DISSERTATION

THE USE OF HUMAN MONOCLONAL ANTIBODIES TO STUDY THE STRUCTURE AND FUNCTION OF THE WEST NILE VIRUS PRM PROTEIN

Submitted by

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ABSTRACT

THE USE OF HUMAN MONOCLONAL ANTIBODIES TO STUDY THE STRUCTURE AND FUNCTION OF THE WEST NILE VIRUS PRM PROTEIN

Several medically important flaviviruses cause severe disease in humans including West Nile virus (WNV) and dengue viruses (DENVs). No licensed vaccines exist for these viruses, and live-attenuated vaccines may be unsafe in certain populations such as the elderly and immunocompromised. Alternatives to traditional vaccines such as human monoclonal antibodies (hMAbs) would complement prevention and treatment of these diseases. While hMAbs could be used in the prevention and treatment of flavivirus infections, they are also useful tools in expanding our knowledge of the anti-flavivirus human antibody response and the complex antigenic structures of these viruses. In this dissertation production of hybridomas producing hMAbs were attempted, and hMAbs reactive to WNV were used to determine epitopes on the prM protein important in human infection. These epitopes were also studied for their unique involvement in particle secretion and prM presentation.

In order to produce hMAbs to flaviviruses a competent human fusion partner cell line, MFP-2, was fused with several different sources of B cells including peripheral blood lymphocytes (PBLs) from people with previous vaccinations or infections with flaviviruses, and splenocytes from humanized mice vaccinated or infected with DENV. hMAbs secreted from hybridomas were able to secrete IgG and IgM antibodies; however, none was specific for anti-flavivirus antibody. Vaccinated and infected humanized mice produced very low and variable levels of

virus specific antibody which did not class switch from IgM to IgG even after repeated booster immunizations or secondary infections, a feature consistent with a T-cell independent response.

Hybridoma cell lines (2E8, 8G8 and 5G12) producing fully human monoclonal antibodies (hMAbs) specific for the prM protein of WNV were developed using MFP-2 cells and PBLs from a blood donor diagnosed with WNV fever in 2004. Using site-directed mutagenesis of a WNV-like particle (VLP), 4 amino acid residues in the prM protein unique to WNV were identified as important in the binding of these hMAbs to the VLP. Residues V19 and L33 were important amino acids for the binding of all three hMAbs. Mutations at residues T20 and T24 affected the binding of hMAbs 8G8 and 5G12 only. These hMAbs did not significantly protect AG129 interferon-deficient mice or Swiss Webster outbred mice from WNV infection, which was consistent with their inability to neutralize virus infectivity *in vitro*.

While producing mutant WN VLPs to map epitope specificity of these hMAbs, 4 mutations (T20D, K31A, K31V, or K31T) resulted in undetectable VLP secretion from transformed COS-1 cells. K31 mutants formed intracellular prM-E heterodimers; however, these proteins remained in the endoplasmic reticulum, ER-Golgi intermediary compartments and Golgi of transfected cells. The T20D mutation affected glycosylation, heterodimer formation, and WN VLP secretion. When infectious viruses bearing the same mutations were used to infect COS-1 cells, K31 mutant viruses exhibited delayed growth and reduced infectivity compared to WT virus; however, the effect of these mutations on infectious virus was not as dramatic as what was shown in WN VLP. Epitope maps of WN VLP and WNV prM were also different. These

results suggest that while mutations in the prM protein can reduce or eliminate secretion of WN VLPs, they have less effect on virus. This difference may be due to the quantity of prM in WN VLPs compared to WNV or to differences in maturation, structure, and symmetry of these particles.

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Introduction

Emerging diseases are described as those diseases that are new to a population or those that have previously existed but have increased occurrences or geographic range (Morse, 1995). In recent years several flaviviruses have been defined as causing emerging and re-emerging diseases including Japanese encephalitis virus (JEV), West Nile virus (WNV) and dengue viruses (DENVs). These viruses have re-emerged due to changes in human activity such as urbanization, transportation and land use, as well as biological changes including genetic changes within the viruses, host-vector relationships, bird migration and climate change (Gould et al., 2003; Mackenzie, Gubler, and Petersen, 2004). While reliable vaccines exist for some flaviviruses such as JEV and yellow fever virus (YFV), vaccines for other flaviviruses are still in development. A high incidence of severe disease with flavivirus infection in certain populations including the elderly and immunocompromised occurs, and alternatives to traditional vaccines would complement prevention and treatment of these diseases. Human monoclonal antibodies (hMAbs) offer another method of prophylaxis to flaviviruses for persons unable to receive vaccination or for those diseases in which no vaccine exists. While these hMAbs have practical applications in flavivirus disease prevention and treatment, they are also useful tools in expanding our knowledge of the anti-flavivirus human antibody response and the complex antigenic structures of these viruses.

Flavivirus

The genus *Flavivirus*, in the family *Flaviviridae*, consists of 53 virus species divided into three groups: mosquito-borne viruses (27), tick-borne viruses (12), and viruses with no known vector (14) (Gould et al., 2003). Most flaviviruses are maintained in a cycle between blood-feeding arthropods and susceptible vertebrate hosts. Encephalitic flaviviruses of public health concern transmitted by ticks include tick-borne encephalitis virus (TBEV), Powassan virus (POWV), Langat virus (LGTV), and Louping ill virus (LIV). The crystal structure of the envelope (E) protein of TBEV was the first flaviviral protein to be solved (Rey et al., 1995). This structure has been the foundation in understanding the functional and structural importance of the flavivirus E protein in many studies.

The mosquito-borne flaviviruses important in human disease can be divided into two major groups by their disease manifestations and ecology. Encephalitic flaviviruses in the Japanese encephalitis sero-group are maintained in nature with *Culex* species mosquitoes as vectors and birds as vertebrate hosts. These viruses include JEV, WNV, Murray Valley encephalitis virus (MVEV), and St. Louis encephalitis virus (SLEV). WNV is known to circulate throughout Africa, the Middle East, parts of Europe, south and central Asia and Australia. Since its introduction into the Western hemisphere in 1999, WNV has spread throughout North America with a serological response in healthy horses and birds being documented in Central America and the Caribbean. WNV is currently the leading cause of mosquito-borne human encephalitis in North America (Mackenzie, Gubler, and Petersen, 2004).

Flaviviruses causing viscerotropic disease include YFV and DENVs. These viruses are maintained in a forest cycle between lower primate vertebrate hosts and *Aedes* species mosquitoes as vectors, although the forest cycle is no longer a requirement for maintenance of DENVs since they have adapted to humans as vertebrate hosts in urbanized areas. DENVs consist of 4 antigenically closely related viruses (DENV1-4). DENVs circulate in all tropical regions of the world including Central and South America, Africa, the Middle East, south and east Asia and Australia. The production of a DENV vaccine is difficult since infection with one serotype does not elicit long-term cross-protective immunity against the other serotypes, and may, in fact, increase the severity of disease upon infection with a heterologous serotype. DENV is the most important vector-borne viral disease of humans due to the high morbidity rate and economic burden placed on countries where the viruses are endemic. It is estimated that 3.6 billion people around the world are at risk of infection with DENV, with 2 million severe cases of disease and 21,000 deaths occurring annually (Gubler, 2012).

Flavivirus Structure and Genome Characteristics

Flaviviruses are small icosahedral enveloped viruses. The virion consists of the genomic RNA held within a nucleocapsid core surrounded by a lipid envelope (Zhang et al. 2003). Flaviviruses exist in two states of maturation: mature and immature, although evidence suggests other intermediate forms exist (Cherrier et al., 2009; Junjhon et al., 2010). The mature virion is approximately 500 Å in diameter with 90 closely packed dimers of the E protein forming a herringbone patterned shell around the nucleocapsid (Kuhn et al., 2002). The immature virion is

approximately 600Å in diameter containing 60 trimer spikes of pre-membrane (prM) and E heterodimers (Zhang et al., 2003; Zhang et al., 2007).

The flavivirus genome is a linear single stranded RNA of positive sense polarity with a length of approximately 11 kb. The genome encodes 3 structural proteins (capsid (C), prM and E) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Genes for the structural proteins are located at the 5' end while the nonstructural proteins are encoded at the 3' end of the genome (Westaway et al., 1985). The 5' end of the genome is capped (m7GpppAmp), but the 3' end is not polyadenylated with the exception of some strains of TBEV (Mandl, Kunz, and Heinz, 1991). Nontranslated regions (NTR) flank each end of the open reading frame (ORF). These conserved regions form secondary structures leading to cyclization of the genome important in regulation, translation, replication and packaging (Chambers et al., 1990).

Flavivirus Infection in the Cell

Flaviviruses enter the cell by receptor-mediated endocytosis via the E protein interacting with a cellular receptor (Ng and Lau, 1988). Once the virion attaches to a receptor clathrin coated pit on the cell surface the virus/receptor complex are opsonized in clatherin coated vesicles which uncoat before fusing with Rab5 positive early endosomes. These early endosomes mature into late endosomes resulting in accumulation of Rab7 followed by loss of Rab5 (van der Schaar et al., 2008). In the acidic environment of the late endosome, the viral E glycoprotein undergoes complex structural rearrangements that lead to fusion of the virion envelope with the endosomal

membrane resulting in the viral RNA genome being released into the cytoplasm of the cell (Stiasny and Heinz, 2006).

Following uncoating of the nucleocapsid, the RNA genome is released into the cytoplasm. RNA replication occurs in the cytoplasmic replication complexes derived from ER membranes (Gillespie et al., 2010; Welsch et al., 2009). The viral RNA template is copied into complimentary negative strands by the viral polymerase NS5 and remains bound to the replication complex by base pairing in the replicative form. The replicative form is converted to the replicative intermediate when the replication complex begins to synthesize positive-sense RNA via asymmetric and semi-conservative replication. Positive-sense RNA stays associated with the replicative form by base pairing until it dissociates by synthesis of the next strand of positive-sense RNA. The newly synthesized positive-sense molecules are used for translation of the structural and non-structural polypeptides, production of negative-sense RNA for RNA replication or encapsidated into virions as genomic RNA (Westaway, Mackenzie, and Khromykh, 2003).

During virion assembly, the core protein and viral genomic RNA interact to form the nucleocapsid precursor. The polyprotein encoded by the flavivirus RNA genome is translated in association with the rough endoplasmic reticulum (ER). The polyprotein traverses the ER membrane several times and is cleaved both co- and post-translationally by the host enzyme signalase and the virally encoded protease NS2B-3 (Lindenbach and Rice, 2003). The viral structural proteins prM and E form dimers on the ER membrane, producing an icosahedral

scaffold that may or may not enclose the viral nucleocapsid (Konishi et al., 1992; Schalich et al., 1996). The virus particles acquire their lipid bilayer envelopes as they bud into the lumen of the ER and are transported through the trans-Golgi network (TGN) for further envelope protein modification, including glycosylation of the structural proteins and cleavage of the pr peptide from the prM protein by host furin protease. Virus particles contained in vesicles are released from the cell through the exocytic pathway (Mackenzie and Westaway, 2001).

