVLLTMKER------SWLVHKQWFLDLPLPWTSGASTS 227
D3-PhMH-J1-97      ILPEYGTLGLECSPRTGLDFNEMILLTMKNK------AWMVHRQWFFDLPLPWTSGATTE 225
D4-Thailand/1985   ELPDYGELTLDCEPRSGIDFNEMILMKMKKK------TWLVHKQWFLDLPLPWTAGADTS 227
YFV-Asibi          EFTGYGKATLECQVQTAVDFGNSYIAEMEKE------SWIVDRQWAQDLTLPWQSGSGG- 223
YFV-17D            EFTGYGKATLECQVQTAVDFGNSYIAEMETE------SWIVDRQWAQDLTLPWQSGSGG- 223
JEV-Nakayama       KLGDYGEVTLDCEPRSGLNTEAFYVMTVGSK------SFLVHREWFHDLALPWTPPSST- 231
MVEV-NG156         KMGDYGEVAVECEPRSGLNTEAYYVMTIGTK------HFLVHREWFNDLLLPWTSPSST- 231
WNV-NY99           KLGEYGEVTVDCEPRSGIDTNAYYVMTVGTK------TFLVHREWFMDLNLPWSSAGST- 231
SLEV-Laderle       DMGEYGTVTIDCEARSGINTEDYYVFTVKEK------SWLVNRDWFHDLNLPWTSPATT- 231
TBEV-Neudoerfl     TMGEYGDVSLLCRVASGVDLAQTVILELDKTVEHLPTAWQVHRDWFNDLALPWKHEGAQ- 233
POWV-LB            RLGDYGDVSLTCKVASGIDVAQTVVMSLDSSKDHLPSAWQVHRDWFEDLALPWKHKDNQ- 233
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D2-16681           GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-PUO-218         GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-M1              GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAIHTALTGATEIQMSSG----NLLFTGHL 283
D2-New Guinea C    GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-PL046           GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-K0005           GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-Jamaica 1409    GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-13382/Tizimin   GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-China-04        GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-PR159           GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-Ven2            GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-IQT2913         GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-SL714           GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-CAMR5           GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-CAMR16          GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D2-DAKAr578        GSNWIQKEMLVTFKNPHAKRQDVVVLGSQEGAMHTALTGATEIQMSLG----NILFMGHL 283
D2-PM33974         GSNWIQKEMLVTFKNPHAKRQDVVVLGSQEGAMHTALTGATEIQMSLG----NILFMGHL 283
D2-P8-1407         GSNWIQKEMLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283

D2-16681           GSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSG----NLLFTGHL 283
D1-16007           QETWNRQDLLVTFKTAHAKKQEVVVLGSQEGAMHTALTGATEIQTSGT----TTIFAGHL 283
D3-PhMH-J1-97      TPTWNKKELLVTFKNAHAKKQEVVVLGSQEGAMHTALTGATEIQTSGG----TSIFAGHL 281
D4-Thailand/1985   EVHWNHKERMVTFKVPHAKRQDVTVLGSQEGAMHSALTGATEVDSGDG----NHMFAGHL 283
YFV-Asibi          --VWREMHHLVEFEPPHAATIRVLALGNQEGSLKTALTGAMRVTKDTNDNNLYKLHGGHV 281
YFV-17D            --VWREMHHLVEFEPPHAVTIRVLALGNQEGSLKTALTGAMRVTKDTNDNNLYKLHGGHV 281
JEV-Nakayama       --AWRNRELLMEFEEAHATKQSVVALGSQEGGLHQALAGAIVVEYSSS----VKLTSGHL 285
MVEV-NG156         --EWRNREILMEFEEPHATKQSVVALGSQEGALHQALAGAVPVEFASST---LKLTSGHL 286
WNV-NY99           --VWRNRETLMEFEEPHATKQSVIALGSQEGALHQALAGAIPVEFSSNT---VKLTSGHL 286
SLEV-Laderle       --DWRNRETLVEFEEPHATKQTVVALGSQEGALHTALAGAIPATVSSST---LTLQSGHL 286
TBEV-Neudoerfl     --NWNNAERLVEFGAPHAVKMDVYNLGDQTGVLLKALAGVPVAHIEGTK---YHLKSGHV 288
POWV-LB            --DWNSVEKLVEFGPPHAVKMDVFNLGDQTAVLLKSLAGVPLASVEGQK---YHLKSGHV 288
                     *   . :: *  .**    *  **.* . :  :*:*.             :  **:

