Development of Improved Dengue 2 Antigen Configurations for a Venezuelan Equine Encephalitis Virus Replicon Particle-Launched Dengue Vaccine

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ABSTRACT

Drue Webb: Development of Improved Dengue 2 Antigen Configurations for a Venezuelan Equine Encephalitis Virus Replicon Particle-Launched Dengue Vaccine
(Under the direction of Laura White)

Dengue has become one of the most important vector-borne diseases. There is currently no licensed dengue vaccine. The envelope glycoprotein is the major target of neutralizing antibodies. Researchers have tried to identify the most antigenic configuration of the envelope protein in different vaccine platforms. In this study, six dengue 2 E configurations, including full-length E, truncated forms of E and the domain III subunit, were expressed as antigen using the Venezuelan equine encephalitis virus replicon paricle system and characterized *in vitro* and *in vivo*. Each construct was immunogenic in mice; however, there was a correlation between protein secretion and neutralizing responses. Two truncated and soluble configurations of E encoding amino acid changes from the previously evaluated DEN2 prME-VRP sequence elicited significant neutralizing immune responses. These amino acid changes may be important in the structure of neutralizing epitopes or for proper folding, processing and secretion, leading to improved neutralizing antibody responses.

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LIST OF ABBREVIATIONS AND SYMBOLS

DEN: dengue

DIII: domain III of the envelope protein

E: envelope

F-Neut$_{50}$: the dilution of serum at which 50% of dengue infection is neutralized

hpi: hours post infection

hpc: the time of chase with unlabeled methionine and cysteine referred to as hours post chase

MAb: monoclonal antibody

s.c.: subcutaneous

VLP: virus-like particle

VRP: Venezuelan equine encephalitis replicon particle

Δ: indicates changes to the amino acid sequence in reference to the amino acid sequence of full-length envelope encoded by the prME construct

CHAPTER I

INTRODUCTION

Dengue viruses are members of the *Flaviviridae* family. They are enveloped, single-stranded, positive sense RNA viruses. The viral genome is packaged inside an isometric nucleocapsid, which is surrounded by a lipid bilayer. The virus is approximately 500 angstroms in diameter. Humans are the major natural host of dengue viruses. The virus is transmitted primarily in urban epidemic/endemic cycles by the mosquito vector *Aedes aegypti*.

Epidemiology and Disease

Dengue has become one of the most important vector-borne viral diseases and a serious health risk world wide. Approximately one-fifth of the world's population, 2.5 billion people, is currently at risk for dengue infection. Areas at greatest risk include urban and suburban areas in the tropics and subtropics, mainly the Americas, South-east Asia, the Western Pacific, and the Eastern Mediterranean. Rural areas in Africa are also at risk. The World Health Organization estimates that 50-100 million cases occur annually[1]. Dengue infection can result in a range of illnesses, from dengue fever to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Dengue fever is a self-limiting febrile illness with symptoms including mild to high fever, severe headaches, arthralgia, rash and retro-orbital pain. It normally does not result in death. DHF is a more severe illness that involves

thrombocytopenia, hemorrhagic tendencies and plasma leakage and can be fatal. DSS is another form of severe illness with symptoms of circulatory failure, low pulse pressure and hypotension in addition to all the symptoms exhibited in DHF. Of the estimated annual dengue infections, as many as 500,000 are DHF cases, and up to 20,000 cases are fatal[1].

There are four antigenically related dengue viruses: dengue 1, dengue 2, dengue 3 and dengue 4. These four serotypes are thought to play an important role in the development of more severe illnesses. Epidemiological studies have shown a correlation between secondary dengue infection and development of DHF/DSS[2, 3]. The dominant theory in the field is that while infection with one dengue serotype provides life-long protection against a homologous secondary infection, it only confers short term protection against heterologous infection. In the long term, low neutralizing antibody titers or cross-reactive, non-neutralizing antibodies against the other serotypes may enhance a heterologous secondary infection. Results from earlier *in vitro* and *in vivo* studies support this theory, referred to as antibody dependent enhancement (ADE). When dengue viruses were combined with sub-neutralizing levels of antibody, *in vitro* infection was enhanced compared to infection without antibody present[4-7]. This was also shown in rhesus monkeys by injecting dengue-immune human cord-blood serum intravenously prior to infection with dengue 2 virus, resulting in higher viremia compared to monkeys that received normal cord-blood serum[8]. Results from a more recent study also provide evidence that increased viral titers were observed in juvenile rhesus monkeys with a range of subneutralizing antibody titers[7]. Kontny et al. Made the observation that interferon-gamma further enhances dengue infection in the presence of antibody in correlation with an increase in IgG FC receptors (FCγRI), suggesting that FCγRI may act as an ADE mediator[9]. The same group published results

showing that FCγRII also mediates enhancement in dengue infection in the erythroleukemia cell line K562, which expresses FCγRII[10]. This process of enhanced infection via ADE might explain the increased risk of severe disease in populations in developing sub-tropical and tropical areas where all four serotypes are prevalent, and the chances of second infection with heterologous serotypes are high.

Cases of DHF/DSS are reported with primary infections as well[11, 12]. A significant portion of these cases occur in infants, which make up five percent of all cases of DHF/DSS[13]. Five percent of children hospitalized with DHF in Asia are infants younger than one year old[14], which has been attributed to dropping levels of maternal antibodies after birth[15, 16]. Maternal antibodies are transferred through the placenta during pregnancy. Therefore, infants born to dengue-immune mothers receive anti-dengue antibodies. Placentally-transferred immunity remains at protective levels for approximately three months, but protective titers have been detected as late as nine months after birth[16, 17]. During this period of antibody decline, infants are at the greatest risk for dengue infection and possible development of DHF/DSS[15].

Other theories for the pathogenesis of severe dengue infections have been proposed that suggest a role for the adaptive and innate immune responses in dengue immunopathogenesis. These include the involvement of complement in mediating dengue infection enhancement and a possible role for apoptosis in vascular leakage[6, 18]. There is evidence suggesting that T cell activation may also play a role in severe dengue pathogenesis. Secondary infection with a different serotype results in profound activation of T cells with a higher affinity for the serotype of the previous infection[19], which is thought to explain the delay in viral suppression that results in severe illness. Although these

mechanisms may play a role in severe dengue pathogenesis, the evidence for ADE has been one of the largest concerns in vaccine development. The role of pre-existing immunity, not only in lack of protection but also in pathogenesis of DHF, represents an immense challenge for dengue vaccine development.

Protein Processing and Viral Structure

The viral genome is 11 kilobases in length. The positive sense, single-stranded RNA has a single open-reading frame, which is translated into a single polyprotein that is co- and post-translationally cleaved by host and viral proteases[20]. The amino-terminus of the polyprotein encodes three structural proteins: capsid, the precursor for membrane protein (prM), and envelope (E). prM is cleaved during maturation resulting in membrane (M). The remainder of the polyprotein encodes seven non-structural proteins that are important for viral RNA replication and processing. A hydrophobic signal sequence at the carboxy-terminus of the capsid protein and two transmembrane domains in prM, including a stop transfer sequence and a signal sequence, allow prM and E to be translocated across the endoplasmic reticulum (ER) membrane during translation while anchored to the ER membrane. Once co-translational cleavage occurs between capsid, prM, E, and the non-structural proteins, the capsid protein remains in the cytosol and associates with the viral RNA genome and the ER membrane in the cytoplasm. Each of the membrane proteins, prM and E, remain anchored in the ER membrane through their carboxy-terminal transmembrane domains, with their ectodomains forming stable heterodimers on the luminal side of the ER. Studies with Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBE) have shown that prM may act as a chaperone during translation and processing, assuring proper

folding of E and maturation of the virion[21, 22]. Viral particles bud into the lumen of the ER, which provides a host derived lipid bilayer, resulting in immature virions. Budding of empty particles can occur when prM and E are expressed in mammalian cells in the absence of other viral proteins[23]. The outer layer of immature virions consists of 60 surface spikes, which contain trimers of the prM and E heterodimers. The presence of spikes causes the external diameter to be slightly larger than mature virions. The fusion peptide of E is covered by the three prM proteins of the spike. It is thought that this association of prM with E during particle trafficking protects E in the acidic vesicles of the exocytic pathway from premature fusion[24]. The immature virions are trafficked through the trans-Golgi network, during which cleavage of prM by furin-like host protease results in maturation of the particles into infectious virions released by exocytosis.

Much of what is known about flavivirus E structure stems from structural analysis of the tick-borne encephalitis virus E glycoprotein[25-27]. More recent crystal structures and cryo-electron microscopy studies have provided more detailed structure analysis of the dengue E protein[28-32]. The E glycoprotein consists of the ectodomain and a stem-anchor region. The ectodomain includes domains I, II, and III (Figure I.2). Domain I is encoded at the amino-terminus of E. It is located at the center of the monomer structure. Domain II is known as the fusion/dimerization domain and contains the fusion loop, a hydrophobic peptide involved in fusion of the virion membrane to cellular membranes. Domain III folds into an IgG-like domain and has been identified as the putative receptor-binding domain[31-33]. The stem region includes two consecutive amphipathic helices, designated E-H1 and E-H2, flanking a highly conserved sequence[30]. These helices connect the ectodomain and the transmembrane region that anchors the protein to the ER membrane. They are arranged

so that the hydrophobic sides of the helices associate with the hydrophobic residues of the membrane[30]. The transmembrane region consists of two antiparallel alpha-helices that are embedded in the ER membrane[30]. Two monomers assemble head to tail to form the homodimers present on mature virions. Three sets of □immers, referred to as "rafts", arrange in a nearly parallel manner, resulting in a herringbone configuration on the surface of the virion[34]. This arrangement results in a mostly smooth viral surface covered with 30 "rafts", with the exception of domain III. The protrusion of domain III from the viral surface may aide in receptor binding[35].

Current Vaccine Strategies

Despite many decades of research directed towards the development of a dengue vaccine, there is still no licensed vaccine. Earlier replication competent vaccine candidates for dengue have been attenuated by serial passage[36-39] or genetic engineering[40]. These vaccine designs were based on the success of the live-attenuated yellow fever (YF) 17D vaccine[41, 42]. Several groups of researchers continue to focus on developing live-attenuated vaccines by formulating tetravalent designs[43-45] to induce simultaneous immune responses to the four serotypes. There are a number of live-attenuated dengue vaccine candidates that have entered phase I clinical trials. While some candidates were considered safe for further testing[37, 44, 46], others were reactogenic in human volunteers and did not induce balanced responses to all four serotypes[47-50]. Other live-attenuated vaccine candidates involve chimeric dengue viruses[51-54] or chimeric designs encoding dengue structural proteins in the backbone of other flaviviruses[55-61]. Some of these

chimeric vaccine designs have entered phase I clinical trials as well, exhibiting low reactogenicity and detectable immune responses in human volunteers[54, 58].

Due to difficulty of tetravalent formulations of live-attenuated viruses to induce simultaneous immune responses against all four serotypes after a single dose, other gene-based vaccine platforms are being evaluated as second generation candidates. They include DNA vectors[62-67], recombinant protein, including subunit designs, expressed from bacteria[68, 69], yeast[70, 71] or baculovirus[72-74], and viral vectored vaccines expressing recombinant or subunit proteins[75-78] . These gene-based platform studies have evaluated different combinations and configurations of the structural proteins as antigen. C.-J. Lai et al. Have experimented with expressing several recombinant dengue immunogen configurations from vaccinia viral vectors[76, 79-83]. Initial results showed that expression of E alone or concomitantly with other structural and non-structural proteins from vaccinia provided some, if not complete, protection against lethal challenge in mice[79, 82, 83]. These results suggest it is not necessary to express more than the prM and E glycoprotein to induce protective immune responses, allowing for the possibility of smaller immunogens in recombinant vaccine designs.

Several groups examined at immunogenicity and protection elicited by expression of full-length prM and E from chimeric flavivirus particles[51, 55, 84], DNA vectors[64, 66, 85], or viral vectors[75, 83]. Konishi et al. Have shown that expression of prM and E in a tetravalent, DNA-based dengue vaccine results in the induction of a long lasting antibody response in mice, with neutralizing antibody titers that are low, but detectable[66]. Monkeys immunized with prM and E expressed from a DNA plasmid or from chimeric particles produced detectable levels of neutralizing antibody and were partially or completely

protected against lethal challenge[60, 85, 86]. Co-expression of prM with chimeric or truncated forms of E has been examined to determine if modifications to E would improve immune responses compared to expression of full-length E with prM. Chang et al. Expressed prM with truncated dengue 2 E and replaced the deleted dengue sequences with the corresponding sequences of the JEV E[87]. In this study, chimeric E behaves more like full-length E with a transmembrane domain. Immunized mice had detectable virus neutralization titers and were protected against lethal dengue challenge. A DNA vaccine design expressing prM and 92% of the amino acid sequence of the dengue 2 E from the amino-terminus induced antibodies in mice, including detectable neutralization titers, but did not provide protection against lethal challenge[67].

Although these studies have shown some level of immune induction from modified E expressed with prM, immune responses observed in other studies towards C-terminally truncated E without expression of prM have been more protective and in many cases induce higher neutralizing responses. A comprehensive panel of C-terminally truncated E proteins expressed from vaccinia virus showed that expression of 79-81% of the amino acid sequence of E resulted in soluble protein that was detected in the intracellular and extracellular fractions[76]. These conformations were the only truncated antigens that induced detectable antibody levels in mice and provided equivalent or better protection in comparison to the longer E proteins[76]. Other studies have supported this data, showing that expression of truncated E proteins (80-85%) results in antibody induction, low but detectable neutralizing antibody titers, and some or complete protection in mice[70-72, 74, 77]. Similar results have also been observed in monkeys[63, 80, 88]. These studies have demonstrated that the expression of prM and E or truncated E without prM can induce detectable and protective

immune responses in mice and non-human primates, making it an attractive antigen for vaccine design.