Flavivirus Structural Proteins Important in Infection and Immunity

The E protein (53-54 KDa) is the major virion glycoprotein that forms 90 homodimers on the surface of the virion in a head to tail conformation making 30 rafts of 3 parallel dimers in the mature virion (Kuhn et al., 2002; Zhang et al., 2004). The structure of E is stabilized by twelve conserved cysteine residues forming six disulfide bonds (Nowak and Wengler, 1987). It is a class II fusion protein with three structural domains, I, II and III, corresponding to antigenic domains, C, A, and B (Mandl et al., 1989; Modis et al., 2003; Rey et al., 1995). Domain I (DI; amino acid (AA) residues 1-51, 137-189 and 285-302), the central domain consisting of an 8 stranded β barrel, contains the amino terminus of the protein. It contains the N-linked glycosylation site (N-X-S/T) at N156 that is present in DENVs and most JE and tick-borne encephalitis (TBE) antigenic complex viruses, but is not found in YFV (Winkler, Heinz, and Kunz, 1987). Domain III (DIII; AA residues 303-395), containing the carboxyl terminal immunoglobulin-like portion of the protein forms a 10 stranded β barrel and contains the cellular receptor binding motifs (Chen et al., 1997; Crill and Roehrig, 2001; Hung et al., 2004; Lee and Lobigs, 2002). Domain II (DII; AA residues 52-136 and 190-284), the dimerization domain, is

an elongated structure that makes most of the contacts to the opposite subunit in the homodimer. In DENV DII contains an N-linked glycan at N67 that has been shown to play a role in cellular binding through interactions with DC-SIGN, a c-type lectin receptor present on dendritic cells (Pokidysheva et al., 2006).

Three regions in the E protein play important roles in virus-membrane fusion: the hinge region, the fusion loop and the stem anchor. A flexible area in DI and II, known as the hinge region, allows DII to pivot outward from the virion during exposure to low pH in the endocytic vesicle. This reorganization of the homodimer exposes the internal fusion loop in DII, which is normally hidden in a hydrophobic pocket made up by DI and DIII of the opposite subunit (Stiasny and Heinz, 2006). This exposure allows the fusion loop to come into contact with the target membrane so that fusion can occur. The stem anchor of the E protein ectodomain connects to the transmembrane domains and anchors the E protein within the viral membrane. This region is thought to be involved in E protein rearrangements that occur during the fusion process by bringing the stem-anchor transmembrane regions of the E proteins into contact with DII forming a lipidic fusion pore mediating virus entry into the cytoplasm of the cell (Allison et al., 1999; Modis et al., 2004).

The humoral immune response to the E protein of flaviviruses has been studied extensively. It is the target of most neutralizing antibodies, which predominately recognize the upper lateral ridge of DIII that extends from the virion surface (Modis et al., 2005; Oliphant et al., 2005). However, anti-DIII antibodies represent a very small portion of the serotype specific antibody

response to DENV in humans and mice (Crill et al., 2009; Wahala et al., 2009). Antibodies to DI and DIII are mostly flavivirus sub-complex and type specific, while antibodies to DII are major flavivirus group and subgroup cross-reactive antibodies (Mandl et al., 1989; Rey et al., 1995; Roehrig, Bolin, and Kelly, 1998). Epitopes in the fusion peptide in DII and near the hinge regions between DI and DII are recognized by cross-reactive moderately neutralizing antibodies (Oliphant et al., 2005; Rey et al., 1995). Several studies involving isolation and characterization of human monoclonal antibodies (hMAbs) have shown that many of the antibodies produced by humans in flavivirus infections recognize the fusion peptide in DII (Beltramello et al., 2010; de Alwis et al., 2011; Throsby et al., 2006).

Neutralizing antibodies to flaviviruses have two main mechanisms of action. They may block attachment of the virion to the cell surface by steric hindrances, or they may block steps in fusion of the virion envelope to the endosomal membrane by preventing conformational rearrangements of the E proteins (Crill and Roehrig, 2001; Kaufmann et al., 2006; Nybakken et al., 2005). The highly neutralizing MAb E16 to WNV targets the lateral ridge of DIII and requires a low occupancy of bound sites in order to inactivate virus (Pierson et al., 2007). At relatively low concentrations this antibody blocks the conformational changes in the E protein required for fusion; however, when virions become saturated it can also block attachment (Nybakken et al., 2005). Some MAbs able to neutralize flaviviruses may do so by blocking fusion. MAb 1A1D-2, which also targets the lateral ridge of DIII, recognizes an epitope buried on the E protein at 4^{o}C. At higher temperatures the virus "breathes", changing the structure dynamically, and revealing more of the target epitope on the surface of the E proteins. Thus, at higher temperatures more antibodies bind to the virion and are able to lock the virion into a conformational structure unable

to undergo E protein rearrangements necessary for fusion (Lok et al., 2008; Roehrig, Bolin, and Kelly, 1998).

The pre-membrane (prM) protein (18.1-19.0 KDa) of flaviviruses is a glycosylated precursor to the membrane (M) protein (7-9 KDa) that forms a heterodimer with E protein. Fifteen amino acid residues at the carboxy-terminus of the core protein make up a hydrophobic domain not found in the mature virion that represents a signal sequence for the insertion of prM across the membrane and into the lumen of the ER. This hydrophobic domain is flanked by an N-terminal and C-terminal section in the peptide. Cleavage at the (+) charged N-terminal is mediated by the cytoplasmic viral protease NS2B-3, allowing for efficient signalase cleavage of prM at the polar C-terminal region (Stocks and Lobigs, 1998; Yamshchikov and Compans, 1993). A stop transfer sequence for prM and a signal sequence for the translocation of E protein are located at the carboxy-terminus of prM, exhibited by the hydrophobic stretches interrupted by a charged residue (Chambers et al., 1990). Six conserved cysteine residues form 3 disulfide bridges that act to stabilize the structure of the protein (Nowak and Wengler, 1987). Potential N-linked glycosylation sites vary depending on the antigenic complex of the virus. DENVs have an N-linked glycosylation site at N69, while a glycosylation site is located at N15 for viruses in the JE antigenic complex. YFV and SLEV viruses have additional sites located in the hydrophobic C-terminal domain of M (Chambers et al., 1990).

The prM protein functions as a chaperone protein to E. Co-expression of prM with E is required for proper folding, maturation and assembly of E (Konishi and Mason, 1993). It is also

important in protecting the fusion loop of the E protein from pre-mature fusion before exiting the cell. The pr peptide is the amino terminal part of the prM protein that is positioned on top of the fusion loop of E protein. It is cleaved from prM during virion maturation by the multibasic residue-recognition host protease furin in the TGN. The pr remains associated with the virion to protect the fusion loop of the E protein from premature fusion until reaching an extracellular neutral pH environment. Once the virion reaches a neutral pH, the pr peptide dissociates from the virion. This results in a fusion-competent particle able to undergo E protein rearrangements in an acidic environment that exposes the fusion loop to cellular membranes (Yu et al., 2009). Mutation of the AA residue H244 on the E protein in the pr-E interface blocks pr peptide and E interactions and reduces DEN VLP release, while having no effect on protein folding, membrane insertions and trimerization of E protein (Zheng, Umashankar, and Kielian, 2010). The cleavage of prM on the virion takes place independently of the other proteins, and not all virus particles contain solely M. Particles may consist of mixed populations of virions containing only M, prM or a mixture of the two proteins on the surface of the virion (Cherrier et al., 2009; Junjhon et al., 2010). In fact, only 30-40% of pr is believed to be cleaved in the extracellular particle of DENV following replication in mosquito cells (Junjhon et al., 2008).

The prM may also play a functional role in the virus life cycle by interacting with key cellular factors during virus entry. In DENV, prM was shown to interact with claudin-1 during virus infection, facilitating entry of immature DENV particles into susceptible cells (Gao et al., 2010). Claudin-1 is a member of a family of tight junction membrane proteins that form a barrier in epithelial and endothelial cells as protection from the external environment (Angelow, Ahlstrom, and Yu, 2008). Other viruses have been shown to interact with this cellular factor including

WNV, adenovirus and hepatitis C virus (HCV), which requires claudin-1 as a co-factor during virus entry (Benedicto et al., 2009; Gralinski et al., 2009; Medigeshi et al., 2009).

The prM protein may interact with key cellular factors during virus maturation and secretion from the infected cell. Flaviviruses associate with calnexin and calreticulin, lectins found in the ER, although the exact mechanism is not known. These cellular factors assist in the proper folding of glycoproteins and then chaperone them through the ER while retaining incompletely folded proteins (Courageot et al., 2000; Lorenz et al., 2003; Mackenzie and Westaway, 2001; Wu et al., 2002). DENV prM associates with ADP ribosylation factor (Arf) proteins (Kudelko et al., 2012). This family of proteins is involved in cellular trafficking and is also important in regulating cellular membrane curvature – a function that is critical to flavivirus assembly and budding from the ER membrane (Beck et al., 2008; Garoff, Hewson, and Opstelten, 1998). The prM protein of DENV was shown to interact with vacuolar-ATPases (V-ATPases) in infected cells. These enzymes acidify intracellular organelles, including those in the secretory pathway, and pump protons across the plasma membrane of the cell. This interaction could be significant in both virus entry and membrane fusion via acidified endosomes and secretion of newly assembled virus particles from the cell (Duan et al., 2008).

During infection, prM and M proteins of flaviviruses induce a significant immune response in mice (Bray and Lai, 1991; Kaufman et al., 1989). In humans, anti-prM antibodies are a major component of the immune response during flavivirus infections. The anti-prM antibodies isolated from humans vary in their neutralization activity (Beltramello et al., 2010; Dejnirattisai

et al., 2010; Schieffelin et al., 2010; Smith et al., 2012). These antibodies may be successful at neutralizing virus due to their close proximity to epitopes on E involved in receptor binding, resulting in blocking virus entry into the cell (Vazquez et al., 2002). Dejnirattisai *et al.* (2010) found a substantial anti-prM antibody response in hMAbs isolated from DENV infected patients. The majority of the antibody response was to prM with 60% of the antibodies isolated specific for this protein, while only 40% were specific to E. The anti-prM antibodies were also shown to be less flavivirus group reactive with only 3% of the antibodies isolated cross-reacting with JEV, compared to 64% of anti-E antibodies showing cross reactivity with this virus (Dejnirattisai et al., 2010).

While these anti-prM antibodies may have some capacity for neutralization, they may also be contributing to the progression of disease during DENV secondary infections through antibody dependent enhancement (ADE), the theory that pre-existing antibodies either at sub-neutralizing or non-neutralizing concentrations bind to the virus particle, enhancing viral uptake into the target cell (Hawkes, 1964). Anti-prM antibodies have been implicated in susceptibility of infants to severe dengue (Chau et al., 2009). hMAbs to prM displaying neutralizing and sub-neutralizing activity produced from patients with previous DENV infections have shown enhancement activity over a broad range of concentrations *in vitro* (Beltramello et al., 2010; Dejnirattisai et al., 2010; Smith et al., 2012).