D2-16681           KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D2-PUO-218         KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D2-M1              KCRLRMDKLQLKGMSYSMCTG-KFKVVEEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D2-New Guinea C    KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D2-PL046           KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVVRVQYEGDGSPCKIPFEIMD- 341
D2-K0005           KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVIRVQYEGDGSPCKVPFEIMD- 341
D2-Jamaica 1409    KCRLRMDKLQLKGMSYSMCTG-KFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D2-13382/Tizimin   KCRLRMDKLQLKGMSYSMCTG-KFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEITD- 341
D2-China-04        KCRLRMDKLQLKGMSYSMCTG-KFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D2-PR159           KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D2-Ven2            KCRLRMDKLQLKGMSYSMCTG-KFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D2-IQT2913         KCRLRMDKLQLKGMSYSMCTG-KFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D2-SL714           KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D2-CAMR5           KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D2-CAMR16          KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D2-DAKAr578        KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVIRVQYEGDDSPCKIPFEIMD- 341
D2-PM33974         KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVIRVQYEGDDSPCKIPFEIMD- 341
D2-P8-1407         KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVIRVQYEGEGAPCKIPFEIMD- 341

D2-16681           KCRLRMDKLQLKGMSYSMCTG-KFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMD- 341
D1-16007           KCRLKMDKLTLKGMSYVMCTG-SFKLEKEVAETQHGTVLVQVKYEGTDAPCKIPFSTQD- 341
D3-PhMH-J1-97      KCRLKMDKLELKGMSYAMCLN-TFVLKKEVSETQHGTILIKVEYKGEDAPCKIPFSTED- 339
D4-Thailand/1985   KCKVRMEKLRIKGMSYTMCSG-KFSIDKEMAETQHGTTVVKVKYEGTGAPCKVPIEIRD- 341
YFV-Asibi          SCRVKLSALTLKGTSYKMCTD-KMSFVKNPTDTGHGTVVMQVKVP-KGAPCKIPVIVADD 339
YFV-17D            SCRVKLSALTLKGTSYKICTD-KMFFVKNPTDTGHGTVVMQVKVS-KGAPCRIPVIVADD 339
JEV-Nakayama       KCRLKMDKLALKGTTYGMCTE-KFSFAKNPADTGHGTVVIELSYSGSDGPCKIPIVSVAS 344
MVEV-NG156         KCRVKMEKLKLKGTTYGMCTE-KFTFSKNPADTGHGTVVLELQYTGSDGPCKIPISSVAS 345
WNV-NY99           KCRVKMEKLQLKGTTYGVCSK-AFKFLGTPADTGHGTVVLELQYTGTDGPCKVPISSVAS 345
SLEV-Laderle       KCRAKLDKVKIKGTTYGMCDS-AFTFSKNPTDTGHGTVIVELQYTGSNGPCRVPISVTAN 345
TBEV-Neudoerfl     TCEVGLEKLKMKGLTYTMCDKTKFTWKRAPTDSGHDTVVMEVTFSGT-KPCRIPVRAVAH 347
POWV-LB            TCDVGLEKLKLKGTTYSMCDKAKFKWKRVPVDSGHDTVVMEVSYTGSDKPCRIPVRAVAH 348
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D2-16681           LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIG 399
D2-PUO-218         LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIG 399
D2-M1              LEKRHVLGRLITVNPIVTE--KDSPVNVEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIG 399
D2-New Guinea C    LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIG 399
D2-PL046           LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYVIIGVEPGQLKLNWFKKGSSIG 399
D2-K0005           LEKRYVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIG 399
D2-Jamaica 1409    LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIG 399
D2-13382/Tizimin   LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIG 399
D2-China-04        LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWVKKGSSIG 399
D2-PR159           LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLDWFKKGSSIG 399
D2-Ven2            LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLDWFRKGSSIG 399
D2-IQT2913         LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGAEPGQLKLDWFKKGSSIG 399
D2-SL714           LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLSWFKKGSSIG 399
D2-CAMR5           LEKRHVLGRLITVNPIVTG--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLSWFKKGSSIG 399
D2-CAMR16          LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLSWFKKGSSIG 399
D2-DAKAr578        LEKKHVLGRLITVNPIVTE--KDNPINIEAEPPFGDSYIVIGVEPGQLKLNWFKKGSSIG 399
D2-PM33974         LEKKHVLGRLITVNPIVTE--KDSPINIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIG 399
D2-P8-1407         LEKKHVLGRLITVNPIVTE--KDSPINIEAEPPFGDSYIVIGVEPGQLKLNWFKKGSSIG 399