Recent results involving domain III of the E glycoprotein have important implications for vaccine design. Studies on antibody binding and epitope mapping showed that some neutralizing epitopes mapped to the ~100 amino acid sequence encoding domain III[89-92]. Recombinant domain III also blocks dengue virus binding to mammalian and insect cells[93, 94]. These studies suggested that immunizing with domain III may be sufficient to induce neutralizing antibodies that block binding and entry by the virus. Crill et al. Showed that monoclonal antibodies that map to domain III are able to block virus binding to mammalian cells, providing supporting evidence that domain III contains the receptor-binding motif[95]. To test this, Simmons et al. Designed plasmid vectors that expressed domain III of E for each of the four serotypes fused to the maltose binding protein (MBP) of *E. Coli*, referred to as DEN-(B)/MBP[96, 97]. The MBP was cleaved in order to administer DEN-(B) as a subunit vaccine. Groups of mice immunized with DEN1-, DEN2-, DEN3-, or DEN4-(B) had significant serotype-specific antibody titers, with neutralizing antibody titers from PRNT 80-1024, and 80% of the mice were protected from lethal challenge. Several groups are now expressing domain III from different platforms and evaluating the induction of immune responses *in vivo*[98-105]. Immunization of mice with viral vectors such as adenovirus and tobacco mosaic virus (TMV) encoding domain III resulted in antibody induction and low but detectable neutralizing antibody titers, suggesting that expression of only domain III induced immune responses comparable to immune responses elicited by truncated or full-length E[100, 106]. Zhang et al. Showed that domain III expressed from *E. Coli*, purified and administered to mice induced low but detectable neutralizing antibody titers and provided

complete protection against lethal challenge[99]. Serotype-specific neutralizing antibodies were detected in mice immunized with domain III expressed from the pediatric live-attenuated Schwarz measles vaccine, which supports previous reports that domain III contains more serotype-specific epitopes[105]. These studies have demonstrated neutralizing immune responses and protection in response to domain III immunization in mice, but they were unable to induce neutralizing antibody titers similar to the titers reported by Simmons et al[96].

Although much of the work published is on expression of domain III of a single serotype that is purified and administered as a subunit vaccine, some studies have tried designing bivalent and tetravalent formulations. Expression of a bivalent antigen consisting of the domain III regions of dengue 2 and 4 connected by a flexible peptide linker in *E. Coli* resulted in specific antibody responses and detectable neutralizing antibodies to the two serotypes in mice immunized with purified protein and Freund's complete adjuvant[102, 107]. Serotype-specific antibodies and low but detectable neutralizing antibody titers were also induced by the individual domain III regions of each of the four serotypes expressed from a plasmid, purified and administrated as a tetravalent vaccine in mice[101]. Other groups have not seen a very significant immune induction with tetravalent domain III expressed from a plasmid, with unbalanced antibody induction and neutralization and minimal protection from lethal challenge[104]. These findings support the use of domain III of the E glycoprotein as an antigen configuration that induces immune responses in mice and monkeys and may minimize cross-reactivity. Similar studies have been conducted involving the domain III of West Nile virus (WNV), and the results show that WNV domain III induces virus-specific neutralizing antibodies and elicits protection from lethal challenge in

mice[108]. This serotype-specific response is thought to be due to the larger proportion of serotype-specific epitopes present in domain III. Because sub-neutralizing levels of cross-reactive antibodies may lead to dengue infection enhancement, employing antigens that induce lower levels of cross-reactive antibodies while inducing a neutralizing and protective serotype-specific response may allow a more effective and safe vaccine design.

Table 1.1 Dengue vaccine candidates.

Vaccine Candidates	**Design**	**Stage of Development**
Live, attenuated Vaccines		
WRAIR and GSK[109]	Dengue virus	Phase I
Mahidol University (Bangkok)[110]	Dengue virus	Preclinical
NIH, Biologicals E (India), Panacea (India)[51, 111]	Dengue-dengue chimera	Phase I
CDC, Invirage, Shantha (India)[51]	Dengue-dengue chimera	Preclinical
Acambis and Sanofi Pasteur[58]	Dengue-yellow fever chimera	Phase II, entering phase III
Gene-Based Vaccines		
U.S. Navy[62]	DNA: DEN E	Preclinical
U.S. Navy[58, 97]	Subunit: E DIII	Preclinical
RGP group[112]	Viral Vector: adenovirus encoding E DII	Preclinical

Project Rationale

There is currently no licensed vaccine for dengue. There are several complications associated with vaccine design. Because a large part of the population in endemic areas has previously been infected with at least one of the four serotypes or has maternally-transferred anti-dengue antibodies in the case of infants, it is important to use a delivery method that will not be inhibited by antibody recognition. The use of viral vectors and DNA vaccines may help to bypass pre-existing antibodies. These delivery methods are not packaged in dengue

structural proteins and are, therefore, not recognized and neutralized by anti-dengue immune responses. However, with viral vectors there may be other complications with pre-existing immunity in humans towards the vector of choice, as well as safety issues regarding possible recombination. To avoid the occurrence of antibody dependent enhancement after immunization, a vaccine would need to induce a strong and balanced immune response to all four serotypes. If a tetravalent vaccination resulted in a lower antibody titer for any given serotype, it might result in severe disease. It is important that the immune response includes a strong induction of neutralizing antibody levels, since non-neutralizing antibodies may also result in the development of severe dengue disease.

Employing a non-propagating Venezuelan equine encephalitis virus (VEE) replicon particle (VRP) system as a delivery system provides a way to circumvent the complications associated with dengue vaccine development. The replicon vector encodes a complete cDNA copy of the VEE genome in which the VEE structural genes have been replaced with a multiple cloning site in the VEE replicon vector downstream of a 26S promoter[113]. Linearized vector is used to make infectious RNA, capable of replication and transcription, which is introduced into cells with two helper RNAs that encode the E1 and E2 glycoproteins and the capsid protein downstream of a 26S promoter. These helper RNAs do not encode the nonstructural proteins or cis-acting elements. Once in the cell, the replicon RNA is packaged by the structural proteins translated from the helper RNAs, resulting in VEE replicon particles. By providing the structural elements in trans during packaging, the resulting replicon particles are limited to a single round of replication.

This system provides some solutions to the complications involved in dengue vaccine design. Because the replicon is packaged in the VEE structural proteins, it avoids any

blockage by pre-existing dengue immunity, likely to occur with a live dengue virus vaccine. Certain strains of VEE replicate in lymphoid tissue and in the context of replicon particles the glycoproteins will target lymphoid tissue, delivering antigen directly to antigen presenting cells[114-116]. The VEE replicon particles are also capable of high-level expression of heterologous genes during a single round of replication[114, 115]. These characteristics make the VRP system an effective alternative in vaccine development. Several *in vivo* studies have shown that the VEE replicon particle system is safe in animals, including rodents, horses and monkeys[117-119]. A potential HIV vaccine encoding HIV Gag expressed from VRP was safe in human volunteers in phase I clinical trials in the U.S. and South Africa[120].

Our laboratory has shown that expression of the dengue 2 prM and E genes from VRPs results in neutralizing antibody responses and protection in mice[121]. The goal of this project is to design an optimal immunogen by modifications to the dengue structural genes expressed from VRPs. We have designed five additional constructs expressing different truncations of E. We wanted to test whether truncations at the C-terminus will enhance neutralizing immune induction in mice compared to neutralizing immune responses induced by full-length E in the presence of prM. Two truncations were designed to determine whether the presence of the H1 domain and the transmembrane domain play a role in the immunogenic properties of E. These constructs encode C-terminally truncated E expressing 85% (E85) or 81% (E81) of the amino acid sequence of the protein. Two other constructs encode the same truncated E proteins with specific point mutations. The E85 constructs encode the ecotodomain and the H1 domain in the stem region. The E81 constructs encode the ectodomain but lack the H1 domain. The final construct encodes

domain III of the E protein (amin acids 296-400). We hypothesize that E configurations expressed both inside and outside of the cell will be more immunogenic in mice, and while expressing domain III alone may not provide a stronger immune response than the C-terminally truncated forms of E containing domains I, II, and III, it may provide a more serotype-specific immune response, minimizing cross-reactive antibody induction. Each construct is downstream of the tissue plasminogen activator (tPA) signal sequence to promote protein secretion. Our aim is to characterize these five constructs in comparison to the DEN2 prME-VRP construct *in vitro* and *in vivo* to determine the best and most practical immunogen design for the VRP system.

CHAPTER II

MATERIALS AND METHODS

Cells

BHK-21 cells were obtained from the American Type Culture Collection (ATCC) and maintained in alpha minimal essential medium containing 10% tryptose phosphate broth, 0.29 mg/ml of glutamine and either 10% donor calf serum or 10% fetal calf serum. Vero-81 cells were obtained from the ATCC and maintained in D-MEM/F12 medium containing 10% fetal calf serum, 0.29 mg/ml of glutamine, non-essential amino acids (1X), penicillin (100U/ml) and streptomycin (0.05 mg/ml) and 0.2% sodium bicarbonate. Insect C6/36 cells from the ATCC were maintained in alpha minimal essential medium containing 10% fetal calf serum.

Virus

The mouse-adapted, neurovirulent New Guinea C (NGC) strain of dengue 2 (DEN2) virus was provided by the late Robert Shope, UTMB, Galveston, TX. The stock virus used for neutralization assays was propagated in C6/36 cells, titrated on Vero cells and stored at -80°C at a concentration of 10^7 PFU/ml. The WHO reference strains of DEN1, DEN2, DEN3 and DEN4, provided by Aravinda de Silva, were used for ELISA antigen and were propagated in C6/36 cells, concentrated by sedimentation at 72,000 x g for 5 hours through a 5 ml cushion of 20% (w/v) sucrose dissolved in PBS, and stored at -80°C.

Antibodies

Flavivirus envelope specific 4G2 mouse monoclonal antibody (MAb) was purified from the HB-112 hybridoma cell line obtained from the ATCC and concentrated to 0.1mg/ml. For neutralization assays, MAb 4G2 was conjugated to Alexa-Flour 488 (Invitrogen) and was used at a concentration of 1mg/ml. Mouse MAb 3H5-1, specific to DEN2 domain III, was obtained from Chemicon International. Biotinylated anti-mouse IgG (H+L), made in horse, was at a concentration of 1.5mg/ml (Vector Laboratories). Anti-mouse IgG (gamma-chain specific)-peroxidase antibody was obtained from Sigma-Aldrich.

Cloning of dengue envelope immunogens

Each dengue 2 envelope configuration was cloned into the multiple cloning site of the VEE replicon vector, pVR21[122], to generate pVRDEN2prM/E, containing the prM signal sequence at the C-terminus of the capsid gene and the prM and E genes, pVRDEN2E85, containing the tPA signal sequence (5'ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTTTCG) and amino acids 1-424 of the E gene, which Wahala Wahala helped construct, pVRDEN2E81, containing the tpa signal sequence and amino acids 1-397 of the E gene, and pVRDEN2EDIII, containing the tpa signal sequence and amino acids 296-400 of the E gene. cDNA of the E genes for prME and E85Δ was amplified from dengue 2 NGC strain viral RNA by RT-PCR. cDNA of the E genes for E81Δ was amplified from isolated pVRprME DNA. The primer sequences were as follows:

prME forward primer (5'AGTCTAGTCCGCCAAGATGTTGAACAGGAGACGCAGAACTGCAGG), prME reverse primer (5'GGCGCGCCTTAGGTCTGCACCATAACTCCCAAATACAGCGT), E forward primer (5'GAGTCTTCGTTTCGATGCGTTGCATAGGA), E85 reverse primer

(5'GGCGCGCCTTAGGATCCAAAATCCCA), E81 reverse primer (5'GGCGCGCCTTAAGAACTTCCTTTCTTAAACCAGTTGAGCTTC), EDIII forward primer (5'GTCTTCGTTTCGATGTCATACTCTATGTGCAC), And EDIII reverse primer (5'GGCGCGCCTTATTGGCCGATAGAACTTCC). The E genes were cloned into the multiple cloning site of pVR21 by further amplification in overlapping extension PCR reactions and using the ApaI and AscI sites, upstream and downstream, respectively, of the 26S subgenomic RNA transcription start site. The vectors were sequenced to confirm the gene sequence and that no changes occurred in the VEE nsp region, the 26S promoter or the 3'UTR.

VRP packaging

All pVR21 clones were linearized at a unique NotI site located downstream of the VEE 3' untranslated region and poly(A) sequence. The mMessage mMachine kit (Ambion) was used to generate full-length T7 RNA transcripts as previously described [123]. Two helper T7 transcripts were generated: one encoding the VEE capsid gene and the other encoding the VEE glycoproteins from a cDNA clone of VEE, V3000. The replicase genes and the cis-acting packaging signal have been deleted from the helper RNAs. Each of the pVR transcripts (pVRprM/E, pVRE85, pVRE81, pVRDIII, pVREs or pVRE81mut) was mixed with the two helper RNAs and were co-transfected into BHK cells by electroporation. At 22-24 hours post-electroporation the culture medium was harvested. Each VRP was safety tested for the absence of replication competent virus using a BHK based CPE test, as described previously [113, 123].

VRP Purification

The harvested VRP culture medium was clarified by centrifugation at 12,000 x g for 30 minutes. The VRPs were then partially purified and concentrated by sedimentation at 72,000 x g for 3 hours through a 5 ml cushion of 20% (w/v) sucrose dissolved in PBS. The pelleted VRPs were incubated at 4°C overnight in endotoxin-free PBS with 1% donor calf serum, resuspended and stored at -80°C. VRP titers were determined by IFA. BHK cells were seeded in 8-well chamber slides and infected the next day with serial dilutions of the concentrated VRPs. Infected cells were incubated for 18 hours at 37°C and fixed in methanol for 20 minutes at 4°C. The cells were permeabilized with 0.1% TritonX-100 and incubated sequentially with mouse polyclonal anti-VEE nsp antibody, biotinylated anti-mouse IgG, and avidin conjugated to FITC. Infectious units (IU) per ml were determined by counting replicon-infected fluorescent cells using fluorescent microscopy.