Anti-prM antibodies assist immature DENV particles in cell entry via Fc receptor-bearing cells. Immature virus particles coupled with antibodies are able to enter the cell and undergo the

conformational change and cleavage of prM protein by furin in the acidic endosome, making an otherwise non-infectious virus particle infectious (Rodenhuis-Zybert et al., 2010). In fact, prM antibodies to WNV were shown to cause fully immature virus particles to become infectious *in vivo,* increasing the number of infectious particles present during infection and thereby increasing the severity of disease in mice (Colpitts et al., 2011b). Antibodies targeted at the E protein are able to enhance infectivity of immature virus particles in the same furin-dependent manner, but much higher antibody concentrations are needed for this to occur (da Silva Voorham et al., 2012). Weakly neutralizing antibodies against the WNV fusion loop in the E protein have also been shown to enhance infectivity (Rodenhuis-Zybert et al., 2011). These pre-existing anti-prM antibodies and anti-E antibodies in high concentrations may be an important component of antibody dependent enhancement along with immature virus particles in secondary infections by increasing the viral load and thus increasing the burden of disease (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010).

Anti-prM antibodies may also be working as auto-antibodies during DENV infection. The theory of molecular mimicry has been proposed as a mechanism of pathogenesis and disease in several viruses causing human disease (Oldstone, 1998). Antibodies to the prM protein can enhance DENV infection of non-Fc receptor bearing cells, suggesting a mechanism different from that using the Fc receptor on target cells (Huang et al., 2008; Huang et al., 2006). Anti-prM antibodies have been shown to cross-react with epithelial, endothelial and T cells *in vitro* (Huang et al., 2006). This anti-prM antibody interaction in the human host may be contributing to pathogenesis of DHF/DSS by damaging cells through complement mediated cytotoxicity or antibody dependent cell phagocytosis (Huang et al., 2006).

Development of hMAbs for Flavivirus Immunotherapy

Antibodies and their use as a therapeutic agent to treat viral diseases has grown in popularity since the development of murine monoclonal antibodies (Kohler and Milstein, 1975). Currently, there are dozens of therapeutic antibodies approved for clinical use for a number of different treatments by the Food and Drug Administration (FDA) and hundreds more in development (An, 2010). The use of hMAbs in prophylaxis and treatment of flaviviruses is applicable for a number of reasons. Few approved vaccines exist for these viruses, and outbreaks for some of these agents can be sporadic. Flaviviral encephalitis usually only severely affects certain populations including the elderly and immunocompromised. While an effective vaccine exists for YFV, post-vaccine adverse events have been reported, making it unsuitable for certain at-risk populations (Barrett, Niedrig, and Teuwen, 2008; Doblas et al., 2006; Engel et al., 2006; Ferguson et al., 2010). Development of a safe and effective vaccine against DENV has remained difficult due to the risk of ADE (Guzman et al., 2010). Antibodies are not only useful tools as prophylaxis and treatment of diseases, but can also be used to improve and standardize diagnostic tests throughout the world. In addition, the study of the native human antibody response can be applied to increase our understanding of the human immunological response to these viruses. This new knowledge of the viral epitopes important in human disease can improve vaccine design and development.

The first antibody therapy developed for use in humans was the use of hyper-immune intravenous immunoglobulin (IVIg) of animal or human origin (Behring and Kitasato, 1965). IVIg is a preparation of purified immunoglobulins (Ig) from pooled human serum containing

polyvalent Ig from donors with high titer antibody responses to specific antigens. IVIg has been used to treat a number of viral infections in humans including parvovirus B19, hepatitis A, hepatitis B, cytomegalovirus, varicella zoster virus, vaccinia virus, rabies and respiratory syncytial virus (Dessain, Adekar, and Berry, 2008). IVIg has also been used in prophylaxis and treatment of TBEV and WNV (Ben-Nathan et al., 2009; Kunz et al., 1981; Makhoul et al., 2009; Morelli et al., 2010; Rhee et al., 2011). Treatment with IVIg of neuroinvasive WNV in patients resulted in reduced severity of neurological symptoms, especially when administered early in infection (Hamdan et al., 2002; Makhoul et al., 2009; Rager-Zisman and Ben Nathan, 2003; Shimoni et al., 2001). Commercial anti-TBEV IVIg is used in Europe for prophylactic treatment of humans following suspected tick-bite exposure in endemic areas (Kunz et al., 1981). Failures to passively immunize children and rare adverse effects have been reported (Arras, Fescharek, and Gregersen, 1996; Kluger et al., 1995; Waldvogel et al., 1996).

In the animal model, passive immunization with immune sera or neutralizing antibodies has been shown to protect against flavivirus challenge (Bosco-Lauth, Mason, and Bowen, 2011; Julander, Trent, and Monath, 2011; Kimura-Kuroda and Yasui, 1988; Kreil and Eibl, 1997; Roehrig et al., 2001; Schlesinger, Brandriss, and Walsh, 1985). While polyclonal antibodies may help in preventing infection with homologous virus challenge, they may not necessarily have the ability to cross-protect against other flaviviruses, and treatment with polyclonal antibodies may even exacerbate the progression of disease (Broom et al., 2000; Roehrig et al., 2001). Reports of ADE following passive immunization in mice have occurred with encephalitic flaviviruses (Broom et al., 2000; Pierson et al., 2007). To overcome the risk of ADE, directed re-engineering of the Fc

region of antibodies was developed to prevent Fc receptor binding (Balsitis et al., 2010; Goncalvez et al., 2007).

While IVIg administration may be beneficial in prophylaxis and treatment of flaviviruses, there are limitations to this technology. Since IVIg is obtained from persons with previous infections, there may be variability in lots that can affect the quantitative titer of antibody and functional activity that would in turn affect the therapeutic efficacy of the preparations. Since IVIgs are purified from human serum, there is the chance that known or unknown infectious agents are being transmitted to the recipient. IVIg also requires a large volume to be administered to the patient, which may cause complications (Diamond, 2009). To overcome these obstacles chimeric, humanized and human antibodies have been developed for the prophylaxis and treatment of many diseases including flavivirus infections.

Chimeric or humanized antibodies have the murine variable regions or only the complementarity determining regions (CDRs) of a murine antibody cloned into the backbone of a human antibody, respectively (Laffly and Sodoyer, 2005). E16 is the first humanized MAb developed for the treatment of WNV infection currently in human clinical trials (Beasley, 2011). This strongly neutralizing antibody targets amino acid residues 307, 330 and 332 on the lateral ridge of DIII of the E protein and is able to penetrate the central nervous system and protect mice and hamsters from lethal viral infection even 5 days after infection (Morrey et al., 2006; Morrey et al., 2007; Nybakken et al., 2005; Oliphant et al., 2005). In Phase I clinical trials MAb E16 (MGAWN1) was shown to be well tolerated among human subjects at a concentration of 30

mg/kg which would provide an excess of virus neutralizing antibody 3 to 4 weeks after treatment (Beigel et al., 2010).

Chimeric and humanized antibodies have been developed to combat other flaviviruses including DENV, JEV and YFV. The highly neutralizing chimeric IgG MAb 2C9 (2C9-cIgG) to YFV was shown to prophylactically protect interferon deficient AG129 mice similarly to its murine counterpart when administered 24 hours before lethal virus challenge at antibody concentrations greater than 1.27 μg per mouse. However, the IgM isotype of the chimeric antibody was unable to protect mice under these same conditions even at a dose of 127 μg per mouse. When the 2C9-cIgG was administered therapeutically to AG129 mice following lethal YFV 17D-204 vaccine strain challenge it was only effective 24 hours after challenge at a dose of 127 μg (Thibodeaux et al., 2012). Surprisingly, 2C9-cIgG had dramatically different results when tested for its therapeutic activity in the YFV hamster model using Jimenez hamster adapted strain. When hamsters were treated with 380 μg/kg of 2C9-cIgG 48 hours post-infection 100% of animals survived lethal YFV challenge. Earlier administration at 4 and 24 hours post-infection provided protection to 90% of challenged animals (Julander, personal communication). These results show that 2C9-cIgG has the potential to be an effective therapeutic antibody in the treatment of YFV infection or during an adverse event following vaccination.

Chimpanzee MAbs to JEV and DENV have been humanized and evaluated as treatment against these viruses in mice and monkey models. The highly neutralizing chimpanzee MAb 5H2 against DENV4 was humanized and shown to be prophylactically protective in 50% of suckling

mice challenged 24 hours after administration. In the rhesus monkey model, animals were completely protected against lethal DENV4 challenge (Lai et al., 2007). A humanized antibody derived from Fab fragments isolated from DENV-infected chimpanzees was able to cross-neutralize DENV1 and DENV2 *in vitro* and may prove to be a reliable antibody for prophylaxis and treatment (Goncalvez et al., 2004). These researchers also humanized Fab fragments from JEV-infected chimpanzees and found that 100% of mice were prophylactically protected when they received a dose of 40 μg of MAb B2 per mouse 24 hours prior to lethal JEV challenge. Fifty percent of mice were therapeutically protected when they received a dose of 200 μg of MAb B2 per mouse 24 hours after lethal JEV challenge (Goncalvez et al., 2008).

As a diagnostic tool in serological assays, chimeric and humanized antibodies are useful for a number of reasons. There are no standardized positive human control sera reactive with all flaviviruses available. These controls are often made from serum pools that may vary in their reactivity between batches, leading to constant recollection and standardization of test parameters, and antibody-positive control sera may not exist for rare or emerging flaviviral infections (Hackett et al., 1998; Martin et al., 2000; Thibodeaux, Panella, and Roehrig, 2010). Also, since these positive controls are made from different batches of human immune sera, diagnostic reagents cannot be standardized between public health laboratories. To resolve this issue, chimeric IgM and IgG isotypes of the murine MAb (mMAb) 6B6C-1, which is a broadly cross-reactive anti-flavivirus antibody, were constructed to be used as positive controls in IgM antibody capture ELISA (MAC-ELISA) and indirect IgG ELISA assays. These chimeric MAbs (cMAbs) were shown to have the same activities as the 6B6C-1 mMAb in a number of

serological assays with several members of the flavivirus genus including WNV, SLEV, YFV, DENV and JEV (Thibodeaux, Panella, and Roehrig, 2010; Thibodeaux and Roehrig, 2009).

With the development of technologies to isolate and immortalize hMAbs, much has been learned about the human antibody response to flaviviruses during infection. As described previously, a large proportion of the hMAbs isolated from patients with previous DENV infections were found to be highly cross-reactive and mapped to the DI/DII of the E protein. Only a small proportion of hMAbs isolated reacted with DIII of E protein, and these were found to be highly neutralizing and virus specific (Beltramello et al., 2010; Dejnirattisai et al., 2010; Kreil and Eibl, 1997; Smith et al., 2012). A large number of hMAbs reacting to the prM protein of DENV have been isolated. These hMAbs tend to vary in their neutralization activity, and may be contributing to the severity of disease (Dejnirattisai et al., 2010). These studies have highlighted the importance of prM protein in the human immune response, which was not previously known. They have also emphasized the importance of producing highly neutralizing virus-specific antibodies to DIII of E protein in order to produce lasting immunity after vaccination. Future studies with hMAbs like these will contribute further to our knowledge of the human antibody response and the factors important for effective and safe flavivirus vaccine development.