D2-16681           LEKRHVLGRLITVNPIVTE--KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIG 399
D1-16007           EKGATQNGRLITANPIVTD--KEKPVNIEAEPPFGESYIVVGAGEKALKLSWFKKGSSIG 399
D3-PhMH-J1-97      GQGKAHNGRLITANPVVTK--KEEPVNIEAEPPFGESNIVIGIGDKALKINWYKKGSSIG 397
D4-Thailand/1985   VNKEKVVGRIISSTPFAEN--TNSVTNIELEPPFGDSYIVIGVGDSALTLHWFRKGSSIG 399
YFV-Asibi          LTAAINKGILVTVNPIAST--NDDEVLIEVNPPFGDSYIIVGTGDSRLTYQWHKEGSSIG 397
YFV-17D            LTAAINKGILVTVNPIAST--NDDEVLIEVNPPFGDSYIIVGRGDSRLTYQWHKEGSSIG 397
JEV-Nakayama       LNDMTPVGRLVTVNPFVATSSANSKVLVEMEPPFGDSYIVVGRGDKQINQHWHKAGSTLG 404
MVEV-NG156         LNDMTPVGIMVTANPYVASSTANAKVLVEIEPPFGDSYIVVGRGDKQINHHWHKEGSSIG 405
WNV-NY99           LNDLTPVGRLVTVNPFVSVATANAKVLIELEPPFGDSYIVVGRGEQQINHHWHKSGSSIG 405
SLEV-Laderle       LMDLTPVGRLVTVNPFISTGGANNKVMIEVEPPFGDSYIVVGRGTTQINYHWHKEGSSIG 405
TBEV-Neudoerfl     GSPDVNVAMLITPNPTIEN---NGGGFIEMQLPPGDNIIYVG----ELSHQWFQKGSSIG 400
POWV-LB            GVPAVNVAMLITPNPTIET---NGGGFIEMQLPPGDNIIYVG----DLSQQWFQKGSTIG 401
                          . ::: .*       :    :* : * *:. : :*     :.  * : **::*

D2-16681           QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-PUO-218         QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-M1              QMFETTMIGAKRMAILRDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-New Guinea C    QMIETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-PL046           QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-K0005           QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-Jamaica 1409    QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-13382/Tizimin   QMFEITMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-China-04        QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-PR159           QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-Ven2            QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-IQT2913         QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-SL714           QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-CAMR5           QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-CAMR16          QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-DAKAr578        QMFETTMRGAKRMAILGDTAWDFGSIGGVFTSVGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-PM33974         QMFETTMRGAKRMAILGDTAWDLGSIGGVFTSVGKALHQVFGAIYGAAFSGVSWTMKILI 459
D2-P8-1407         QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSVGKALHQVFGAIYGVAFSGVSWTMKILI 459