Site-directed mutagenesis PCR

Site directed mutagenesis was conducted on pVRE85Δ and pVRE81Δ to change the indicated residues to the amino acids present in the pVRprME sequence. Site-specific mutations were introduced by using a QuickChange® II Site-Directed Mutagenesis kit, following the manufacturers recommended protocol (Stratagene). Mutagenic primers 25-45 nucleotides in length were designed for each point mutation. The sequences of the primers are: A33T forward primer (5'GGAAGCTGTGTGACGACGATGGAAAAAACAAACC), A33T reverse primer (5'GGTTTGTTTTTTGCCATCGTCGTCACACAGCTTCC), V54A and K58E forward primer (5'CAGAAGCCAAACAACCTGCCACTCTAAGGGAGTACTGTATAGAGG), V54A and K58E reverse primer (5'CCTCTATACAGTACTCCCTTAGAGTGGCAGGTTGTTTGGCTTCTG), G112S

forward primer (5'GGATTATTTGGAAAAGGAAGCATTGTGACCTGTGCTATG), G112S reverse primer (5'CATAGCACAGGTCACAATGCTTCCTTTTCCAAATAATCC), N124I forward primer (5'GTTCACATGCAAAAAGATCATGAAAGGAAAAGTCGTGC), N124I reverse primer (5'GCACGACTTTTCCTTTCATGATCTTTTTGCATGTGAAC), V197A forward primer (5'CGACTTCAATGAGATGGCGTTGCTGCAAATGG), V197A reverse primer (5'CCATTTGCAGCAACGCCATCTCATTGAAGTCG). Between 50 and 75ng of pVRE85Δ or pVRE81Δ template DNA was added to a PCR reaction mixture containing 0.2μM of the appropriate sense and antisense primers, 1X Pfu Turbo buffer (Stratagene), 0.5mM dNTPs, and 2.5 U of Pfu Turbo (Stratagene). The PCR reaction mixture was then digested with 20 U of DpnI (New England Biolabs) for 3 hours at 37°C to eliminate any template DNA. The cDNA was then precipitated with 40% isopropanol and 0.2M sodium acetate for 15 minutes at -80°C. Precipitated cDNA was concentrated at 14,000rpm for 15 minutes and then washed with 70% ethanol. The pellet was resuspended in 2XYT broth and transformed into DH5α chemically competent cells (Invitrogen).

In vitro VRP infections, radiolabeling, immunoprecipitations, SDS-PAGE

BHK cells were seeded in 24 well plates. Monolayers were mock infected or infected the next day with the DEN2 prME-, E85Δ-, E81Δ-, E85-, E81- and DIII-VRP at an MOI of 10. Four hours post infection, the media was removed, replaced with methionine and cysteine free medium (MP Biomedicals, Inc.), and cells were starved for 1 hour. The monolayers were metabolically radiolabeled with 50-100μCi of [^{35}S] methionine and cysteine/ml (Pro-Mix, Amersham) for 5 hours. The media was harvested and clarified at 13,000 rpm for 15 minutes at 4°C. Cells were lysed in NP-40 lysis buffer (1X TNE with 0.2% Igepal from Sigma and the Complete Mini protease inhibitor cocktail from Roche). Lysates were

clarified at 13,000 rpm for 15 minutes at 4°C. Envelope proteins were immunoprecipitated from the harvested supernatants and cell lysates with MAb 4G2 or MAb 3H5-1 using protein A-Sepharose CL-4B beads (Sigma) according to standard protocols. Immunoprecipitated proteins were denatured in 1% SDS containing 50mM 2-β-mercaptoethanol and electrophoresed in 12% or 15% SDS-Polyacrylamide gels (SDS-PAGE) with molecular weight markers (14.3 to 220 kDa, Amersham). Radio-labeled and banded proteins were visualized using a phosphorimager.

Indirect Immunofluorescence assay (IFA) for DEN envelope protein

BHK cells were seeded in eight-well chamber slides. Monolayers were mock infected or infected with DEN2 prME-, E85Δ-, E81Δ-, E85-, E81- or DIII-VRP at an MOI of 5. 10hpi, cells were rinsed with PBS and fixed either in cold methanol for 20 minutes or in 2% paraformaldehyde at room temperature for 20 minutes. Some cells were further permeabilized with TBS containing 0.1% TritonX-100 and 0.1% BSA for 15 minutes at room temperature. The cells were stained with the primary DEN2 MAb 3H5-1 (1:400) and the secondary biotinylated anti-mouse IgG antibody made in horse (1:1000). Alexa Fluor® 594-conjugated streptavidin (1mg/ml, Molecular Probes) was used as the reporter (1:500). Stained cells were observed by fluorescent microscopy.

Immunization of BALB/c mice

Specific pathogen-free adult (5 weeks old) BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). Groups of six mice were physically restrained and immunized at 6 weeks of age by subcutaneous (s.c.) inoculation in both rear footpads with a volume of 10 μl in each footpad. The mice were observed for 7 days for any adverse reactions. Mice were bled prior to and periodically after immunization by tail vein puncture.

Sera was separated and stored at -20°C for later analysis by virus neutralization assays and ELISAs.

Dengue 2 Neutralization Assay

DEN2 specific neutralizing antibody titers in immunized mouse sera were determined by a flow cytometry-based dengue neutralization assay (FRNT) as described earlier[124, 125]. Briefly, equal volumes of DEN2 NGC virus and heat-inactivated sera diluted two-fold with DMEM-F12 containing 1% bovine serum albumin were combined and incubated for 1 hour at 37°C. Vero cells seeded in 24 well plates were infected with the serum/virus mixtures and incubated at 37°C. At 24 hours post infection, the monolayers were trypsanized and washed with PBS. The cells were fixed and permeabilized with Cytofix/Cytoperm (BD-PharMingen, San Diego, CA) for 20 minutes at 4°C. After fixation the cells were washed with Cytowash (BD-PharMingen, San Diego, CA), resuspended in 50µl of a 1:600 to 1:1000 dilution of ALEXA 488-conjugated monoclonal antibody 4G2 for 1 hour at 4°C, and then washed with and resuspended in Cytowash. Fixed and stained cells were analyzed by flow cytometry using a CyAn ADP analyzer (Dako). Neutralizing antibody titers (F-$Neut_{50}$) were determined based on end-point titers resulting in 50% or more reduction in the number of cells positive for dengue infection.

ELISA for dengue specific IgG

The antigen used to coat the ELISA plates was purified virus from WHO reference strains of dengue 1, 2, 3 and 4 that were propagated in C6/36 cells. Media were harvested at 7 and 9 days post-infection, clarified by centrifugation at 12,000 x g for 30 minutes, and concentrated by sedimentation at 72,000 x g for 5 hours through a 5 ml cushion of 20% (w/v) sucrose dissolved in PBS. The concentrated viruses were resuspended in PBS and used as antigen.

Protein concentrations were determined using the Coomassie Plus-The Better Bradford™ Assay Kit (Pierce, Rockford, IL) according to the protocol. Microtiter 96 well ELISA plates were coated with 500μg/well of dengue 1, 2, and 3, 1000μg/well of dengue 4 and an equal volume of mock antigen (approximately 10μg) obtained from uninfected C6/36 culture medium in carbonate buffer overnight at 4°C. The wells were thoroughly washed with PBS containing 0.05% Tween-20. Sera collected from immunized mice were serially diluted in ELISA wash buffer (PBS and 0.05% Tween-20) containing 10% Sigma Blocking Buffer (Sigma), added to the corresponding wells and incubated at 4°C for 2 hours. After thoroughly washing with ELISA wash buffer, HRP-conjugated mouse anti-IgG (1:2000) was added to each well. Antibody bound to antigen was visualized by addition of substrate (equal volumes of 0.1 M sodium citrate and 0.1 M citric acid, 2 o-phenylenediamine dihydrochloride pellets (Sigma) per 10 ml of substrate and 0.02% hydrogen peroxide). The reaction was stopped after 30 minutes with 0.1 M sodium fluoride and read at 450 nm (single wavelength) on automated plate reader.

CHAPTER III

RESULTS

***Design and construction of the DEN2 envelope constructs*.**

The design of the different DEN2 E configurations was based on the following rationale. When DEN E protein is co-expressed with prM, they form heterodimers that may stay associated with the ER membrane but mostly bud into the lumen of the ER as recombinant subviral particles (VLPs)[23]. While in other flaviviruses VLPs are efficiently secreted, in the case of DEN these proteins are predominantly found inside the cell and may or may not be in the form of VLPs. One way of increasing the immunogenicity of viral membrane proteins that are associated with intracellular membranes has been to express them as secreted proteins by removing sequences that anchor them to the plasma membrane. The construct E81 was designed to express the ectodomain in the absence of any membrane anchor or membrane associating sequences, preceded by a signal sequence for efficient secretion (Figure 3.1). A second construct, E85, was designed to express the ectodomain in the absence of the transmembrane anchor sequences but to retain the H1 amphypathic alpha helix (Figure 3.1) that has been hypothesized to associate with the hydrophobic residues of the membrane[30]. A third construct, EDIII, was designed to express domain III of the ectodomain of E (Figure 3.1), to which murine serotype-specific neutralizing MAbs have been mapped and which has been shown to elicit a more serotype-specific neutralizing antibody response *in vivo*

[97, 101]. The two other constructs characterized in this study, E85Δ and E81Δ, were intermediate clones that contained amino acid changes with respect to the parental prME clone. Because they showed interesting phenotypes early on, they were further characterized.

The six DEN2 VRP constructs expressing different configurations of E are shown in Figure 3.1. Each construct contained E structural genes from the DEN2 NGC strain. We had previously constructed a VRP expressing prM and full-length E, referred to as DEN2 prME-VRP. The C-terminal signal sequence of the capsid protein was included to promote insertion of prME into the ER membrane during translation. The DEN2 E85-VRP construct encodes 85% of the E amino acid sequence (amino acids 1-424), which includes the ectodomain and the H1 domain in the stem region. DEN2 E85Δ-VRP also expresses 85% of the E amino acid sequence, but with three amino acid changes with respect to the prME construct: T33A in domain I and D58K and A197V in domain II. The DEN2 E81-VRP expresses 81% of the E amino acid sequence (amino acids 1-397), which only includes the ectodomain. DEN2 E81Δ-VRP encodes 81% of the E amino acid sequence with four amino acid changes in domain II with respect to DEN2 prME-VRP: A54V, S112G, I124N and A197V. DEN2 DIII-VRP only expresses domain III of the envelope glycoprotein (amino acids 296-400). To ensure efficient secretion of the truncated E proteins, a signal sequence from tPA was included at the N-terminal end in frame with the DEN E sequence.

Each envelope configuration was inserted into the multiple cloning site of the VEE replicon vector, pVR21, downstream of the nonstructural proteins under the control of the VEE 26S subgenomic promoter. Recombinant vectors were sequenced confirming

no changes in the VEE non-structural region, 26 S subgenomic region and 3' untranslated region. Linearized recombinant vector was transcribed *in vitro* to make infectious RNA that was introduced into BHK cells along with two helper RNAs encoding the VEE structural proteins, glycoproteins and capsid. VEE replicon particles secreted into the medium were purified, concentrated and titrated.

Expression and secretion of full-length E, truncated E and the DIII subunit in DEN2 VRPs infected cells.

The immunogenicity of a viral membrane antigen can be affected by the expression levels and secretion efficiency[76]. *In vitro* studies were conducted to compare the full-length and truncated E constructs in their expression and secretion. E protein expression and secretion was evaluated in VRP infected cell monolayers. BHK cells were infected at a high MOI with each of the six DEN2 VRP constructs and metabolically radiolabeled with [^{35}S]-methionine and cysteine for 5 to 10 hours post infection (hpi). Harvested cell lysates and supernatants were analyzed for protein expression by 15% SDS-PAGE (Figure 3.2A) or proteins immunoprecipitated with dengue-specific monoclonal (Figure 3.2B) and anti-dengue polyclonal sera (Figure 3.2C) to assess specific expression of E and expression of any associating proteins.

To compare the levels of E protein synthesized from each construct, total cell lysates and concentrated supernatants were analyzed by 15% SDS-PAGE (Figure 3.2A). The major radiolabeled protein in each cell lysate migrated at the apparent molecular weight for E in its full-length or truncated forms. A second prominent band migrating at the apparent molecular weight of prM was detected in cell lysates infected with DEN2

prME-VRP. A band was not detected at the apparent molecular weight of M after cleavage of the pre peptide, suggesting that prM expressed from DEN2 prME-VRP is mostly uncleaved. Because cleavage of prM occurs later in the transport process, it is not surprising that prM is mainly detected in the uncleaved form. However, M is only 8KDa and may not be detected in using 12% SDS-PAGE analysis. Quantitative analysis of the density of each E band indicates similar expression levels within approximately 1-fold among the DEN2 prME-, E85-, E85Δ-, E81 and E81Δ-VRP infected cell lysates, indicating that truncations to E did not have a significant effect on intracellular expression levels. The band detected in DEN2 prME-VRP infected cell lysates appeared less intense, approximately 1.5-fold lower, than the truncated E configurations. This may be due to lower levels of expression from DEN2 prME-VRP but could also be explained by lower stability of full-length E co-expressed with prM. Similar expression levels among E85 and E85Δ and among E81 and E81Δ suggest that the amino acid changes did not have a significant effect on the expression of truncated E. In cell lysates infected with DEN2 DIII-VRP, a band migrating at the apparent molecular weight of DIII was detected with less intensity, at least 3-fold, compared to bands detected for the other constructs. This is most likely due to weaker signal because of the limited number of cysteines and methionines in the short, ~100 amino acid sequence rather than lower levels of protein expression. Due to some distortion of the lanes in this particular gel, it is difficult to draw any conclusions about protein secretion.

To confirm identity of the most abundant radiolabeled protein synthesized in DEN2 VRP infected cells (Figure 3.2A) as dengue E, DEN-specific protein present in the cell lysates and supernatants were immunoprecipitated with MAb (4G2 or 3H5-1) or with

mouse anti-DEN2 NGC polyclonal sera. Analysis of immunoprecipitated proteins by 12% SDS-PAGE showed DEN-specific E proteins migrating at the predicted apparent molecular weight (Figure 3.2B and 2C). A very prominent band migrating at the apparent molecular weight of the E85Δ protein expressed from DEN2 E85Δ-VRP was observed by immunoprecipitation with MAb (Figure 3.2B) and with polyclonal sera (Figure 3.2C). Since this difference was not apparent in the analysis of total protein (Figure 3.2A), it suggests that increased intensity of the E85Δ band is likely due to antibody affinity. Because we did not observe similar increased levels of E85 expressed from DEN2 E85-VRP in infected cell lysates by immunoprecipitation (Figure 3.2B and 2C), it is possible that the amino acid changes in E85Δ resulted in improved antibody affinity for E85Δ. Higher levels of secreted E85Δ were detected in supernatants immunoprecipitated with MAb compared to supernatants collected from DEN2 E85-VRP infected cells (Figure 3.2B). Similar results were observed in analysis of supernatants immunoprecipitated with polyclonal sera, where E85Δ was detected at very low levels and E85 was undetectable (Figure 3.2C). Although these results suggest that the amino acid changes in E85Δ promote protein secretion, increased E85Δ in the supernatants may also be due to increased protein stability.