Aims of Study

Initially, the main objective of this project was to produce and characterize hMAbs for prophylaxis and treatment of flavivirus infections, as well as investigate the human antibody

repertoire to the antigenic structure of these viruses. The original hypothesis of the dissertation was the epitope specificity of the human anti-flavivirus antibody response is similar to the murine anti-flavivirus antibody response, and therefore hMAbs specific for critical flavivirus epitopes can be isolated and will be capable of preventing, reducing, or abrogating flavivirus infection in an animal model.

While producing mutant WN VLPs for epitope mapping of the three available hMAbs (2E8, 8G8 and 5G12 generously provided by Dr. Trakht and Dr. Kalantarov, Columbia University), specific mutations made in the prM protein resulted in loss of WN VLP secretion. This result prompted an evaluation of this region in the prM protein of WNV and its effect on VLP and virion secretion with the hypothesis being mutations made in the WN VLP that affect secretion will have the same affect when introduced into the virion.

The specific aims of the project were 1) to attempt to produce hMAbs to flaviviruses using two different sources of B cells and the MFP-2 cell line; 2) characterize the 3 available WNV prM-specific hMAbs; and 3) determine the effects of prM mutations made important in VLP secretion.

Chapter 2: Human Monoclonal Antibody Development to Flaviviruses Using MFP-2 Cells

Introduction

Since the development of murine monoclonal antibodies (mMAbs) by Köhler and Milstein in 1975, scientists have worked to expand this technology to develop human monoclonal antibodies (hMAbs) for use as therapeutics in a variety of diseases (Steinitz, 2009). Many methods are available to produce hMAbs, each with varying degrees of success. Chimerization of antibodies, the replacement of murine constant regions of an immunoglobulin gene with those of the human, results in antibodies that have improved interactions with human effector cells and the complement system. These antibodies are perceived as less foreign than full mMAbs; however, human anti-chimeric antibody responses have been reported (Laffly and Sodoyer, 2005). In order to improve on this technology humanization of murine MAbs (mMAbs) was developed. These antibodies only contain the murine complementarity determining regions (CDRs) on a human antibody backbone (Tsurushita, Hinton, and Kumar, 2005). While these antibodies are much less likely to induce a human anti-chimeric antibody response, the construction of the antibody may affect its binding properties to the antigen or the conformation of the CDR loops (Laffly and Sodoyer, 2005). Humanized MAbs against flaviviruses and alphaviruses have been constructed for use in diagnostic assays (Thibodeaux, Panella, and Roehrig, 2010).

The transformation of human B cells with Epstein Barr (EBV) virus results in the immortalization of these cells. In the past this method produced cells that were difficult to clone and only produced low levels of immunoglobulin. Improvements have been made on this method by including CpG DNA upon infection of cells, an activator of B cells (Traggiai et al., 2004a). Using EBV to immortalize B cells has been shown to be an effective way to produce hMAbs to DENV (Lanzavecchia et al., 2010; Schieffelin et al., 2010; Smith et al., 2012).

Phage display is a well-established method of screening human antibodies (McCafferty et al., 1990). In this system a large collection of the Fab portions of antibody genes are cloned into bacteriophage vectors so that the Fab fragment will be expressed on the surface of the phage. Phage-expressed Fab fragments are rapidly screened by an affinity selection technique called bio-panning. This allows for rapid screening against antigens of interest and selection of many epitope-specific antibodies. The genes for Fab fragments of interest can then be cloned into a human IgG backbone to produce fully hMAbs (Steinitz, 2009). This system has been used to produce hMAbs to arthropod-borne viruses, including Venezuelan equine encephalitis virus (VEEV), and West Nile virus (WNV) (Hunt et al., 2006; Oliphant et al., 2005).

Transgenic mice engineered to have their immunoglobulin genes replaced with the human version of the genes are another source of human antibodies (Lonberg, 2008). There are some disadvantages to using these mice as a source of B cells for hybridoma production. The antibodies produced in these mice can be distinguished from human antibodies produced in human cells due to differences in glycosylation between the two species. The Galα1-3Gal

glycosylation found in non-primate mammals is present on antibodies isolated from these mice. Humans produce anti-Galα1-3Gal antibodies making up about 1% of the circulating immunoglobulin (Laffly and Sodoyer, 2005). If antibodies produced from these mice were to be used in therapeutics they may be susceptible to rapid clearance (Wright and Morrison, 1997). Humanized mice have also been used as a source of B cells for hMAb production. Human immune system (HIS) mice are generated by engrafting human hematopoietic stem cells (HSCs) from fetal human lymphoid tissues onto immunodeficient mice such as BALB/c $Rag2^{-/-}$IL-2R$\gamma c^{-\backslash-}$ (RAGHu) or NOD-SCID mice (Legrand et al., 2009). Using RAGHu mice, Becker *et al*. (2010) were able to humanize these mice with an engraftment of human $CD34^{+}CD38^{-}$ HSC. These mice were immunized with commercially available vaccines against hepatitis B virus (HBV) and tetanus, and vaccinated mice produced high levels of antigen-specific IgM antibody. Antigen-specific B cells were isolated and cloned to generate hMAbs (Becker et al., 2010).

Others have tried to use different myeloma and lymphoblastoma cell lines as effective fusion partner cell lines with human lymphocytes. Mouse myelomas have been shown to fuse well with human lymphocytes; however, the resulting hybridomas rapidly lose the ability to produce antibody due to the elimination of the human chromosomes from the genome (Abrams et al., 1983; Alkan et al., 1987). The common murine myeloma cell line, SP2, used to make murine hybridomas has been used to fuse with human lymphocytes. The resulting hybridomas grow well but do not retain human immunoglobulin production (Ostberg and Pursch, 1983). The human myeloma cell line Karpas707H fuses well with human lymphocytes; however, this cell line produces an endogenous λ chain that affects normal production of hMAbs (Karpas, Dremucheva, and Czepulkowski, 2001; Vaisbourd et al., 2001).

The MFP-2 cell line developed by Gary Kalantarov and Ilya Trakht at Columbia University was used in experiments discussed in this chapter in an attempt to produce hMAbs to flaviviruses. The MFP-2 cell line was developed by first generating a heteromyeloma (B6B11) by fusing a murine myeloma cell line with a human myeloma cell line. B6B11 was subsequently fused with human lymph node lymphocytes to produce the trioma cell line MFP-2 (Kalantarov et al., 2002). MFP-2 cells have been used previously to produce hMAbs to breast cancer (Kirman et al., 2002). This cell line was also fused with the peripheral blood lymphocytes (PBLs) from a person who had been infected with WNV in 2004 to produce hMAbs that will be discussed in the next chapter (Calvert et al., 2011).

In this chapter, MFP-2 cells were fused with a variety of different sources of human B cells in order to develop hybridomas producing hMAbs to flaviviruses. PBLs from persons exposed by previous vaccinations or infections with flaviviruses were used as a source of B cells for fusions along with splenocytes from humanized RAGHu mice either vaccinated with inactive DENV2 or infected with DENV1 or DENV2. These mice were previously shown to produce a robust and mature human antibody response to DENV2 infection (Kuruvilla et al., 2007). While the fusions carried out were unproductive for hMAbs to flaviviruses, new understanding was gained into the factors necessary to create a successful system using the MFP-2 cells.

Results

Fusions of MFP-2 cells with human peripheral blood lymphocytes. Fusions with MFP-2 cells were performed with PBLs from persons working at the CDC who had previous exposure to flaviviruses. Subject A had an anti-DENV1 IgG ELISA titer of 4.6 $\log_{10}$, and neutralization titers to DENV1 and DENV2 of 2.8 $\log_{10}$ and 1.9 $\log_{10}$, respectively. Subject B had an anti-DENV2 IgG ELISA titer of 1.6 $\log_{10}$, however, neutralization tests were ambiguous. Subject C, who had been vaccinated against JEV and YFV and had a natural exposure to WNV, had neutralization titers to JEV, YFV and WNV of 1.8 $\log_{10}$, 3.1 $\log_{10}$ and 4.0 $\log_{10}$, respectively.

Six independent fusions were performed with both fresh and frozen PBLs isolated from these subjects, and hybridoma supernatants were tested for the production of non-specific human IgG antibody, as well as IgG antibody specific for the flaviviruses to which the subjects were exposed. All fusions yielded hybridoma clones able to secrete human IgG antibody, and the average number of wells secreting antibody was approximately 14.5%. However, when these hybridomas were screened for antibody to flaviviruses, all were negative.

Fusion of MFP-2 cells with splenocytes from RAGHu mice vaccinated with inactivated DENV2. Splenocytes from RAGHu mice vaccinated with inactivated DENV2 were used as a source of B cells in fusions with MFP-2 cells in order to produce hybridomas secreting human anti-DENV2 antibody. Two mice were vaccinated a total of 3 times at 2 week intervals with 25 μg of inactivated virus for the initial vaccination and 50 μg of inactivated virus for subsequent

boosts. Five days before splenocytes were harvested mice were boosted a final time with 50 μg of inactivated virus. After the second vaccination serum was taken and tested for anti-DENV2 antibody in an ELISA. While human IgG titers were greater than 3.0 $\log_{10}$, anti-DENV2 IgG titers were undetectable. After the third vaccination anti-DENV2 IgG titers rose for only one mouse to 1.2 $\log_{10}$. Splenocytes from vaccinated mice were harvested 8 weeks after the first vaccination. The number of human IgG secreting wells was determined to be 12.8%. Hybridomas were tested for anti-DENV2 IgG and IgM antibodies and all tested negative.

A second set of mice was immunized in the same way as before, but was also given an injection of murine anti-CD40 antibody as well as increasing the initial dose of inactivated DENV2 to 50 μg. Murine anti-CD40 antibody has been shown to enhance B cell proliferation *in vitro* as well as increase B lymphocytes in mice treated with the antibody before harvesting the animals' spleens for fusion and hybridoma production (Rycyzyn et al., 2008). When fusions were performed with the spleens from these mice 48.8% of the hybridomas expressed human IgM antibody. This was a significant increase over the percent of antibody secreting hybridomas from previous fusions. Initial tests with DENV2 antigen suggested that 74% of these hybridomas were positive for anti-DENV2 IgM antibody. However, upon closer evaluation it was determined that these hybridomas were in fact secreting human antibody to the murine anti-CD40 antibody that was given to the mice before harvest, since hybridoma culture supernatant reacted in the ELISA only when the murine capture antibody, 4G2, was present and not when purified DENV2 was directly coated onto the plate.