D2-16681           QMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILI 459
D1-16007           KMFEATARGARRMAILGDTAWDFGSIGGVFTSMGKLVHQVFGTAYGVLFSGVSWTMKIGI 459
D3-PhMH-J1-97      KMFEATARGARRMAILGDTAWDFGSVGGVLNSLGKMVHQIFGSAYTALFSGVSWIMKIGI 457
D4-Thailand/1985   KMFESTYRGAKRMAILGETAWDFGSVGGLLTSLGKAVHQVFGSVYSTMFGGVSWMVRILI 459
YFV-Asibi          KLFTQTMKGAERLAVMGDAAWDFSSAGGFFTSVGKGIHTVFGSAFQGLFGGLNWITKVIM 457
YFV-17D            KLFTQTMKGVERLAVMGDTAWDFSSAGGFFTSVGKGIHTVFGSAFQGLFGGLNWITKVIM 457
JEV-Nakayama       KAFSTTLKGAQRLAALGDTAWDFGSIGGVFNSIGKAVHQVFGGAFRTLFGGMSWITQGLM 464
MVEV-NG156         KAFSTTLKGAQRLAALGDTAWDFGSVGGVFNSIGKRVHQVFGGAFRTLFGGMSWISQGLL 465
WNV-NY99           KAFTTTLKGAQRLAALGDTAWDFGSVGGVFTSVGKAVHQVFGGAFRSLFGGMSWITQGLL 465
SLEV-Laderle       KALATTWKGAQRLAVLGDTAWDFGSIGGVFNSIGKAVHQVFGRAFRTLFGGMSWITQGLL 465
TBEV-Neudoerfl     RVFQKTKKGIERLTVIGEHAWDFGSAGGFLSSIGKAVHTVLGGAFNSIFGGVGFLPKLLL 460
POWV-LB            RMFEKTRRGLERLSVVGEHAWDFGSVGGVLSSVGKAIHTVLGGAFNTLFGGVGFIPKMLL 461
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D2-16681           GVIITWIGMNSRSTSLSVTLVLVGIVTLYLGVMVQA 495
D2-PUO-218         GVIITWIGMNSRSTSLSVSLVLVGIVTLYLGVMVQA 495
D2-M1              GVIITWIGMNSRSTSLSVSLVLVGIVTLYLGVMCQA 495
D2-New Guinea C    GVIITWIGMNSRSTSLSVSLVLVGVVTLYLGVMVQA 495
D2-PL046           GVIITWIGMNSRSTSLSVSLVLVGVVTLYLGVVVQA 495
D2-K0005           GVIITWIGMNSRSTSLSVSLVLVGIVTLYLGVMVQA 495
D2-Jamaica 1409    GVIITWIGMNSRSTSLSVSLVLVGVVTLYLGAMVQA 495
D2-13382/Tizimin   GVIITWIGMNSRSTSLSVSLVLVGVVTLYLGAMVQA 495
D2-China-04        GVIITWIGMNSRSTSLSVSLVLVGVITLYLGAMVQA 495
D2-PR159           GVIITWIGMNSRSTSLSVSLVLVGIVTLYLGVMVQA 495
D2-Ven2            GVIITWIGMNSRSTSLSVSLVLVGIVTLYLGVMVQA 495
D2-IQT2913         GVIITWIGMNSRSTSLSVSLVLVGIVTLYLGVMVQA 495
D2-SL714           GVVITWIGMNSRSTSLSVALVLVGIVTLYLGVMVQA 495
D2-CAMR5           GVVITWIGMNSRSTSLSVSLVLVGVVTLYLGVMVQA 495
D2-CAMR16          GAVITWIGMNSRSTSLSVSLVLVGVVTLYLGVMVQA 495
D2-DAKAr578        GVVITWIGMNSRSTSLSVTLVLVGFVTLYLGVMVQA 495
D2-PM33974         GVVITWIGMNSRSTSLSVTLVLVGIVTLYLGVMVQA 495
D2-P8-1407         GVIITWIGMNSRSTSLSVTLVIVGIVTLYLGVMVQA 495

D2-16681           GVIITWIGMNSRSTSLSVTLVLVGIVTLYLGVMVQA 495
D1-16007           GILLTWLGLNSRNTSLSMMCIAVGMVTLYLGVMVQA 495
D3-PhMH-J1-97      GVLLTWIGLNSKNTSMSFSCIVIGIITLYLGTVVQA 493
D4-Thailand/1985   GFLVLWIGTNSRNTSMAMSCIAVGGITLFLGFTVHA 495
YFV-Asibi          GAVLIWVGINTRNMTMSMSMILVGVIMMFLSLGVGA 493
YFV-17D            GAVLIWVGINTRNMTMSMSMILVGVIMMFLSLGVGA 493
JEV-Nakayama       GALLLWMGVNARDRSIALAFLATGRVLVFLATNVHA 500
MVEV-NG156         GALLLWMGVNARDKSIALAFLATGGVLLFLATNVHA 501
WNV-NY99           GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA 501
SLEV-Laderle       GALLLWMGLQARDRSISLTLLAVGGILIFLATSVQA 501
TBEV-Neudoerfl     GVALAWLGLNMRNPTMSMSFLLAGGLVLAMTLGVGA 496
POWV-LB            GVALVWLGLNARNPTMSMTFLAVGALTLMMTMGVGA 497
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