Differences in the levels of E81 and E81Δ immunoprecipitated from infected cell lysates were not observed (Figure 3.2B and 2C), suggesting that the amino acid changes in E81Δ did not affect antibody affinity. Differences in secreted protein levels were observed in E81 and E81Δ immunoprecipitated from supernatants, similar to the differences observed between secreted E85 and E85Δ. Detectable levels of E81Δ were observed after immunoprecipitation by MAb (Figure 3.2B) and polyclonal sera (Figure

3.2C), while we were unable to detect secreted E81 (Figure 3.2). As we observed with E85 and E85Δ, the analysis of total protein suggests that more E81Δ is present in the supernatants at the time of harvest compared to E81, and this difference is not due to antibody affinity (Figure 3.2A). Increased E85Δ and E81Δ protein levels in the supernatant were also confirmed by precipitation with glycoprotein binding lectin beads (data not shown).

Although domain III was detected at low levels in the analysis of total protein (Figure 3.2A), intense bands migrating at the predicted apparent molecular weight (~12KDa) were detected in MAb immunoprecipitated cell lysates and supernatants from DEN2 DIII-VRP infected cells (Figure 3.2B), indicating that DIII is expressed intracellularly at high levels and efficiently secreted. Recognition of expressed DIII by MAb 3H5-1, which recognizes a conformational epitope in domain III, suggests that the protein retains this epitope in its structure. Bands migrating at the predicted apparent molecular weight were detected with very low intensity in cell lysates and supernatants immunoprecipitated by anti-DEN2 polyclonal sera. Epitopes in domain III may not be immunodominant in DEN2 NGC immunized mice, resulting in lower levels of antibodies that recognize domain III epitopes in mouse polyclonal sera.

***Differences in protein secretion and stability detected during expression of DEN2 VRPs in vitro*.**

To examine the kinetics of protein secretion and stability *in vitro*, pulse-chase labeling experiments were conducted where DEN2 VRP infected cells were metabolically radiolabeled with [^{35}S]-methionine and cysteine for one hour and chased

with excess unlabeled methionine and cysteine for time intervals of 0, 2, 6, 8 or 12 hours before harvesting the cell lysates and supernatants. The lysates and supernatants were immunoprecipitated with MAbs 4G2 or 3H5-1 and analyzed by SDS-PAGE (Figure 3.3). Strong bands were detected after 0 and 2 hours post chase (hpc) in DEN2 prME-VRP infected cell lysates, and protein levels were low to undetectable by 6 hpc. In the initial immunoprecipitation it appeared that lower levels of E were expressed in DEN2 prME-VRP infected cell lysates compared to expression from the other constructs (Figure 3.2). However, the results of the pulse-chase suggest that this difference is due to instability of E expressed from DEN2 prME-VRP rather than deficient expression. E protein was not detected at any time point in the supernatants, which suggests that the decrease in cellular levels of protein is not due to transport out of the cell.

E85Δ expressed from DEN2 E85Δ-VRP was detected up to 12 hpc in the cell lysates. Levels begin to decrease by 6 hpc, which could be due to secretion more than protein instability. E85 expressed from DEN2 E85-VRP was detected in the cell lysates up to 6 hpc and dropped to very low levels by 8 hpc. At 12 hpc, E85 was no longer detectable. E85 was not detected at any time point in the supernatants. Therefore, this decrease in protein levels in the cell lysates was most likely due to protein instability, suggesting that the amino acid changes in E85Δ may confer increased stability to the truncated form of E. Secreted E85Δ was barely detectable at 0 hpc, but peaks at 2 hpc and remains relatively stable up to 12 hpc. These results indicate that the difference in E85Δ and E85 secretion is not likely due to degradation of E85 in the supernatants prior to harvesting at 10hpi. Rather, the amino acid changes in E85Δ resulted in increased

efficiency of E85Δ secretion. The mechanism behind this difference remains to be elucidated.

E81Δ expressed from DEN2 E81Δ-VRP was detected in the cell lysates up to 8 hours post-chase and dropped to barely detectable levels by 12 hours post-chase. Similar results were observed in DEN2 E81-VRP infected cell lysates. However, levels of E81 were still detected at 12 hours post-chase. The difference in protein levels at 12 hpc indicates that E81 may be slightly more stable. However, because we detect more E81Δ in the supernatants compared to E81, the difference at 12 hpc is most likely due to secretion of E81Δ rather than instability of E81Δ. E81Δ secretion from infected cells peaked at 2 hpc and gradually decreased. The delay in secretion of E81Δ is similar to the delay observed with secretion of E85Δ, suggesting that processing and trafficking of the truncated forms of E takes approximately 2 hours. Secreted E81Δ was detected up to 12 hpc, indicating the protein remains relatively stable in the media. Very low levels of E81 were detected in the supernatants up to 12hpc. These results indicate that, similar to the amino acid changes in E85Δ, the amino acid changes in E81Δ result in increased efficiency of protein secretion.

Analysis of DIII immunoprecipitated from cell lysates showed two bands at the end of the 1 h pulse. One band migrated at a higher molecular weight, while the other band migrated approximately at the predicted apparent molecular weight of DIII. The larger band may contain the uncleaved tPA signal sequence. It is thought that the signal sequence is cleaved during transport from the ER to the Golgi apparatus. At early time points some of the DIII may still be associated with the tPA signal peptide, which may explain the presence of two bands. The levels of DIII gradually decrease but are still

detectable by 12 hpc. Because high levels of DIII were detected in the supernatants at each time point, the decrease in cellular DIII is most likely due to secretion rather than instability of DIII. Detection of DIII in the supernatants up to 12 hpc suggests that DIII remains relatively stable.

E85Δ associates with the plasma membrane of infected cells.

One of the questions we wanted to ask was whether the presence of the H1 domain in the E85 constructs, which is predicted to interact with the hydrophobic outer layer of the ER membrane, facilitates the association of the protein with the plasma membrane. A previous study has shown that including residues of the stem region results in E association with the cell membrane [76]. This may affect antigen presentation and immunogenicity. To determine if the E proteins associate with the plasma membrane of infected cells, BHK cells infected with DEN2 VRPs were analyzed by IFA (Figure 3.4). To visualize intracellular protein, the cells were fixed at 10hpi with either methanol or 2% PFA and permeabilized with TritonX-100. Specific staining by MAb 3H5-1 of all six configurations was observed, confirming that each construct expresses E protein recognized by a conformational MAb (Figure 3.4A). In order to visualize extracellular protein, cells were fixed 10hpi in 2% PFA, in the absence of a permeabilizing agent (Figure 3.4B). This excludes the staining Ab from entering the cell and allows specific staining of proteins exposed on the surface of the cell without staining of intracellularly localized protein. Specific staining on the cell surface above background levels was only observed in cells infected with DEN2 E85Δ-VRP, indicating that E85Δ is not only secreted but also associates with the cell membrane. This may aid in immune induction

by increased antigen presentation. Association with the cellular membranes may also promote proper folding. Although E85 also encodes the H1 domain and would be expected to associate with the plasma membrane, inefficient secretion of E85 may prevent E85 from reaching the plasma membrane.

These results indicate that each construct expresses E protein which is recognized by a conformationally dependent monoclonal antibody. They also show that at 10hpi, there are differences in association with the plasma membrane among the six constructs, which may be important for immune induction *in vivo*.

Immunogenicity in mice immunized with DEN2 VRPs.

To determine the optimal configuration of DEN2 E protein, the antibody responses elicited in BALB/c mice immunized with the six DEN2 VRPs were compared. For all the experiments, adult BALB/c female mice were immunized by s.c. injection into both rear footpads with the indicated VRP configuration. Serum samples were collected at different time points for analysis of antibody induction. Neutralizing antibody levels were evaluated by a flow cytometry-based neutralization assay[124] and total IgG production by ELISA.

The first immunogenicity study included three groups of six BALB/c female mice that were immunized at six weeks of age with a total dose of $5x10^5$ IU of DEN2 prME-, E85Δ- and E81Δ-VRP (Figure 3.5A). Mice were boosted at 4 weeks post prime (wpp) with the same doses. Serum samples were collected prior to the prime and every two weeks post prime and post boost. All mice had neutralizing antibodies by 2wpp. By 4wpp, mice immunized with DEN2 E85Δ- and E81Δ-VRP had significantly higher

neutralizing antibody titers compared to titers in mice immunized with DEN2 prME-VRP ($P<0.0001$) by at least 10-fold. Boosting mice with DEN2 E85Δ- and E81Δ-VRP resulted in a further 4-fold increase in neutralizing antibody titers by 2 weeks post boost (wpb). An increase in F-$Neut_{50}$ titers in mice immunized with DEN2 prME also was observed. However, neutralizing antibody titers in DEN2 E85Δ- and E81Δ-VRP were still significantly higher, by at least 16-fold. These titers remained high at 4wpb.

Antibody induction by E85 and E81 compared to prME was evaluated in a similar experiment. Three groups of six BALB/c female mice were immunized at six weeks of age with $5x10^5$ IU of DEN2 prME-, E85- and E81-VRP. Serum samples were collected at 2 and 4wpp assayed for neutralizing antibodies. Similar differences in antibody titers to titers observed in mice immunized with DEN2 E85Δ- and E81Δ-VRP were not observed in mice immunized with DEN2 E85- and E81-VRP (Figure 3.5B). Mice immunized with DEN2 prME-VRP had similar mean F-$Neut_{50}$ titers, compared to the previous *in vivo* studies, at both time points. DEN2 E85-VRP immunized mice had much lower neutralizing antibody titers at 2wpp, 4-fold less than titers induced by prME ($P=0.0065$). Mean F-$Neut_{50}$ titers induced by E85 were at or below the limit of detection by 4wpp. A significant decrease in immunogenicity was not observed in mice immunized with DEN2 E81-VRP at 2wpp, but by 4wpp neutralizing antibody titers were 4-fold lower than titers induced by prME. The difference in neutralizing antibody titers showed trends towards significance ($P=0.0985$). The decrease in neutralizing antibody titers at 4wpp also suggests that the amino acid changes in E85Δ and E81Δ may affect the longevity of neutralizing antibody titers. This would need to be further assessed by evaluating titers at later time points. Evaluating titers post boost for the E85 and E81

would also add to the characterization of the truncated configurations *in vivo*. These results suggest that E85 and E81 were less immunogenic in mice compared to prME, which was the converse of results observed in mice immunized with DEN2 E85Δ- and DEN2 E81Δ-VRP. To further clarify these differences, the truncated E constructs were compared in a head-to-head immunogenicity study.

Four groups of six BALB/c female mice were immunized at six weeks of age with 10^6 IU of DEN2 E85-, E85Δ-, E81- and E81Δ-VRP. Serum samples were collected at 2 and 4 wpp. This study confirmed the results obtained in the two previous studies. At 4wpp, E85Δ and E81Δ induced high mean F-$Neut_{50}$ titers, similar to those observed in the first immunogenicity study (Figure 3.5C). Similarly, mice immunized with DEN2 E85- and E81-VRP had significantly lower mean F-$Neut_{50}$ titers. The mean F-$Neut_{50}$ in mice immunized with DEN2 E85Δ-VRP was at least 12-fold greater than the mean F-$Neut_{50}$ induced in mice immunized with DEN2 E85 (P=0.0022). The mean F-$Neut_{50}$ induced in mice immunized with DEN2 E81Δ-VRP was at least 20-fold greater than the mean F-$Neut_{50}$ induced in mice immunized with DEN2 E81-VRP (P=0.0043). These results indicate that E85Δ and E81Δ induce significantly greater neutralizing antibody titers against DEN2 NGC.

The *in vivo* results indicated that E85Δ and E81Δ were the best antigen configurations, in terms of neutralizing antibody induction. Therefore, a dose response of the two antigens was evaluated. Six groups of three or six BALB/c female mice were immunized at six weeks of age with 10^6, $5x10^5$, or $5x10^4$ IU DEN2 E85Δ- or DEN2 E81Δ-VRP, and serum samples were collected at 4wpp. Although neutralizing antibody titers did seem to be dose dependent, a significant decrease in mean F-$Neut_{50}$ titers did

not occur with decreasing doses of DEN2 E85Δ-VRP (Figure 3.6A). Mice immunized with $5x10^4$ IU of DEN2 E85Δ-VRP had a mean F-$Neut_{50}$ less than 2-fold lower than mice immunized with 10^6 IU. A more significant difference was observed in mice immunized with DEN2 E81Δ-VRP (Figure 3.6B). The mean F-$Neut_{50}$ titer in mice immunized with 10^6 IU was 2-fold higher than the mean F-$Neut_{50}$ titer in mice immunized with $5x10^5$ IU. The mean F-$Neut_{50}$ titer in mice immunized with $5x10^4$ IU was at least 14-fold lower than the mean F-$Neut_{50}$ titer in mice immunized 10^6 IU (P=0.0238) and $5x10^5$ IU (P=0.0238), indicating that DEN2 E81Δ-VRP immunogenicity is more dependent on dose than DEN2 E85Δ-VRP immunogenicity and that E85Δ is an effective immunogen in mice, even at low doses.

In order to evaluate induction of neutralizing antibodies in mice immunized with DEN2 DIII in comparison to the response observed in mice immunized with DEN2 prME-VRP, we immunized groups of four or six BALB/c female mice at six weeks of age with $5x10^5$ IU of DEN2 prME-VRP and 10^6 IU or $5x10^5$ IU of DEN2 DIII-VRP. Serum samples were collected at 2 and 4wpp. Compared to the neutralizing antibody titers observed in mice immunized with DEN2 prME-VRP at 2wpp, the mean F-$Neut_{50}$ titer in mice immunized with $5x10^5$ IU of DEN2 DIII-VRP was almost 3-fold lower and did not increase significantly by 4wpp (Figure 3.7). Antibody induction was not improved with a higher dose. The mean F-$Neut_{50}$ titer in mice immunized with 10^6 IU of DEN2 DIII-VRP was lower at 2 and 4wpp than titers observed in mice that received the lower dose. Although the neutralizing antibody titers were lower, the immunogen design was able to induce a neutralizing response.