Another fusion was performed with 4 RAGHu mice vaccinated with inactivated virus as previously described; however, instead of using alum as an adjuvant, CpG DNA and Titermax Gold were used as adjuvants. CpG DNA has been shown to activate B cells and induce a Th1 response when combined with other adjuvants (Weeratna et al., 2000). Four weeks after the second vaccination only two of the four mice had detectable anti-DENV2 IgG antibody titers (2.0 and 2.6 $\log_{10}$), and at the time of harvest these titers had dropped to 1.7 and 2.3 $\log_{10}$ respectively. This fusion resulted in similar results, with no hybridomas producing anti-DENV2 antibody.

Fusion of MFP-2 cells with splenocytes from RAGHu mice infected with DENV1 and DENV2. A second attempt at using the splenocytes from RAGHu mice was made by infecting the mice with DENV1 or DENV2 and monitoring anti-DENV IgM and IgG titers by ELISA before harvesting the spleens for B cells and subsequent fusion with MFP-2 cells. After mice were infected with DENV2, sera were tested for anti-DENV2 IgM on week 4 and anti-DENV2 IgG on weeks 5 to 13. At week 4, anti-DENV2 IgM titers were detectable for 4 of the 8 mice. At week 5, only 1 mouse had a detectable anti-DENV2 IgG titer (2.9 $\log_{10}$), and on week 6 this animal's anti-DENV2 serum ELISA titer dropped to 2.6 and to 2.3 $\log_{10}$ by week 8. On week 8, two of the mice died. At week 9, since no anti-DENV2 IgG antibody could be detected, mice were re-infected with DENV2 in order to boost antibody titers. On week 10, 4 of the remaining 6 mice had an increase in anti-DENV2 IgG antibody titers (2.6, 2.3, 2.0 and 2.0 $\log_{10}$). However, by week 11 these anti-DENV2 IgG antibody titers had dropped significantly again with only one mouse testing positive for anti-DENV2 IgG antibody (2.3 $\log_{10}$). By week 13, this animal's anti-DENV2 IgG antibody titer had increased to 2.6 $\log_{10}$; however, the remaining mice

had no detectable anti-DENV2 IgG antibodies. Spleens from the mice were harvested at week 13 and fusions were performed with the splenocytes and MFP-2 cells. All hybridomas tested negative for anti-DENV2 IgM and IgG antibodies (Table 2.1).

Another group of mice were infected with DENV2, and IgG titers were monitored weekly beginning on week 5. On week 5 only 1 of 6 mice had detectable anti-DENV2 IgG antibody titers (2.0 $\log_{10}$). By week 6, this animal's anti-DENV2 antibody titer had increased to 2.6 $\log_{10}$, and three additional mice had anti-DENV2 antibody titers of 2.6, 2.0 and 2.0 $\log_{10}$. At week 7, two mice had increased anti-DENV2 antibody titers to 2.9 and 2.3 $\log_{10}$, while the other two animals' antibody titers stabilized. At week 8, only one mouse had detectable anti-DENV2 IgG (2.0 $\log_{10}$), and on week 9 no mice had detectable anti-DENV2 IgG antibody. These mice were re-infected on week 9 and their sera were monitored for an additional two weeks for an increase in anti-DENV2 antibody. On week 11 no mice had detectable anti-DENV2 IgG antibody (Table 2.2). Spleens were harvested on week 11 and fusions were performed with splenocytes from these mice and MFP-2 cells. Hybridomas all tested negative for the production of anti-DENV2 IgM and IgG.

RAGHu mice were also infected with DENV1 as a source of anti-DENV1 activated B cells for fusions with MFP-2 cells. Mice were monitored from week 5 to week 13 for anti-DENV1 IgG antibody. Anti-DENV1 IgG antibody was only detected on weeks 7 and 8, even after re-infection on week 11 (Table 2.3). Spleens were harvested on week 13 and fusions were

Table 2.1. DENV2-specific antibody responses of RAGHu mice following primary and secondary infections with DENV2 strain 16681.

Mouse	IgM[a]	IgG[b]								
	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9*	Week 10	Week 11	Week 12	Week 13
J295	2.0	< 2.0	< 2.0	nt[c]	dead[d]	dead	dead	dead	dead	dead
J302	< 2.0	< 2.0	< 2.0	< 2.0	dead	dead	dead	dead	dead	dead
J347	2.3	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	2.6	< 2.0	nt	< 2.0
J454	≥ 3.5	2.9	2.6	nt	2.3	< 2.0	2.3	2.3	2.3	2.6
J455	2.9	2.0	2.0	nt	< 2.0	< 2.0	2.0	< 2.0	< 2.0	< 2.0
G-30	< 2.0	< 2.0	< 2.0	nt	< 2.0	< 2.0	2.0	< 2.0	2.3	< 2.0
G-31	< 2.0	< 2.0	< 2.0	nt	< 2.0	< 2.0	< 2.0	< 2.0	nt	< 2.0
G-32	< 2.0	< 2.0	< 2.0	nt	< 2.0	< 2.0	< 2.0	< 2.0	2.6	< 2.0

[a]Geometric mean reciprocal IgM ELISA titer ($\log_{10}$).
[b]Geometric mean reciprocal IgG ELISA titer ($\log_{10}$).
[c] nt, not tested.
[d]dead, mice did not survive infection.
*mice were reinfected with DENV2 strain 16681 after ELISA results were obtained on week 9.
Positive antibody titers are shaded.

Table 2.2. DENV2-specific antibody responses of RAGHu mice following primary and secondary infections with DENV2 strain 16681.

Mouse	IgG[a]						
	Week 5	**Week 6**	**Week 7**	**Week 8***	**Week 9**	**Week 10**	**Week 11**
635	2.0	2.6	2.9	2.0	< 2.0	< 2.0	< 2.0
636	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
620	< 2.0	2.6	2.6	< 2.0	< 2.0	< 2.0	< 2.0
623	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
624	< 2.0	2.0	2.3	< 2.0	< 2.0	< 2.0	< 2.0
625	< 2.0	< 2.0	2.0	< 2.0	< 2.0	< 2.0	< 2.0

[a]Geometric mean reciprocal IgG ELISA titer (log_{10}).
*Mice were reinfected with DENV2 strain 16681 on week 8 after ELISA results were obtained.
Positive antibody titers are shaded.

Table 2.3. DENV1-specific antibody responses of RAGHu mice following primary and secondary infections with DENV1 strain 16681.

Mouse	IgG[b]								
	Week 5	**Week 6**	**Week 7**	**Week 8**	**Week 9***	**Week 10**	**Week 11**	**Week 12**	**Week 13**
530	< 2.0	dead[b]	dead	dead	dead	dead	dead	dead	dead
626	< 2.0	< 2.0	3.2	2.3	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
627	< 2.0	< 2.0	2.6	2.6	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
628	< 2.0	< 2.0	2.3	2.3	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
629	< 2.0	< 2.0	2.6	2.3	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0

[a]Geometric mean reciprocal IgG ELISA titer (log_{10}).
[b]dead, mice did not survive infection.
*mice were reinfected with DENV1 strain 16007 after ELISA results were obtained on week 9.
Positive antibody titers are shaded.

performed on splenocytes and MFP-2 cells. Hybridomas were tested for anti-DENV1 IgM and IgG antibodies, however, none tested positive for the production of these antibodies.

Discussion

This chapter describes attempts made to use MFP-2 cells as a fusion partner with different sources of human B cells for isolation of hybridomas producing fully hMAbs against flaviviruses. Various fusions were performed with PBLs from people previously infected or vaccinated with flaviviruses. B cells isolated from RAGHu mice infected or vaccinated with DENV1 and DENV2 were also used in fusions with MFP-2 cells. While fusions did produce hMAbs, none were specific for flaviviruses; however, new insights were made into the technology that can be applied to future experiments.

Continuous serum antibody concentrations are linked to the number of circulating memory B cells in the peripheral blood of persons with previous infections or vaccinations (Traggiai, Puzone, and Lanzavecchia, 2003). While these memory B cells can be isolated, the total number of cells that need to be screened to do so may be high (Bernasconi, Traggiai, and Lanzavecchia, 2002). There are three theories as to how these memory B cells are sustained over long periods of time. First, they may be maintained throughout a person's lifetime by plasma cells persisting in the bone marrow. They may also be constantly stimulated by cross-reactive antigen that puts pressure on these cells to differentiate and proliferate into short-lived plasma cells, or they may be activated by non-specific stimuli such as CpG DNA. Many studies have shown variations in the rate and strength of the antibody response and circulating B cells in response to a variety of

antigens. Memory B cells in circulation have been shown to be long-lived in persons vaccinated with vaccinia, measles, mumps and rubella viruses, tetanus toxoid and diphtheria vaccine; however, high serum antibody titers did not necessarily correlate with high numbers of circulating memory B cells (Amanna, Carlson, and Slifka, 2007). In subjects vaccinated with influenza virus a rapid and strong anti-influenza IgG+ secreting plasma cell (ASC) response was observed. This response peaked at day 7 and made up 6% of the circulating PBLs. This response was different from influenza specific IgG+ memory B cells that peaked 14-21 days after vaccination and made up 1% of the circulating B cells (Wrammert et al., 2008).

PBLs, from persons born before 1915, secreting antibodies to the 1918 influenza virus HA protein could be isolated and immortalized, suggesting that memory B cells can be long-lived (Yu et al., 2008). Memory B cells have been shown to circulate in the blood for around 14 days after smallpox vaccination. The number of these B cells drops somewhat during the first year after vaccination, but the B cells can still be isolated up to 50 years post-vaccination (Crotty et al., 2003). While circulating memory B cell longevity may be achieved with virus infections like influenza and vaccinations like smallpox, not much is known about the life span of peripheral memory B cells secreting antibody to flaviviruses; however, peripheral CD8+ T cells specific to YFV were shown to peak at day 15 and return to baseline by day 30 post-vaccination (Wrammert et al., 2009). Isolations of B cells secreting anti-DENV antibodies from PBLs of persons previously infected has been reported years and in some cases decades after primary or secondary infections (Smith et al., 2012), while others have reported similar findings but with unknown dates of infection (Beltramello et al., 2010; Schiefflin et al., 2010).

In these experiments PBLs from persons working at the CDC who had been exposed to a flavivirus via natural infection or vaccination were fused with MFP-2 cells in order to isolate hybridoma clones producing anti-flavivirus antibodies. Subjects A and B in this study had natural DENV infections; however dates of infections are unknown. Subject C had JEV vaccination in 1999, YFV vaccination in 2009 and a natural WNV infection acquired in 2003. Even though the YFV vaccination and WNV infection were recent, no B cells able to secrete anti-flavivirus antibodies were isolated from PBLs. One way to improve these results might be to find donors who have had more recent flavivirus infections. The number of circulating memory B cells within a month of infection may be much higher, and therefore it may be easier to isolate B cells expressing anti-DENV antibodies from PBLs. The Sentinel Enhanced Dengue Surveillance System (SEDSS) has been established by the Dengue Branch, CDC, San Juan, Puerto Rico in a number of area hospitals in order to conduct population based studies of DENV prevalence on the island. The SEDSS has created a rigorous screening process for inclusion of patients with possible DENV infections. The collaboration between the CDC and hospitals will allow for a reliable source of PBLs from DENV infected patients for fusions to produce hMAbs. Immortalizing B cells from PBLs with EBV before MFP-2 fusions may be another way to increase the efficiency of the process. This method has been proven numerous times as a reliable method to keep B cells in culture while cells are screened for antibody production (Traggiai et al., 2004a). EBV transformation of B cells will make screening B cells for anti-flavivirus antibody production more efficient, and combined with the MFP-2 fusions, will allow for a greater likelihood of isolating hybridomas of interest.

RAGHu mice vaccinated with inactivated DENV2 or infected with DENV1 or DENV2 were used to produce a source of human B cells secreting anti-DENV antibody for fusions with MFP-2 cells. While both vaccinated and infected mice produced very low and variable levels of anti-DENV IgG antibodies, antibody titers were not sustained over even short periods of time. This was in stark contrast to a recent report of DENV2 infections using RAGHu mice, on which these experiments were based. RAGHu mice were infected with DENV2 with viremia lasting up to 3 weeks. Human anti-DENV2 IgM antibodies were detected as early as two weeks post-infection, and about 70% of infected mice were shown to class-switch to anti-DENV2 IgG antibodies around 6-8 weeks post-infection. Only mice infected with a pool of three or more strains of DENV2 were able to produce a strong anti-DENV2 IgG antibody response (Kuruvilla et al., 2007). Others have shown that humanized mice are able to mount a humoral immune response consisting almost entirely of IgM antibody. RAGHu mice vaccinated with commercially available tetanus toxoid or hepatitis B surface antigen vaccines produced an IgM antigen-specific response, but these animals were unable to class-switch to IgG (Becker et al., 2010). Humanized NOD-*scid* IL2 rg^{null} mice infected with DENV2 produced anti-DENV2 IgM antibodies at titers that peaked in sera 1 week post-infection; however, a very low IgG antibody response was detected and only in a minority of the mice between 4 and 6 weeks post-infection. The T cell response was monitored and showed that T cells produced IFNγ, TNFα and IL-2 in response to 3 HLA:A2 restricted DENV specific epitopes (Jaiswal et al., 2009). More recently, humanized NOD.Cg-*Prkdc*^{scid}*IL2rg*^{tm1Wjl}*/SzJ* (hu-NSG), were infected with DENV2 strain K0049 via mosquito bite and shown to only mount a DENV-specific IgM response (Cox et al., 2012).

These findings suggest that the humanized mouse models may be generating a T-cell independent response characterized by low levels of virus-specific antibody class-switching and insensitivity to booster immunizations or repeated infections. This may be due to the xenogeneic environment in the mice that make it inhospitable for the proper generation of human T cells. Although the T cell response to DENV2 was shown to occur in humanized NOD-*scid* mice, this T cell response may still not be optimal, since maturation of T cells occurs in the murine thymus with stimulation from murine cytokines. B cell receptors of B cell clones generated from mice immunized with tetanus and hepatitis B antigens also show similar germ line sequences suggesting that the induction of somatic hypermutation is not optimal in humanized mice (Becker et al., 2010). Poor B cell development may also be due to the inability of murine cytokines to signal efficiently to human B cells in humanized mice (Watanabe et al., 2009). Other humanized mouse models also have shown limited generation of class-switched antigen specific B cell responses (Baenziger et al., 2006; Tonomura et al., 2008; Traggiai et al., 2004b; Yajima et al., 2008).

In order to provide a better environment for the development of human T cells, BLT mice were developed. These mice are engrafted with human fetal thymic and liver tissues as wells as HSC CD24+ cells that result in a strong repopulation of human T cells that mount human MHC class I and II restricted immune responses; however, these mice are still unable to produce an antibody class-switch despite booster immunizations. While the mice were able to produce a better T cell response than traditional humanized mice, they were still only able to produce an anti-DENV2 antibody response restricted to IgM antibody (Jaiswal et al., 2012b). This deficiency may also be attributed to the xenogeneic environment in which the T cells are developed, which may be

overcome by treating with human cytokines to restore T cell function (Biswas et al., 2011; Rajesh et al., 2010).

The results in this chapter demonstrate that while humanized mice may be used for some limited understanding of immunological processes occurring during viral infections, they are not suitable for the development of hMAbs due to their inability to produce a fully mature humoral response. Similarly, finding people with more recent flavivirus infections, along with EBV transformation for immortalization of B cells during screening, may increase the chances of isolating B cells secreting anti-flavivirus antibodies that can be used for fusions with MFP-2 cells.

Materials and Methods

Cells and viruses. MFP-2 cells were cultured as previously described (Kalantarov et al., 2002). Cells were maintained in RPMI (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 110 mg/L sodium pyruvate, 0.1 mM nonessential amino acids, MEM vitamins (Mediatech) and penicillin (100 U/ml)/streptomycin (100 mg/ml). Cells were grown at 37^{o}C with 5% CO_2. DENV2 strain 16681, and DENV1 strain 16007, were obtained from Dr. Claire Huang's laboratory, Virology Activity, CDC. DENV2 was passaged 4 times in C6/36 cells, once in LLC-MK2 cells, and once in Vero cells. Stock virus was grown in C6/36 cells to a titer of 2.5 x 10^8 pfu/ml. DENV1 was passaged twice in mosquitoes, twice in LLC-MK2 cells and once in Vero cells. Stock virus was grown in C6/36 cells to a titer of 1.5 x 10^8 pfu/ml. WNV strain NY99 35262, originally isolated from a Chilean flamingo at the Bronx Zoo (NY,

USA) in 1999, was obtained from the Arbovirus Diseases Branch, Diagnostic and Reference Activity, CDC. The virus was passaged once in suckling mouse brain and twice in C6/36 cells. Stock virus was grown in C6/36 cells to a titer of 1.5×10^9 pfu/ml.

Purified virus was inactivated in the presence of 0.1% beta-propriolactone (BPL) with constant stirring at 4^oC for 48 hours after which an aliquot of inactivated virus was inoculated onto Vero cells and cytopathic effect (CPE) was monitored for 7 days to confirm inactivation of the virus.

Fusion of MFP-2 cells with human lymphocytes. MFP-2 cells were fused with human lymphocytes following the same protocol as previously described (Kalantarov et al., 2002). Briefly, both MFP-2 cells and lymphocytes were washed in serum-free RPMI medium 3 times by centrifugation at 280-300 x *g* at room temperature (RT) for 10 minutes. Medium for washing lymphocytes was supplemented with 0.38% sodium citrate. Cells were counted prior to fusion, and MFP-2 cells and lymphocytes were mixed at a 1:3 ratio, respectively. The mixture of cells was centrifuged once more using the same conditions, after which all of the supernatant was aspirated from the cell pellet. The cell pellet was briefly resuspended in the tube before the addition of 400 μl of pre-warmed PEG-1500 (Sigma) and incubated with constant shaking for 3 minutes. The PEG/cell mixture was diluted by the addition of diluent medium [9 parts DPBS and 1 part 10X Hank's solution (Cellgro)] at a rate of 15 ml dropwise over 15 minutes followed by the addition of RPMI growth medium at a rate of 15 ml dropwise over 2-3 minutes. The concentration of fetal calf serum (FCS) in the cell mixture was increased to 20% as well as the addition of 1X HT solution consisting of hypoxanthine (2.5×10^{-5}M) and thymidine (4×10^{-6}M).

The cell suspension was incubated for 1-3 hours at 37^{o}C with 5% $CO_{2.}$ Cells were collected and resuspended in RPMI growth medium supplemented with 20% FCS and 1X HT solution to a concentration of 0.8-1 x 10^{6} lymphocytes/ml and plated to wells in a 96-well plate. Cells were incubated at 37^{o}C with 5% CO_2 overnight before the addition of RPMI growth medium supplemented with 20% FCS and 4X HAT solution (1 x 10^{-4} M hypoxanthine, 4 x10^{-7} M aminopterin, and 1.6 x 10^{-5} M thymidine). Medium was changed every three to four days for two weeks prior to screening the hybridoma cells for human antibody production.

Vaccination of RAGHu mice with live and inactivated virus. RAGHu mice provided by Dr. Larisa Poluektova at the University of Nebraska were vaccinated with inactivated DENV2 strain 16681 with 1.3% Alhydrogel (Accurate Chemical and Scientific Corp.). Mice were vaccinated using the following schedule: On day 0 mice were vaccinated with 25 μg of inactivated DENV2 subcutaneously followed by 25 μg of inactivated DENV2 subcutaneously on day 14. Serum was collected on day 28 and tested for the presence of human IgG antibody as well as human anti-DENV2 IgG and IgM antibodies. Five weeks after the first injection mice were again vaccinated with 50 μg of inactivated DENV2 intraperitoneally (i.p.), and five days later spleens and bone marrow were harvested from the animals for B cell isolation and fusion with MFP-2 cells.

RAGHu mice provided by Dr. Ramesh Akkina from Colorado State University were infected with DENV2 strain 16681, or DENV1 strain 16007, as described previously (Kuruvilla et al., 2007). Briefly, mice were inoculated i.p. with 10^{6} pfu of virus and monitored for four weeks. At four weeks mice were bled every week for 9 to 13 weeks to monitor development of human

anti-DENV IgM and IgG antibodies by ELISA. Once anti-DENV IgG antibody titers peaked and then waned, mice were challenged again with 10^6 pfu of virus, after which spleens were harvested following the 11^{th} to 13^{th} week of infection.

ELISAs. Hybridoma cells were grown 2-4 weeks after fusions before the cell culture fluid was screened for human antibody. Hybridomas were first screened for the presence of non-specific human antibody. ELISA plates were coated with rabbit anti-human IgG + IgM diluted 1:1600 in carbonate/bicarbonate buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6) and incubated overnight at 4^oC. Plates were washed five times with PBS/0.1% Tween wash buffer with an automatic plate washer. Non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS and incubated for 1 hour at 37^oC. Blocking buffer was removed from the plate before hybridoma supernatant was added in 2-fold dilutions (50µl/well) and incubated for one hour at 37^oC. Plates were washed five times before the addition of goat anti-human antibody conjugated to alkaline phosphatase (50µl/well), diluted 1:1600 in 1% BSA in PBS. After an incubation period of one hour at 37^oC, plates were washed again ten times. Alkaline phosphatase substrate (Sigma) was added to each well of the plate (100µl/well) and incubated in the dark at room temperature for 30 minutes. The plates were read at OD_{405} nm on an automatic plate reader. Positive samples were those determined to have an OD_{405} reading of at least twice that of the mean for the negative control human sera.