Previous studies expressing dengue E as antigen have shown that different E configurations induce high levels of total IgG antibody but fail to induce significant levels of neutralizing antibodies[71, 73, 76]. These studies suggest that, in reference to total IgG, the proportion of antibody that is capable of neutralizing virus induced by E immunogens has historically been low. To evaluate total IgG antibody titers induced by DEN2 VRPs in mice, an ELISA was conducted in which sera collected from mice immunized with the six DEN2 VRPs at 4wpp were tested for their ability to bind to DEN2. Each of the six constructs induced anti-DEN2 IgG mean OD=0.2 titers greater than $\log_{10}3$ (Figure 3.8A). The highest anti-DEN2 titers were observed in mice immunized with DEN2 prME-, DIII- and E81Δ-VRP. To get an idea of what proportion of total IgG antibodies were neutralizing, the total IgG DEN2 ELISA titer (log10) was plotted on the same axis as the F-$Neut_{50}$ titer of each mouse at 4wpp converted to log10 (Figure 3.8B). Although total IgG titers cannot be compared directly to neutralizing antibody titers due to differences in the two assays, it was evident that a greater proportion of the antibodies produced by mice immunized with DEN2 E85Δ- and E81Δ-VRP were neutralizing compared to antibody proportions in mice immunized with the other constructs. To help evaluate any correlation between total anti-DEN2 IgG and neutralizing antibody titers, F-$Neut_{50}$ titers at 4wpp converted to log10 were plotted against the total IgG ELISA titer (log10) against DEN2 (Figure 3.8C). A line of identity where neutralization titers and total ELISA titers have the same value was added to assist in the comparison of the relative amount of neutralizing antibody as a proportion of total IgG between immunogens. The data points for the E81Δ and E85Δ immunogens all lie much closer to the dotted diagonal line than do the data points for the E81 and E85

immunogens or the data points for the prME and DIII immunogens. This suggests that, regardless of the actual quantitative relationship between the sensitivity of the neutralization assay versus that of the virus-specific ELISA assay, a much larger proportion of the total antibody produced in response to immunization with E81Δ and E85Δ has the capacity to neutralize the test DEN2 serotype virus compared to the neutralizing response elicited by immunization with any of the other immunogens.

In order to evaluate cross-reactivity induced by the six VRP constructs, the sera collected at 4wpp were also tested for their ability to recognize the other three serotypes: DEN1, 3, and 4. As expected, prME, E85 and E81 VRPs induced cross-reactive IgG ELISA titers (Figure 3.8D). DEN2 prME-VRP induced significantly lower levels of cross-reactivity compared to titers induced by E81 ($P<0.01$), E81Δ ($P<0.01$) and DIII ($P<0.01$) against DEN1, DEN3 and DEN4. We did not see any significant changes in IgG ELISA titers against the other serotypes between groups immunized with the truncated E constructs, suggesting that the amino acid changes did not affect the cross-reactive antibody response. It would be important to determine if the amino acid changes affected cross-neutralization.

Based on previous results suggesting that expression of domain III induces a more serotype-specific response in mice[96], mice immunized with DEN2 DIII-VRP were expected to have lower heterotypic responses compared to the other constructs. However, DIII induced high titers of IgG against the four serotypes (Figure 3.8D). The high cross-reactive titers observed in mice immunized with DEN2 DIII-VRP may be due to incomplete or improper folding. High heterotypic responses may also be due to

presence of cross-reactive epitopes in DIII or the availability of additional epitopes when DIII is expressed out of context of the native E protein.

Figures

Figure 3.1. Designs of the six DEN2 E configurations. Schematic representations of the gene cassettes expressing 6 different DEN E configurations, cloned into the multiple cloning site of VEE replicon vector pVR21. Capsid signal sequence (□), pr sequence (horizontal stripes), M protein (dark gray), domains I and II of E (light gray), domain III of E (gray), TPA signal sequence (diagonal stripes), transmembrane domains (solid black bars), changes to the amino acid sequences compared to the E sequence in the DEN2 prME-VRP construct(*).

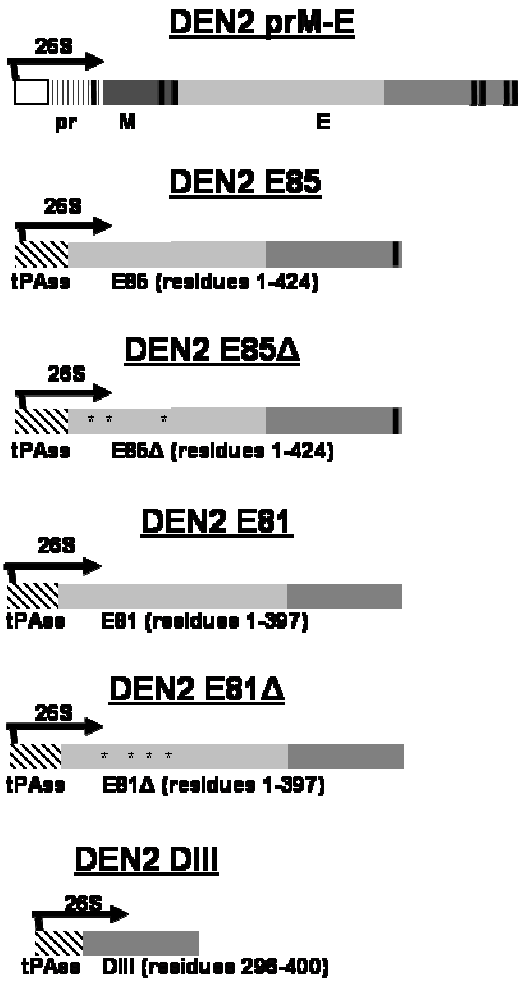
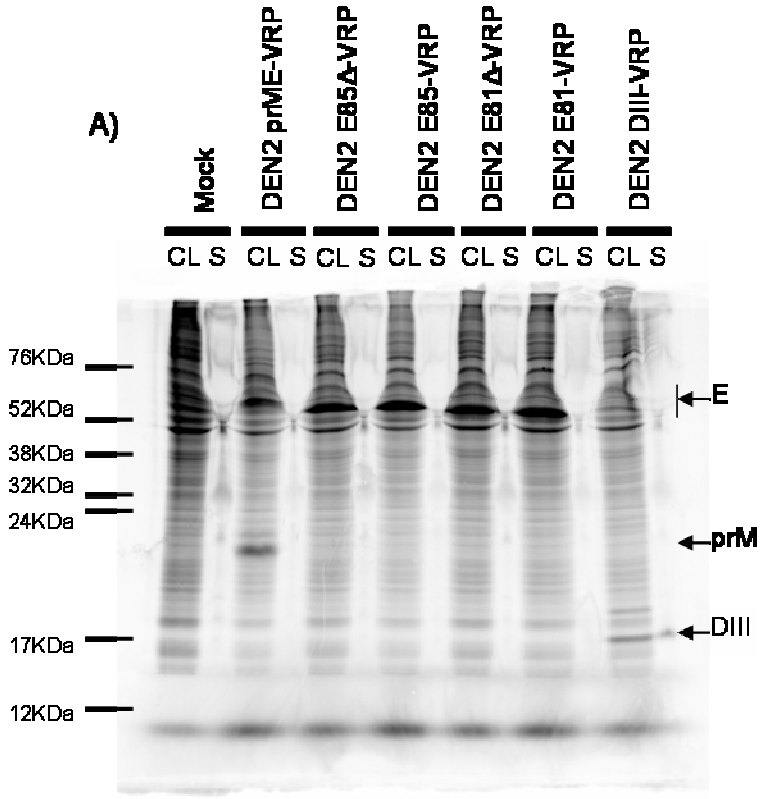
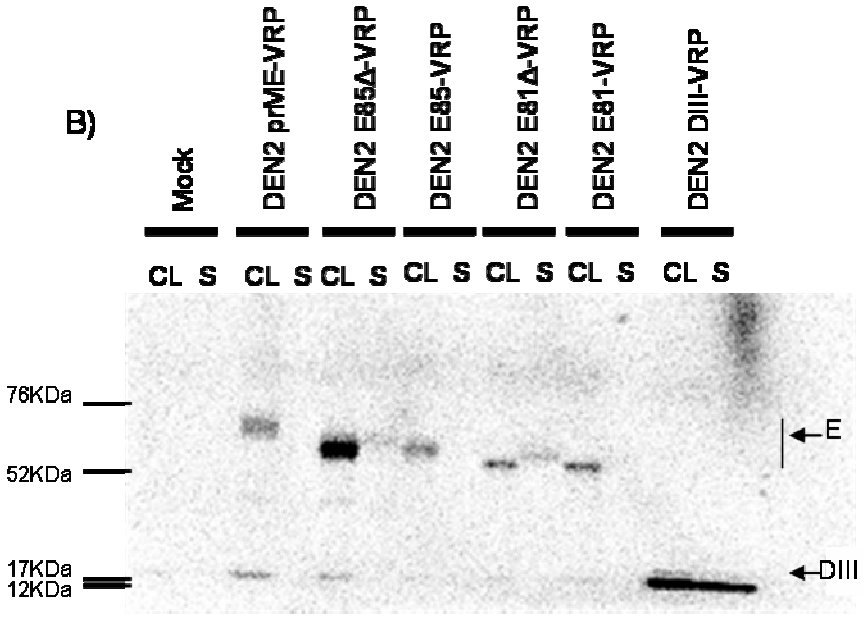

DEN2 prM-E
26S
pr
M
E
DEN2 E85
26S
tPAse
E85 (residues 1-424)
DEN2 E85Δ
26S
tPAse
E85Δ (residues 1-424)
DEN2 E81
26S
tPAse
E81 (residues 1-397)
DEN2 E81Δ
26S
tPAse
E81Δ (residues 1-397)
DEN2 DIII
26S
tPAse
DIII (residues 296-400)

Figure 3.2. Expression and secretion of prME, truncated E and DIII subunit from DEN2 VRP infected cells. BHK cells were mock infected or infected at an MOI of 10 with each of the six DEN2 VRP constructs, starved for 1 hour, and radiolabeled with [^{35}S]-methionine and cysteine at 5hpi. Supernatants and cell lysates were harvested at 10hpi. A) The entire cell lysate (CL) and concentrated supernatant (S) were analyzed by 15% SDS-PAGE. Molecular weight standards are indicated on the left. B) The cell lysates and supernatants were immunoprecipitated with MAb 4G2, or 3H5-1 for DIII, and analyzed by 12% SDS-PAGE. C) The cell lysates and supernatants were immunoprecipitated with pooled polyclonal anti-dengue 2 NGC sera and analyzed by 12% SDS-PAGE. The apparent molecular weights of E, prM and DIII are indicated to the right of the gel.


A)
Mock
DEN2 prME-VRP
DEN2 E85Δ-VRP
DEN2 E85-VRP
DEN2 E81Δ-VRP
DEN2 E81-VRP
DEN2 DIII-VRP
CL S CL S CL S CL S CL S CL S CL S
76KDa
52KDa
38KDa
32KDa
24KDa
17KDa
12KDa
E
prM
DIII


B)
Mock
DEN2 prME-VRP
DEN2 E85Δ-VRP
DEN2 E85-VRP
DEN2 E81Δ-VRP
DEN2 E81-VRP
DEN2 DIII-VRP
CL S CL S CL S CL S CL S CL S CL S
76KDa
52KDa
17KDa
12KDa
E
DIII

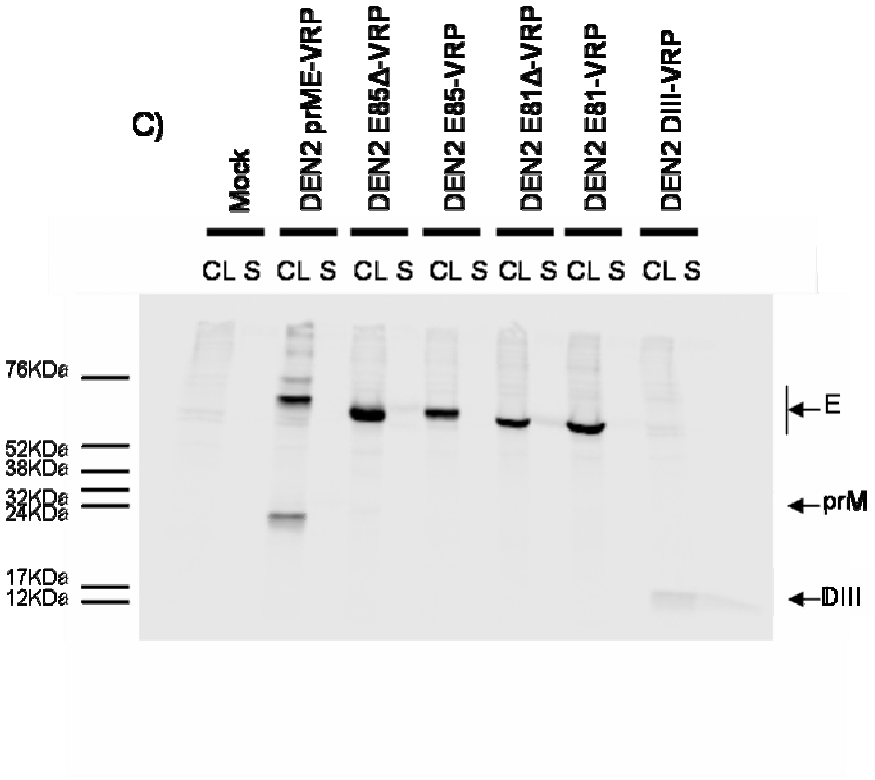

C)
Mock
DEN2 prME-VRP
DEN2 E85Δ-VRP
DEN2 E85-VRP
DEN2 E81Δ-VRP
DEN2 E81-VRP
DEN2 DIII-VRP
CL S CL S CL S CL S CL S CL S CL S
76KDa
52KDa
38KDa
32KDa
24KDa
17KDa
12KDa
E
prM
DIII

Figure 3.3. Protein secretion and stability upon DEN2 VRP infection. BHK cells were mock infected or infected at an MOI of 10 with each of the six VRP constructs, starved for 1 hour, radiolabeled with [^{35}S]-methionine and cysteine for 1 hour at 5hpi, and chased with excess unlabeled methionine and cysteine. Cell lysates (CL) and supernatants (S) were harvested at several time points (0, 2, 6, 8 and 12 hours of chase). Hours of chase are indicated above the lanes. Harvested CL and S were immunoprecipitated with MAb 4G2 or 3H5-1 (DIII). Immunoprecipitated lysates and supernatants were analyzed by 12% SDS-PAGE (15% SDS-PAGE for DIII samples).