Once hybridomas were determined to be positive for production of human antibody their supernatants were screened for anti-flavivirus antibodies using the following protocols. To test

for human anti-flavivirus IgG, wells of a 96 well plate were coated with a capture antibody, flavivirus group-reactive mMAb 4G2 ascitic fluid diluted 1:1000 in carbonate/bicarbonate buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6) and incubated overnight at 4^{o}C. Plates were washed as previously described, and non-specific binding sites were blocked with Pierce Starting Block (PBS) blocking buffer (100µl/well). Blocking buffer was removed immediately before flavivirus antigen diluted in PBS was added to the wells (50µl/well). Various antigens were used in the ELISA to screen for human anti-flavivirus IgG antibody. Purified DENV2 strain 16681 and WNV strain NY99 were used at concentrations of 0.03 µg/well. DENV1 polyethylene glycol-precipitated VLP antigen was obtained from the Diagnostic and Reference Section, ADB, CDC, and used at a dilution of 1:80 in PBS. Yellow fever viral antigen from infected suckling mouse brain was also obtained from the Diagnostic and Reference Section, ADB, CDC and used at a dilution of 1:40 in PBS. Antigens were incubated on the plates for two hours at 37^{o}C, after which the plates were washed. Hybridoma supernatant was added (50µl/well) and incubated for one hour at 37^{o}C. Plates were washed five times before the addition of goat anti-human antibody conjugated to horseradish peroxidase (50µl/well), diluted 1:3000 in 5% skim milk/PBS. After an incubation period of one hour at 37^{o}C, plates were washed again ten times. Enhanced K-blue TMB substrate (Neogen) was added to each well of the plate (100µl/well) and incubated in the dark at room temperature for 10 minutes. The reaction was stopped with the addition of 1N H_2SO_4 (50 µl/well), and the plates were read at 450 nm.

To test for human anti-DENV1 and anti-DENV2 IgM from human hybridomas made from RAGHu B cells, hybridoma supernatant was tested by IgM antibody capture ELISA (MAC ELISA). Briefly, goat anti-human IgM diluted 1:2000 in coating buffer (50 μl/well) was used as the capture antibody, added to 96-well plates and incubated overnight at 4^{o}C. Plates were washed and non-specific binding sites were blocked with Pierce Starting Block (PBS) blocking buffer (100μl/well). Blocking buffer was immediately removed and hybridoma cell culture fluid was added to the plate (50 μl/well) and incubated for one hour at 37^{o}C. Plates were washed and DENV1 or DENV2 PEG-precipitated VLP antigen diluted 1:80 and 1:30, respectively, in PBS were added to the plate (50 μl/well) and incubated overnight at 4^{o}C. COS-1 cell antigen was used as a negative control and incubated with DENV2 IgM positive control sera. Plates were washed and mMAb 6B6C-1 conjugated to HRP diluted 1:3000 in PBS (50 μl/well) was added and incubated for one hour at 37^{o}C. Plates were washed 10 times before the addition of TMB substrate, and plates were read as previously described.

Chapter 3: Human Monoclonal Antibodies to West Nile Virus Identify Epitopes on the prM Protein

Introduction

West Nile virus (WNV) is an emerging global pathogen causing WN fever and meningoencephalitis. Since its introduction into the Western Hemisphere in 1999, WNV has spread throughout North and Central America and the Caribbean and currently is the leading cause of mosquito-borne human encephalitis in the region (Mackenzie, Gubler, and Petersen, 2004). The WNV epidemic in the USA, which peaked in 2002-2003, has resulted in more than 29,000 reported cases of human disease with 40% of those cases being classified as neuroinvasive (Lindsey et al., 2010). WNV is a member of the virus family *Flaviviridae*, genus *Flavivirus*. It is maintained in an enzootic cycle between mosquitoes and birds with humans and other vertebrate animals as incidental hosts (Mackenzie, Gubler, and Petersen, 2004). Other medically important flaviviruses include Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), yellow fever virus, and the four serotypes of dengue virus (DENV).

WNV has a single-stranded positive sense 11kb RNA genome that encodes 3 structural proteins at its 5'-end. The envelope (E) protein is the major virion glycoprotein responsible for viral cell membrane attachment and fusion. The capsid (C) protein binds the genomic RNA to form the nucleocapsid. The pre-membrane (prM) protein is a chaperone protein that assists in the maturation of the E protein, and occurs as a prM/E heterodimer. The pr peptide is the amino

terminal part of the prM protein that is cleaved from prM by the multibasic recognition site protease, furin. The pr stays associated with the virion to protect the fusion peptide of the E protein from premature fusion until reaching a neutral pH environment. Once the virion reaches a neutral pH, the pr peptide dissociates, resulting in the formation of a fusion competent particle (Yu et al., 2009). Recently, anti-prM human antibodies have been shown to be an important factor in DENV infection (Dejnirattisai et al., 2010; Huang et al., 2006). Their ability to render immature virus particles infectious by facilitating entry into Fc-receptor bearing cells has been documented in both DENV and WNV infections *in vitro* (Colpitts et al., 2011b; Rodenhuis-Zybert et al., 2010).

Many technologies have been developed to produce humanized or fully human monoclonal antibodies (hMAbs), including phage display for selection of hFAbs and humanization of murine MAbs (mMAbs) (Tsurushita, Hinton, and Kumar, 2005), directed re-engineering of mMAbs (Thibodeaux and Roehrig, 2009), using transgenic mice that have had the murine genes for antibody production replaced with human genes (Lonberg, 2008), and transformation of activated B cells with Epstein Barr virus (EBV) to immortalize B-cells (Traggiai et al., 2004a).

For arthropod-borne viruses (arboviruses), Hunt *et al*. (2006) humanized the strongly neutralizing Venezuelan equine encephalitis virus (VEEV)-reactive mMAb, 3B4C-4, using combinatorial antibody libraries and phage-display technology. The humanized version of 3B4C-4 has been shown to be protective in mice when given prophylactically (Hunt et al., 2006). The humanized anti-WNV antibody E16 was also developed using phage display technology. This MAb binds to a highly conserved epitope on domain III of the WNV envelope protein and

has been shown to protect mice from lethal virus challenge (Oliphant et al., 2005). The E16 MAb is in commercial clinical trials, and has been shown to be safe for humans (Beigel et al., 2010). By EBV immortalization of the peripheral blood lymphocytes from a person recently infected with DENV, Schieffelin *et al*. (2010) were able to show that this technology is a viable option for the production of MAbs against DENV. Beltramello *et al.* (2010) have recently published their work isolating anti-DENV hMAbs in the same way. Directed humanization of the flavivirus group-reactive mMAb 6B6C-1 converted this mouse IgG MAb to human IgM and IgG (Thibodeaux and Roehrig, 2009). These humanized antibodies can be used as positive control antibodies for all flaviviruses in human antibody-specific diagnostic assays.

Gary Kalantarov and Ilya Trakht at Columbia University created a new technology to develop hMAbs that are produced by fusing a human fusion partner cell line, MFP-2, with human B cells from PBLs. The MFP-2 cell line was developed by first generating a heteromyeloma (B6B11) by fusing a murine myeloma cell line with a human myeloma cell line. B6B11 was subsequently fused with human lymph node lymphocytes to produce the trioma cell line, MFP-2 (Kalantarov et al., 2002). Kalantarov and Trakht fused the MFP-2 cell line with the PBLs from a person who had been infected with WNV in 2004. This chapter discusses the isolation and characterization of three hybridomas (designated 2E8, 8G8 and 5G12) from this fusion producing hMAbs reactive to WNV.

To investigate the protein specificities of these three hMAbs, virus-like particles (VLPs) were used as antigen in ELISA. These VLPs have been used previously to identify binding sites of E

protein-specific mMAbs for DENV, WNV, and SLEV (Crill and Chang, 2004; Crill, Trainor, and Chang, 2007; Trainor et al., 2007). For DENV, JEV, and SLEV the prM->M processing does not occur, and only prM and E occur in VLPs (Hunt, Cropp, and Chang, 2001; Purdy and Chang, 2005; Purdy, Noga, and Chang, 2004). The prM->M cleavage of the WNV VLPs is inefficient, so the WNV VLP contains E, prM, and M proteins (Davis et al., 2001). Interestingly, only TBEV-derived VLPs contain only E and M proteins (Schalich et al., 1996).

The 2E8, 8G8 and 5G12 hMAbs reacted only with WNV, and reacted with the WNV prM protein. Using site-directed mutagenesis of the WNV VLP system, specific amino acid (AA) residues were identified as important in hMAb binding. Residues V19 and L33 of the prM protein were determined to be the important sites for binding of all 3 hMAbs. Mutations at residues T20 and T24 had some effect on binding of two hMAbs. When tested in mice for their ability to inhibit lethal WNV infection, hMAbs, 8G8 and 5G12 slightly increased the median survival time (MST) of WNV-infected AG129 and Swiss Webster (SW) mice, respectively; however, neither hMAb fully protected animals from lethal infection.

Results

Production of hMAbs to WNV. The construction of a unique fusion partner cell line, MFP-2, and its use for the immortalization of human PBLs has been previously described (Kalantarov et.al, 2002). Three independent fully hMAbs, 2E8, 8G8 and 5G12, were generated using this

protocol with PBLs from a donor diagnosed with WN fever in 2004. These hMAbs were found to be IgG_1 λ-chain isotype by ELISA.

Characterization of hMAbs. To determine the protein specificity of these hMAbs, reduced and non-reduced purified WNV NY99 was separated on a 4-12% Bis/Tris polyacrylamide gel and eluted proteins were used as antigen in immunoblots. All three hMAbs reacted specifically with non-reduced prM protein, which is approximately 20 KDa in mass (Fig. 3.1, Table 3.1). The three hMAbs failed to react with reduced prM protein (data not shown), indicating that the reactive prM epitopes were conformational in nature. The hMAbs did not react with the M protein (approximately 6 KDa), suggesting that they recognize the pr portion of the prM. Interestingly, serum from the donor of the PBLs used to make the hMAbs was also tested in the immunoblot and reacted with only the E and C proteins of WNV.