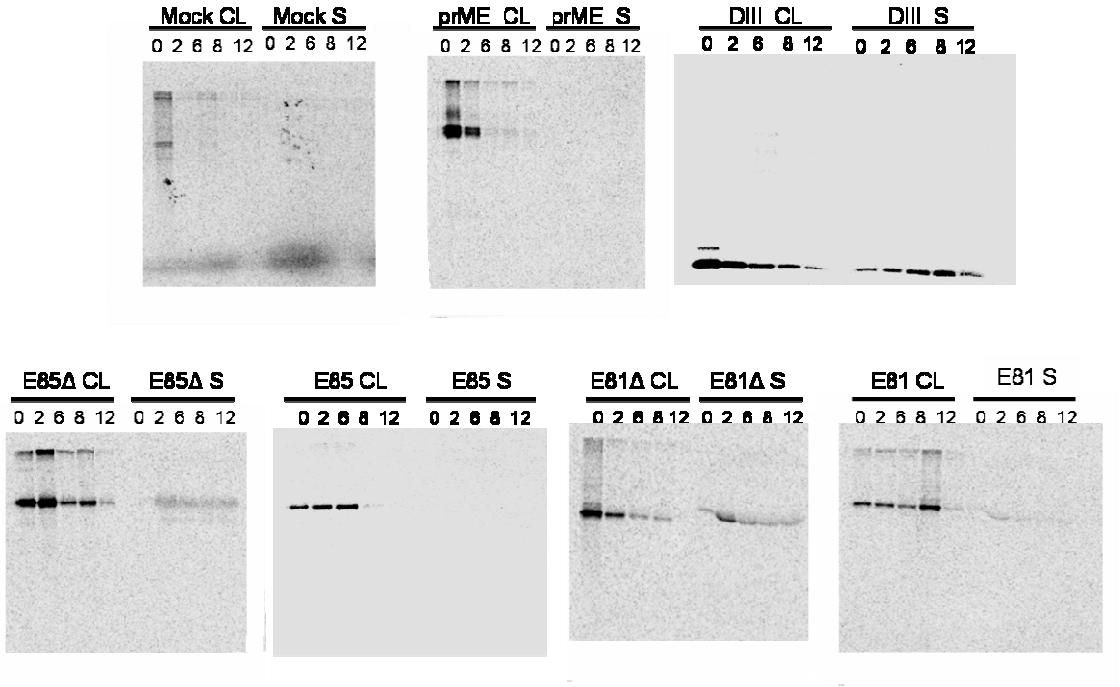


Figure 3.4. Expression and cell membrane association of E in DEN2 VRP infected cells. BHK cells were mock infected or infected at an MOI of 5 and fixed at 10hpi. A) Cells were fixed in methanol or 2% PFA (DIII) and permeabilized with 0.1% TritonX-100. B) Cells were fixed with 2% PFA without permeabilizing. Cells were stained with MAb 3H5-1 and visualized by fluorescence microscopy.

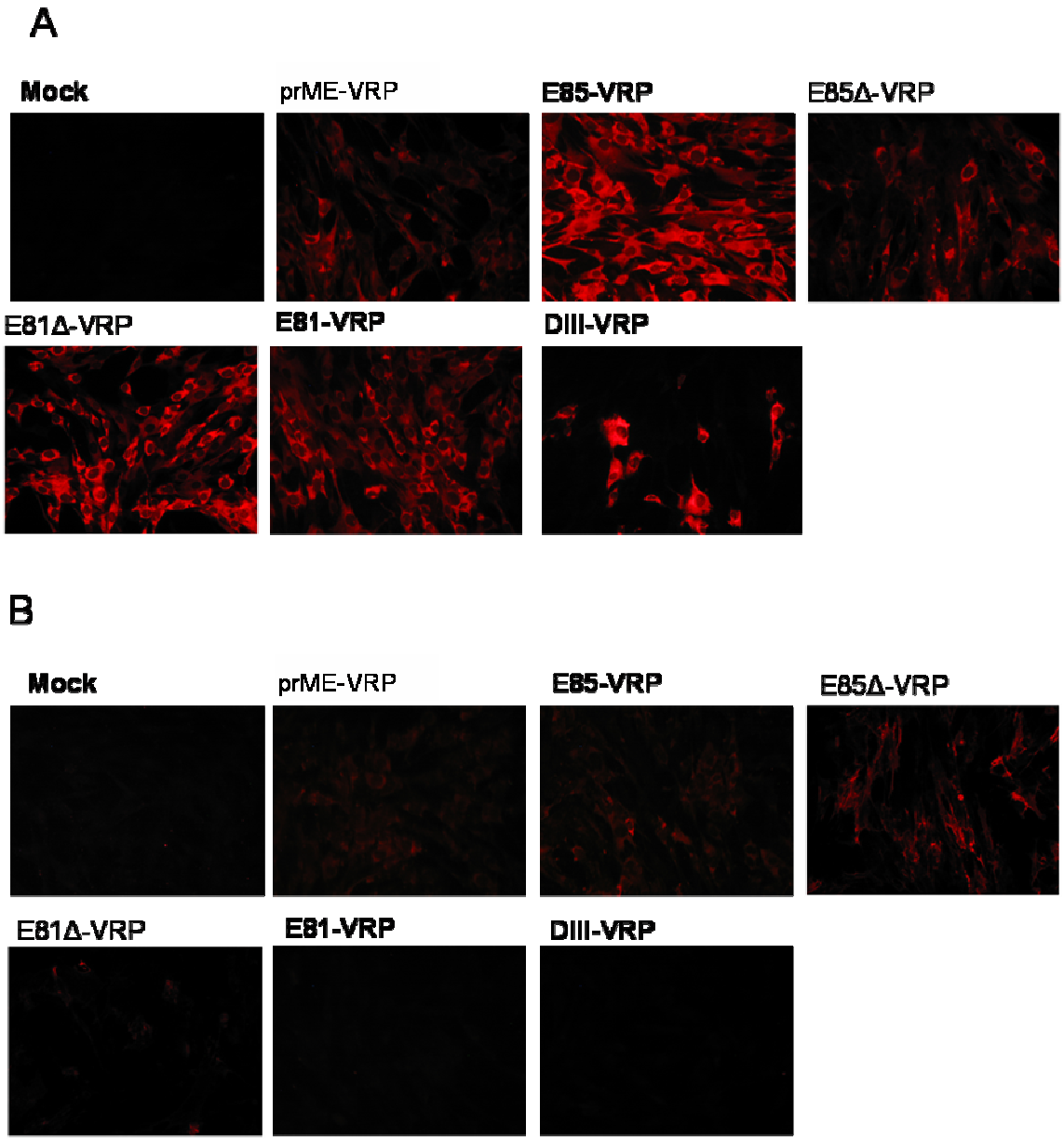
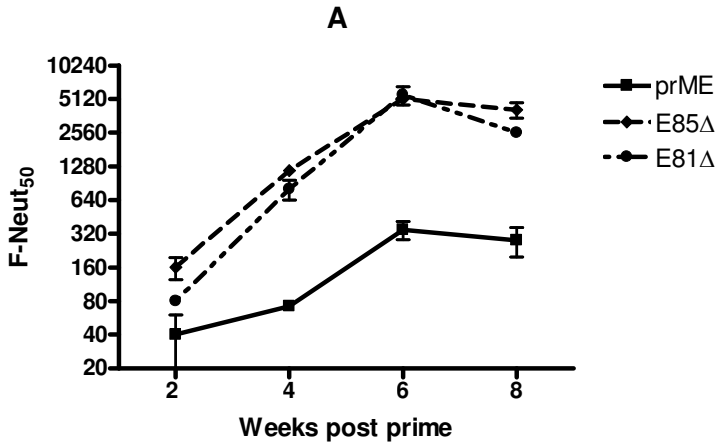
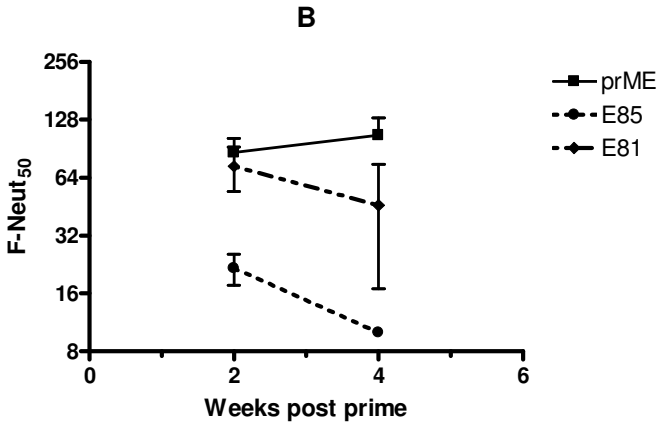


Figure 3.5. Neutralizing antibody titers induced by truncated E expressed from VRP. A) Three groups of 6 BALB/c female mice were immunized by s.c injection with $5x10^5$IU of DEN2 prME-, DEN2 E85Δ- and DEN2 E81Δ-VRP. 10μl was delivered to each rear footpad. The mice were boosted with the same dose at 4wpp. Mice were bled every two weeks from the tail vein. Sera was separated and tested for neutralizing antibody titers by FRNT. B) 3 groups of 6 BALB/c mice were immunized by s.c. injection with $5x10^5$IU of DEN2 prME-, DEN2 E85- and DEN2 E81-VRP. Volumes administered were the same as described above. Mice were bled at 2 and 4 wpp. Sera was separated and tested for neutralizing antibody titers by FRNT. The error bars represent the standard error mean (SEM). C) 4 groups of 6 BALB/c mice immunized by s.c. infection with 10^6IU of DEN2 E85Δ-, DEN2 E81Δ-, DEN2 E85- and DEN2 E81-VRP. Volumes and bleeding were as described above. Sera was separated and tested for neutralizing antibody titers by FRNT. For each group, titers from individual mice are shown and the geometric mean is indicated by the black bar.


A
10240
5120
2560
1280
640
320
160
80
40
20
F-Neut50
2
4
6
8
Weeks post prime
prME
E85Δ
E81Δ


B
256
128
64
32
16
8
F-Neut50
0
2
4
6
Weeks post prime
prME
E85
E81

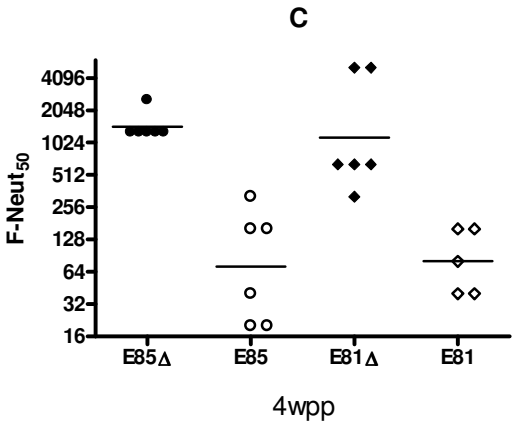

C
4096
2048
1024
512
256
128
64
32
16
F-Neut50
E85Δ
E85
E81Δ
E81
4wpp

Figure 3.6. Dose response for DEN2 E85Δ- and E81Δ-VRP in mice at 4wpp. Three groups of 3 or 6 BALB/c female mice were immunized by s.c. injection in the rear footpads with 10^6, $5x10^5$ and $5x10^4$ IU of DEN2 E85Δ-VRP (A) or DEN2 E85Δ-VRP (B). 10µl was delivered to each rear footpad. Mice were bled from the tail vein at 4wpp. Serum was separated and tested for neutralizing antibody titers by FRNT. Titers from individual mice are shown and the geometric mean is indicated by the black bar.

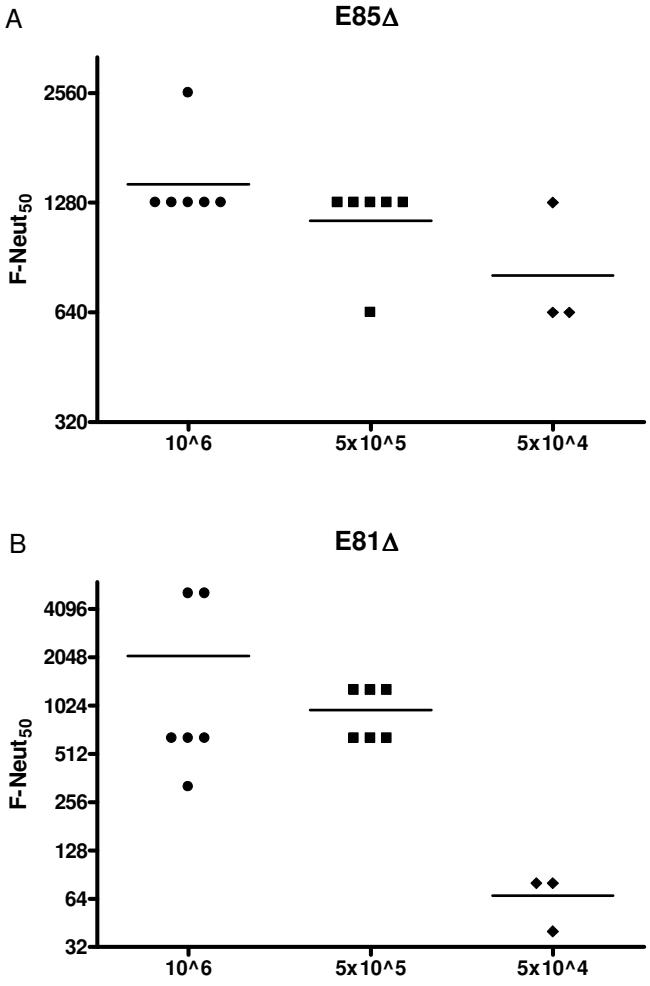


Figure 3.7. Neutralizing antibody titers induced by domain III. Three groups of 4 or 6 BALB/c female mice were immunized by s.c. infection in the rear footpads with $5x10^5$ IU of DEN2 prME- or DIII-VRP or 10^6 IU of DEN2 DIII-VRP. 10µl was delivered to each rear footpad. Mice were bled every two weeks from the tail vein. Serum was separated and tested for neutralizing antibody titers by FRNT. Mean titers and the SEM for each group are represented below.

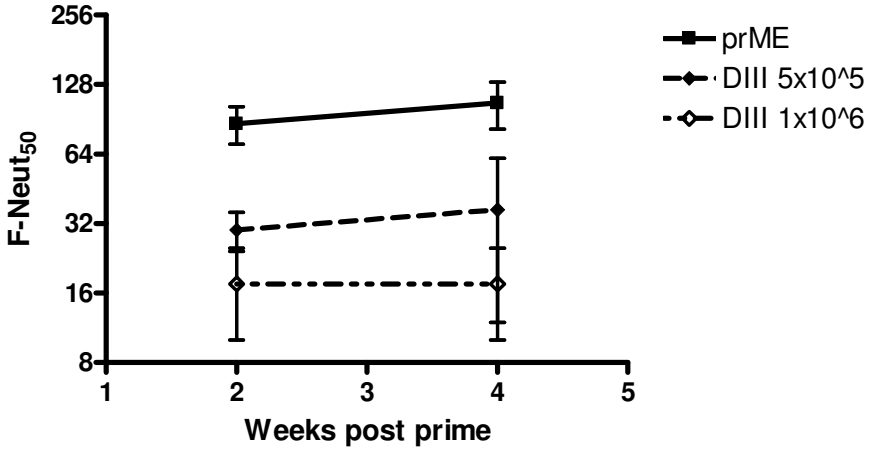
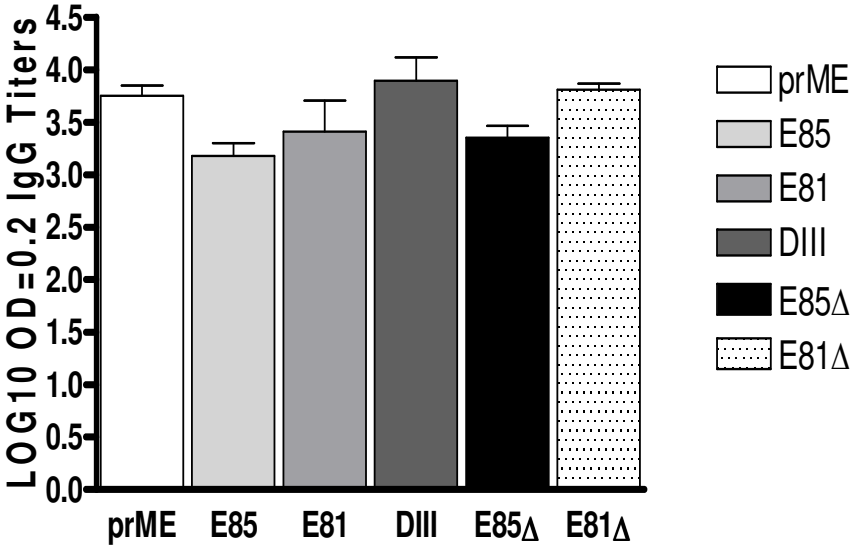
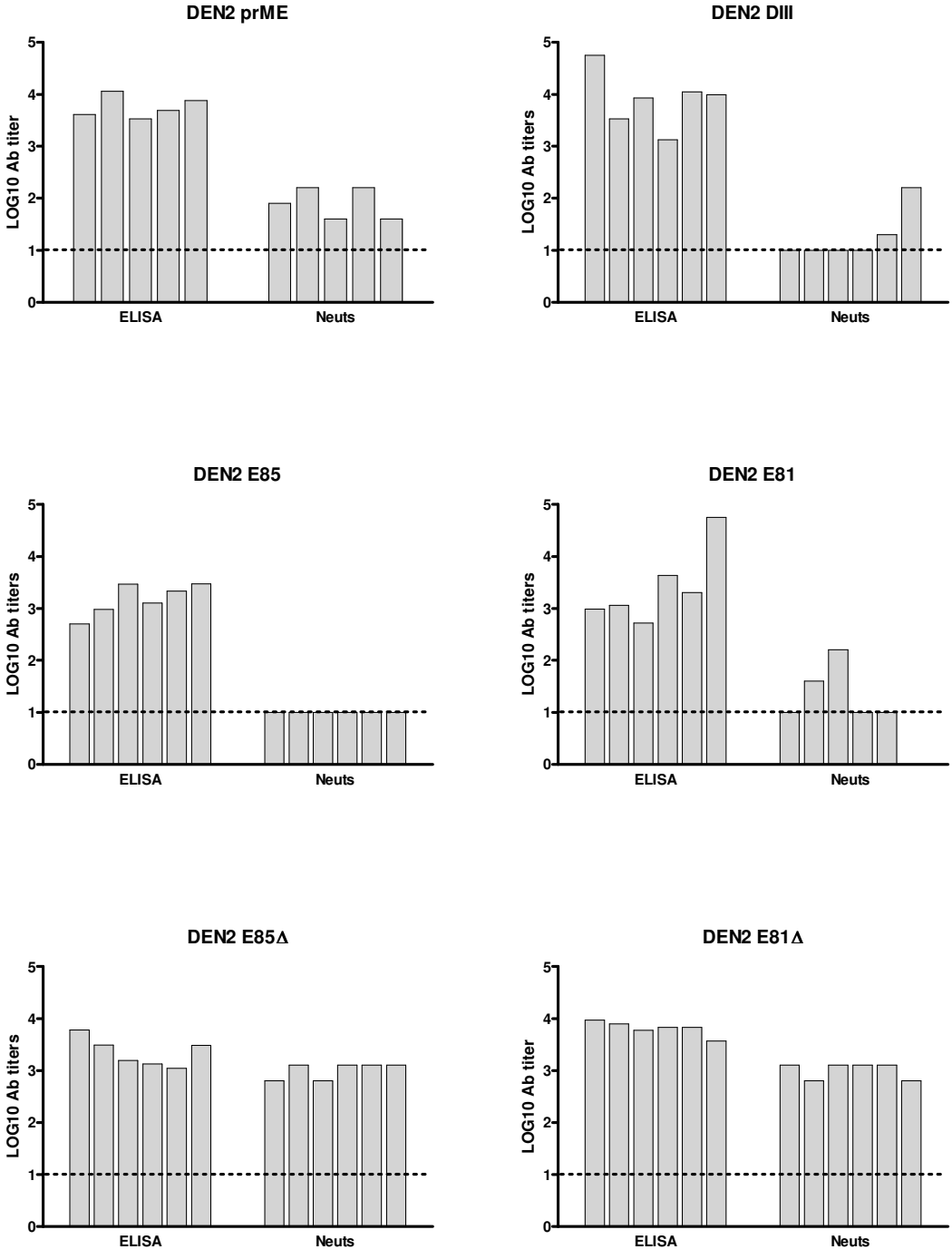


Figure 3.8. Total IgG ELISA titers induced by the DEN2 VRPs. Sera collected from mice immunized with $5x10^5$ IU of DEN2 prME-, E85Δ-, E81Δ-, E85-, E81- or DIII-VRP at 4wpp was tested for total IgG titers against DEN1, 2, 3, and 4. Plates were coated with purified virus. The signal produced by substrate binding of the secondary goat anti-mouse-HRP was measured at 450nm. A sigmoidal dilution curve was fit to the observed data points by regression analysis using least squares fit. The equation describing the curve was solved for the y value=0.2. This x value is considered the OD=0.2 titer. A) The mean total anti-DEN2 IgG antibody titers were ploted as log10. The error bars represent the SEM. B) Anti-DEN2 IgG titers converted to log10 of each individual mouse was plotted on the same axis as log10 converted F-$Neut_{50}$ values for each mouse. The limit of detection for the neutralizing antibody titers is indicated by a dotted line. C) F-$Neut_{50}$ titers of individual mice from 4wpp were converted to log10 were plotted against total IgG ELISA titers against DEN2 converted to log10 in a scatter plot. The dotted line represents identity between ELISA titers and F-$Neut_{50}$ titers, where F-$Neut_{50}$ titers and ELISA titers have the same value. D) Log10 values of the total IgG antibody titers (ELISA) against all four serotypes.

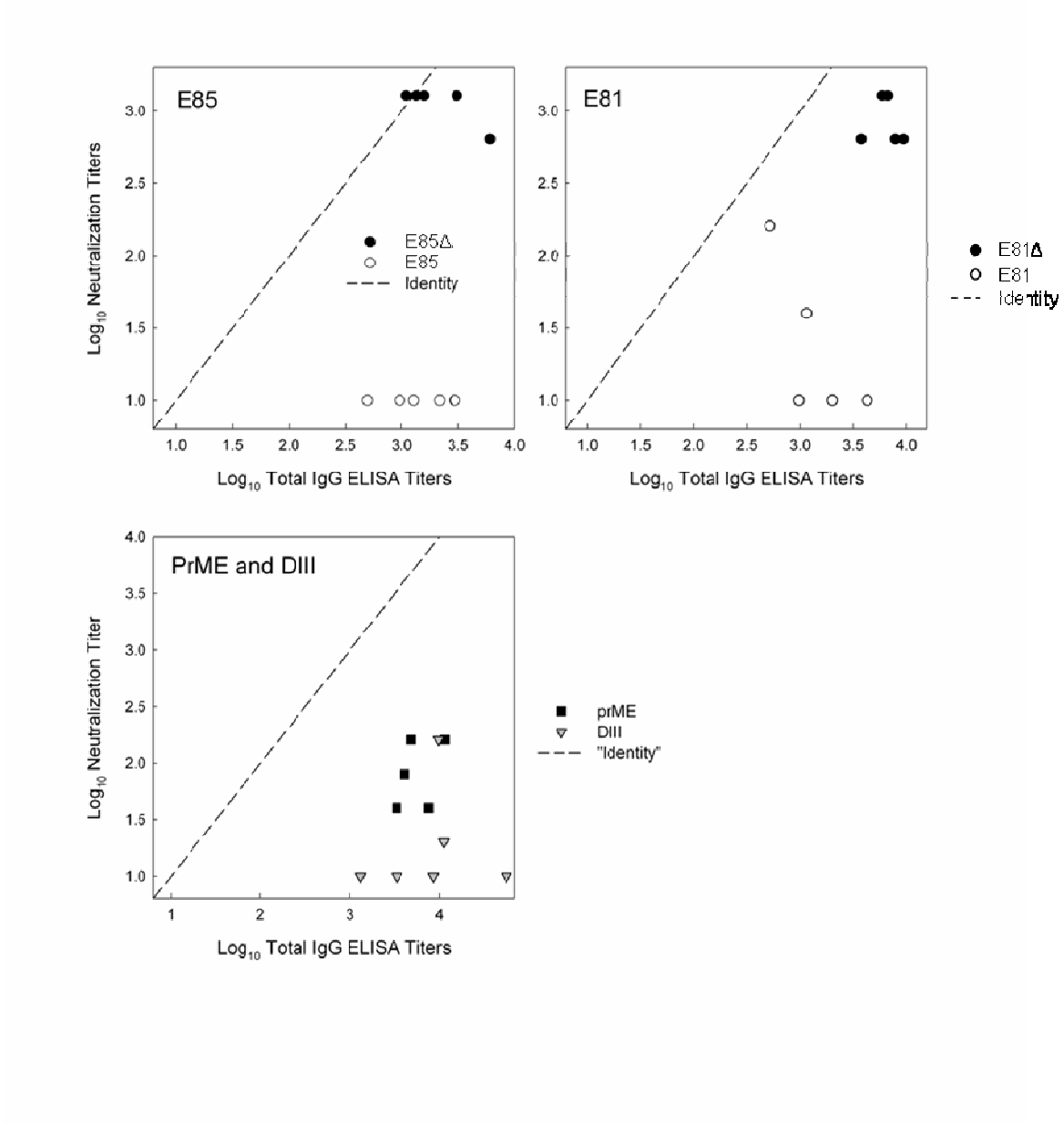
A


LOG10 OD=0.2 IgG Titers
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
prME
E85
E81
DIII
E85Δ
E81Δ
prME
E85
E81
DIII
E85Δ
E81Δ

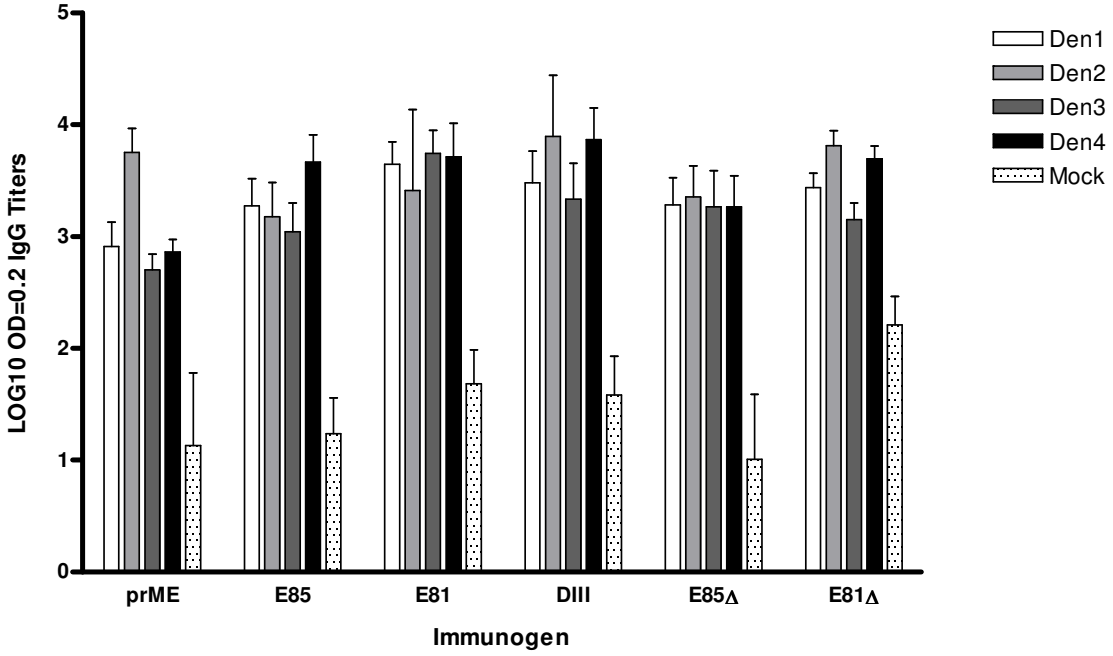
B


DEN2 prME
DEN2 DIII
DEN2 E85
DEN2 E81
DEN2 E85Δ
DEN2 E81Δ
LOG10 Ab titer
LOG10 Ab titers
ELISA
Neuts
0
1
2
3
4
5

C


E85
E85Δ
E85
Identity
Log10 Neutralization Titers
Log10 Total IgG ELISA Titers
E81
E81Δ
E81
Identity
Log10 Total IgG ELISA Titers
PrME and DIII
prME
DIII
"Identity"
Log10 Neutralization Titer
Log10 Total IgG ELISA Titers

D


LOG10 OD=0.2 IgG Titers
5
4
3
2
1
0
prME
E85
E81
DIII
E85Δ
E81Δ
Immunogen
Den1
Den2
Den3
Den4
Mock

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Several characteristics are considered optimal for a dengue vaccine. In terms of the intensity of the antibody response, the ideal vaccine should induce a strong and long lasting neutralizing antibody response against each of the four serotypes simultaneously. In terms of the quality of the immune response, the ideal vaccine should induce antibodies that are associated with protection and avoid the induction of non-neutralizing antibodies that could enhance infection without a strong neutralizing response. Gene-based dengue vaccine research, which has utilized viral vectors, DNA plasmids and subunit proteins, has focused on expressing the major dengue surface glycoprotein E, which is the major target of protective neutralizing antibodies. One important factor in development of these vaccine platforms is to determine the best way to present E to the immune system as well as to demonstrate specific configurations or domains of E that are more immunogenic. This study was aimed at answering this question, using the VEE replicon particle vectors as a vaccine platform.

Previous dengue vaccine studies from our laboratory have shown that expression of prM and E from VRP results in a rapid induction of a long lasting and significant immune response compared to mice immunized with live virus[121]. In order to determine if this immune induction could be further improved upon, truncated and subunit forms of E were characterized *in vitro* and *in vivo*. The current study provides further characterization of full-

length E expressed in the presence of prM and provides evidence that some modifications to the configuration of the E glycoprotein launched from VRP do not significantly affect immunogenicity, while other changes may contribute to better presentation of neutralizing epitopes.

Results from the previous study indicated that full-length E was expressed from DEN2 prME-VRP and could be detected in the cell medium after pelleting, most likely on the surface of VLPs[121]. The formation of VLPs during expression of full-length E in the presence of prM had been previously described for DEN and other flaviviruses[23, 126, 127]. It has been hypothesized that secretion of VLPs may correlate with immune responses. In the current study, full-length E was not detected in the supernatants of cells infected with the prME construct. However, the supernatants were unconcentrated prior to immunoprecipitation, perhaps leading to levels of E below the limit of detection by MAb or polyclonal mouse sera. Most importantly, the results from this study show that full-length E expressed with prM is much less stable compared to the other E configurations. It has been shown that improper folding, glycosylation or processing by glycosidases of prM or E results in instability of the prM and E heterodimers[128], which may explain the shorter half-life of full-length E. This may also explain the absence of prM or E in the supernatants. Although significant neutralizing titers were detected in DEN2 prME-VRP immunized mice compared to titers induced by live virus, these titers were significantly lower than neutralizing titers induced by DEN2 E85Δ-VRP and DEN2 E81Δ-VRP, consistent the observed instability and deficiency in secretion.

Previous characterization of C-terminally truncated E proteins from dengue and Japanese encephalitis provided evidence that expression of the ectodomain results in efficient

extracellular secretion, while secretion was not observed with E proteins encoding additional residues downstream of the ectodomain, responsible for membrane association and anchoring[76]. It was hypothesized in these studies that the residues downstream of S397 of E may be responsible for directing cellular distribution of E, which prevented export and secretion. It was also indicated that configurations expressing the ectodomain and excluding the transmembrane domains were the only E proteins that induced neutralizing antibodies in mice. In the current study, it was thought that employing a strong signal peptide at the N-terminus of the E sequence would result in efficient secretion of the truncated E configurations. *In vitro* expression of E85 and E81 revealed that contrary to initial predictions, these proteins were secreted at very low or undetectable levels and neither was found in association with the plasma membrane. These truncated forms of E, designed to increase secretion (E81) or both secretion and membrane association (E85), did not significantly improve the total IgG antibody or the neutralizing antibody responses, compared to the response elicited by full-length E in the presence of prM. Rather, these constructs elicited reduced neutralizing antibody responses.

The two intermediate configurations E81Δ, with four amino acid changes (A54V, S112G, I124N and A197V), and E85Δ, with three amino acid changes (T33A, D58K and A197V), had more interesting phenotypes. Compared to their counterparts, these proteins appear to have increased intracellular stability and are present at higher levels in the culture media as stable secreted forms. While they induce similar levels of total dengue-specific IgG antibodies, a higher proportion of those antibodies are able to neutralize DEN2 NGC *in vitro*. Similar neutralizing antibody titers induced by E85Δ and E81Δ and by E85 and E81 suggest that presence of the H1 domain does not have a significant affect on neutralizing antibody

responses. However, because the sequences of E85Δ and E81Δ are different, it is difficult to draw final conclusions. It will be important to further evaluate the role of the H1 domain once we have determined the residues responsible for the increase in neutralizing antibody responses. The results observed for the truncated E constructs also suggests that there is a correlation between increased protein secretion observed *in vitro* and induction of increased mean F-$Neut_{50}$ titers. Secretion of antigenic proteins could aide in the presentation of neutralizing epitopes, resulting in a more neutralization oriented humoral response. Antigen secretion may also impact memory responses, which would account for the drop in titers 4 weeks after prime associated with E85 and E81 expression and very low levels of secretion.

The differences observed in protein secretion during *in vitro* expression of the truncated E constructs indicate that specific point mutations in the ectodomain may result in conformational changes that can impact protein export and secretion. However, the mechanism for this is still unknown. Comparison of the envelope sequences of each of the six constructs to the sequence of the DEN2 NGC strain used in the neutralization assays indicates that each construct sequence differs from the parental viral sequence. It has been hypothesized that concerted reorganization across the entire surface of the ectodomain is likely to occur with any conformational changes[32]. Analysis of the published crystal structure of the ectodomain[32] using a molecular visualization system (PyMol) and a protein design simulation program (Rosetta design) supports this hypothesis, indicating that these amino acid changes may result in significant changes in side chain repacking in several regions of the ectodomain. Although these changes do not necessarily affect binding of MAbs used in this study, they may affect other aspects of protein processing and secretion. Some of these changes, specifically residues 112 and 124, are within 4 angstroms of residues

that are located along the dimer interface, suggesting that dimerization of E may have been affected by these residue changes, but this has not been measured directly. Although the role of dimerization in secretion of truncated E proteins has not been fully investigated, it may affect secretion efficiency. Incomplete or inhibited dimerization may prevent complete vesicular trafficking or lead to decreased stability in the low pH environment of the vesicles. Amino acid 197 is within 4 angstroms of residues located in a hairpin shown to affect the pH threshold for membrane fusion[32]. Side-chain reorganization in this region resulting in a change in the pH threshold may also have an effect on secretion of the truncated E configurations.

Changes in post-translational modification may also play a role in the different secretion phenotypes. Lorenz et al. observed a dramatic reduction in the secretion of TBE soluble E when glycosylation or glucose trimming was inhibited[23]. It has been hypothesized that proper glycosylation may affect E homodimer formation. Although glycosylation of the E configurations was not evaluated directly in this study, analysis of whether the E configurations are being differentially glycosylated or glucose trimmed would be important in determining if post-translational modifications are affecting the secretion of soluble E formations.

Although we have observed a strong correlation between protein secretion and neutralizing antibody induction, we also need to take into account possible changes in antigenic structure. Previous studies in which epitopes were mapped by isolating and sequencing neutralization escape variants (reviewed in [129]) have determined six neutralization regions on the E glycoprotein. The residue changes in E85Δ and E81Δ involve amino acids that have been directly associated with neutralization escape or are within 4

angstroms of other amino acids directly involved in neutralization escape. Analysis of the ectodomain by protein modeling programs suggests that the changes in E85Δ and E81Δ result in significant side-chain reorganization in these neutralization regions. Therefore, the increase in neutralizing antibody induction may also be due to the enhancement of neutralizing epitopes promoted by specific amino acid changes in E85Δ and E81Δ. This is supported by the difference in affinity of 4G2 for E85Δ compared to E85. It is uncertain which specific amino acid changes may be responsible for this increase in induction. However, the results of this study indicate that changes to the ectodomain sequence can have a significant impact on neutralizing antibody induction. Analysis of viral neutralization of different dengue 2 strains will be important to determine if this increase is strain specific. To determine if these changes will affect the induction of a neutralizing response in other configurations, the amino acid changes would need to be introduced into the prME sequence of full-length E as well as truncated forms.

It has been proposed for some flaviviruses, including WNV, JEV and DEN, that type-specific neutralizing antibodies map to E DIII[108, 130-132]. The hypothesis that immunizing with DEN DIII results in a type-specific, neutralizing antibody response has been tested by Simmons et al. and Mota et al.[96, 101]. In these studies, immunization of mice with recombinant DIII as a subunit vaccine elicited serotype-specific antibody responses capable of viral neutralization and protection from challenge. Based on these studies, we predicted that expressing domain III in the absence of other DEN2 E sequences from VRPs would induce a strong neutralizing antibody response with limited induction of cross-reactive, non-neutralizing antibodies. If this hypothesis were true, a tetravalent formulation based on all four DIII antigens expressed from the same VRP would be a

promising strategy. The high levels of intracellular and extracellularly secreted DIII proteins detected during *in vitro* infection with DEN2 DIII-VRP suggested that the configuration may induce significant antibody responses in mice, based on the immunogenicity results observed for the truncated Δ configurations. The anti-DEN2 IgG ELISA titers in mice immunized with DEN2 DIII-VRP were as high as or even higher than those detected in mice immunized with all other configurations, indicating that the DIII antigen configuration is highly immunogenic. However, the neutralizing antibody titers were never higher than the mean F-$Neut_{50}$ titers detected in mice immunized with DEN2 prME-VRP, which may be due to improper presentation of neutralizing epitopes without the presence of additional E sequences or other carrier sequences.

Levels of cross-reactive IgG to the other three serotypes induced in mice immunized with DEN2 DIII-VRP were higher than expected, suggesting that our DIII design predominantly presented non-neutralizing epitopes that are common to the other serotypes. Sukopolvi et al. have shown that while serotype and subcomplex-specific MAbs map to epitopes in domain III, a conserved region in domain III predicted to have limited access in the context of the mature virion is recognized by poorly neutralizing and cross-reactive antibodies[91]. This study suggests that expression of domain III alone and out of the context of the ectodomain may result in not only serotype-specific immune responses, but cross-reactive responses as well, due to increased availability to cross-reactive, non-neutralizing epitopes. This may account for the high levels of cross-reactive antibodies elicited in mice by the DIII configuration.

The two bands detected at early time points in DEN2 DIII-VRPs infected cell lysates during pulse-chase experiments may offer an alternative explanation for the unexpected

immunogenicity data. It was hypothesized that the presence of two bands migrating at different molecular weights was due to cleavage of the tPA sequence during transport from the ER to the Golgi apparatus. The predicted molecular weight of the tPA sequence (~2KDa) may account for the band migrating at a higher molecular weight. However, this observation may also be explained by partial degradation of domain III that might occur during transport from the ER. Saejung et al. showed that expression of domain III with a hexahistidine tag and a signal peptide at the N-terminus from a tobacco mosaic viral (TMV) vector in plants resulted in two bands detected by domain III specific monoclonal antibodies[100]. Only the band migrating at the higher molecular weight was detected by an anti-His antibody, suggesting that cleavage at the C-terminus had occurred during expression. Most of the studies showing serotype-specific antibody responses elicited by immunization with domain III involved expression of domain III in association with other proteins[96, 105]. Other studies involving expression of domain III alone from other vectors have not evaluated cross-reactive antibody titers. One group expressing DIII alone from a plasmid observed serotype-specific antibody induction in mice[101]. However, a signal peptide was not included at the N-terminus to promote retention in the ER membrane and secretion. These studies suggest that expression of domain III with an ER retention signal peptide may result in partial degradation at the C-terminus of DIII, which could explain the induction of a more cross-reactive response elicited by the DIII design and may also explain low levels of neutralizing antibodies.

Modifications to the DIII design may result in improved immune responses elicited in mice. Expression of several DIII sequences from the same serotype separated by flexible linkers may allow for expression of a more stable protein similar to its native conformation.

This may promote multimerization, which may aide in proper folding and could protect the C-terminus or other regions of the domain from any cleavage or degradation. However, the presentation of neutralizing epitopes would need to be verified by detection with several monoclonal antibodies mapping to type-specific neutralizing epitopes on domain III. If domain III can be successfully expressed as a multimer, resulting in serotype-specific, neutralizing immune responses *in vivo*, then a tetravalent DIII design may be effectively expressed from VRPs.

This study provided a characterization of several DEN2 E antigenic configurations in a VRP-launched vaccine design. The results show that VRP vectors are capable of expressing different configurations of DEN2 E protein at high levels in the cytoplasm, some of which are efficiently secreted. We have identified two constructs with improved neutralizing responses, which will aide in antigenic design in future studies. These results provide future directions for evaluating antigenic configurations and to determine the most effective immunogen design. We have gained a better understanding of the *in vitro* characteristics and antigenicity of several E configurations and sequences, providing several future directions to further evaluate and optimize dengue envelope expressed from VEE replicon particles.

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