A panel of 7 flavivirus VLP antigens (DENV 1-4, JEV, WNV, and SLEV) was used to test for virus cross-reactivity of the hMAbs (Davis et al., 2001; Hunt, Cropp, and Chang, 2001; Purdy and Chang, 2005; Purdy, Noga, and Chang, 2004). The VLPs were derived from transformed COS-1 cells (Davis et al., 2001). The VLPs were concentrated by ultracentrifugation (Purdy, Noga, and Chang, 2004), and the concentration of VLP bound to a well was standardized by VLP-capture using the broadly flavivirus-reactive mMAb, 4G2. Flavivirus group-reactive human serum was used as a positive control antibody. An OD of two times the background was considered a positive result and endpoints are expressed in μg/ml of hMAb. All three hMAbs

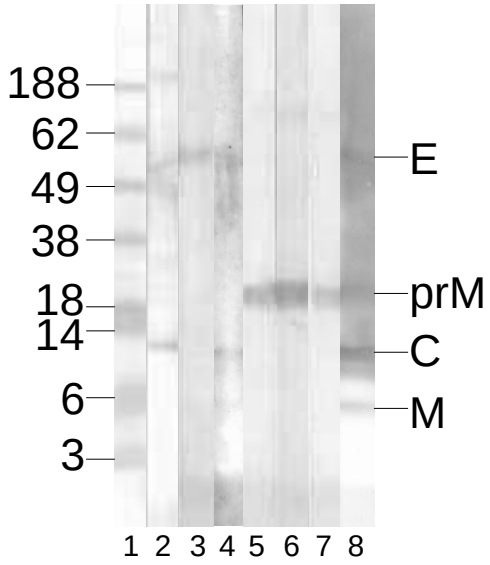

Figure 3.1. **Protein specificity of hMAbs on WNV proteins**. A western blot of WNV proteins was prepared as described in Methods. Lane 1, Bio-Rad broad-range protein standard (sizes are shown in KDa); Lane 2, mouse hyper-immune anti-WNV ascitic fluid; Lane 3, WNV human immune serum; Lane 4, WNV human immune serum from donor of PBLs used to make hMAbs; Lane 5, hMAb 2E8; Lane 6, hMAb 8G8; Lane 7, hMAb 5G12; Lane 8, purified WNV total protein stain.

Table 3.1. Serological and Biological Characteristics of hMAbs

hMAb[a]	Isotype	Immunoblot[b]		PRNT (>90%)[c]	Capture ELISA[d]						
		- ME	+ ME		DEN1	DEN2	DEN3	DEN4	JE	WN	SLE
2E8	IgG1	prM	O	> 6.7	> 0.67	> 0.67	> 0.67	> 0.67	> 0.67	**≤0.004**	> 0.30
8G8	IgG1	prM	O	> 6.0	> 0.60	> 0.60	> 0.60	> 0.60	> 0.60	**≤0.020**	> 0.55
5G12	IgG1	prM	O	> 5.5	> 0.55	> 0.55	> 0.55	> 0.55	> 0.55	**≤0.028**	> 0.75
anti-WN hs[e]	nt[f]	E, C	nt	1:80	nt	nt	nt	nt	nt	nt	nt

[a] Human monoclonal antibodies analyzed.
[b] Protein specificities: E, envelope protein, C, capsid protein, prM, pre-membrane protein; 0, no reactivity detected.
[c] Plaque reduction neutralization activity of hMAb recorded as mg/ml, or anti-WNV human immune serum dilution able to neutralize 90% of virus infectivity.
[d] Endpoint titers expressed in μg/ml of hMAb using VLP as antigen.
[e] anti-WNV human immune sera – sera from donor of PBLs used to make hMAbs
[f] not tested

reacted only with WNV antigen, indicating that these hMAbs are most likely WNV specific (Table 3.1). To assess whether these hMAbs could neutralize virus *in vitro*, hybridoma supernatants were tested in a plaque reduction neutralization test (PRNT) with WNV NY99. None of the hMAbs neutralized virus at a 90% reduction in plaque formation level (Table 3.1). The $PRNT_{90}$ titer of the donor serum was determined to be 1:80.

prM hMAb binding site analysis. To identify the specific binding sites of these hMAbs, VLPs were prepared containing changes in the prM AA sequence using site-directed mutagenesis of the pVAXWN plasmid. Since these hMAbs were found to be specific for WNV, we compared the AA sequence similarity of 10 flaviviruses in the pr portion of the prM protein using GeneDoc (version 2.6.002) software. Nine unique WNV-specific AA residues were identified (Fig. 3.2). Corresponding residues were identified on the crystal structure of the prM-E heterodimer of DENV2 virus at neutral pH using Swiss Pdb Viewer (version 4.0.1) (Fig. 3.3). Five of the WNV-specific AAs were found to occur in surface accessible sites (V19, T20, T24, K31 and L33), while the other 4 sites (T70, S72, R77 and K83) were predicted to be internally located in the protein. Mutagenesis efforts were focused on the 5 surface accessible sites, and 21 mutant plasmids were produced (Table 3.2). The mutant plasmids were transfected into COS-1 cells, and the resulting VLPs were used as antigens in ELISAs as previously described; however, in this assay antigen concentration in the wells was standardized by capture with a polyclonal anti-prM and E WNV rabbit serum raised against WT WN VLP. This more broadly cross-reactive polyclonal rabbit antibody was used as capture antibody in case the prM mutations altered the expression of the E protein epitope reactive with 4G2. A human serum sample containing

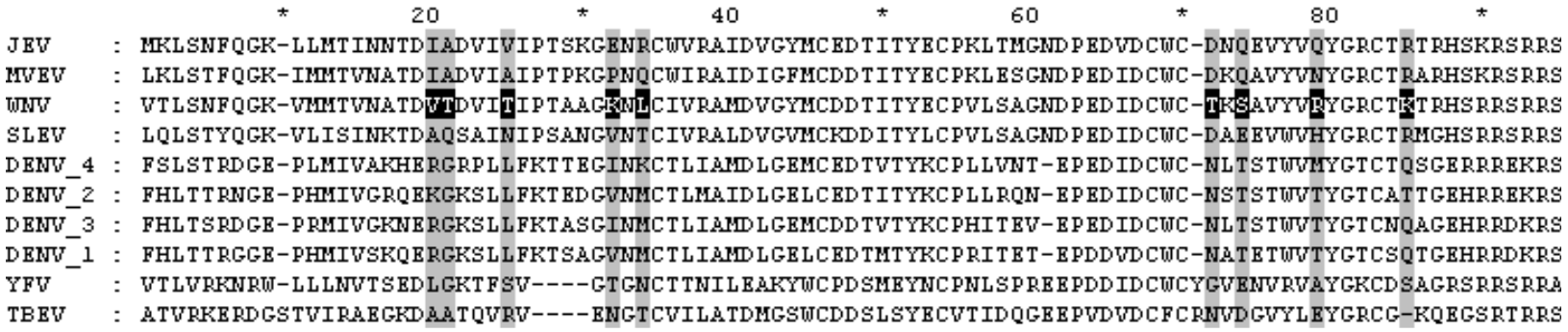

Figure 3.2. **Amino acid sequence homology in the pr peptide of flaviviruses**. The AA sequence homologies of 10 flaviviruses were compared in order to determine the AA residues unique to WNV in the pr peptide. Nine amino acid residues (shown in black with the position highlighted in gray) were identified, including 5 surface accessible residues (V19, T20, T24, K31 and L33) and 4 interior residues (T70, S72, R77 and K83).

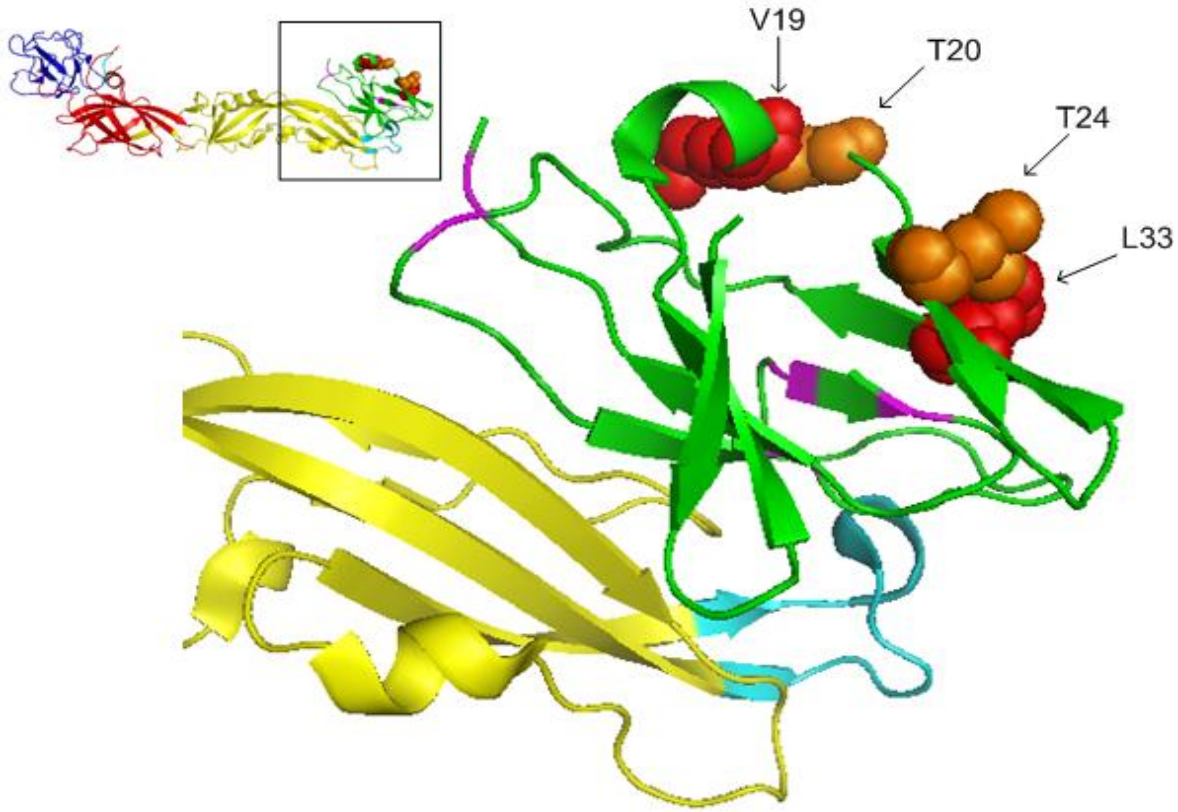

Figure 3.3. **DENV2 prM-E heterodimer**. The image (PDB ID: 3C6E) shows a top view towards the viral surface of the DENV2 prM protein at neutral pH oriented near the fusion peptide in domain II of the E glycoprotein. In the inset, the E protein structural and functional domains I, II and III are shown in red, yellow and blue, with the fusion peptide in domain II shown in cyan. The prM protein is shown in green, with the WNV epitopes important in the hMAbs' binding shown in space-filling representations. V19 and L33 are highlighted in red. T20 and T24 are highlighted in orange. Disulfide bonds in the pr-peptide are shown in magenta.

Table 3.2. Nucleotide sequences of primers used for mutagenesis

Primer	Mutagenic primer sequence (5' - 3')[a]	Nucleotide mutation	Amino acid substitution
V19A	GGTAAATGCTACTGACG**C**CACAGATGTCATCACGA	GTC-GCC	Val-Ala
V19N	GGTAAATGCTACTGAC**AA**CACAGATGTCATCACGA	GTC-AAC	Val-Asn
V19R	GGTAAATGCTACTGAC**AGG**ACAGATGTCATCACGA	GTC-AGG	Val-Arg
V19T	GGTAAATGCTACTGAC**AC**CACAGATGTCATCACGA	GTC-ACC	Val-Thr
T20A	GTAAATGCTACTGACGTC**G**CAGATGTCATCACGATTC	ACA-GCA	Thr-Ala
T20D	GTAAATGCTACTGACGTC**GAC**GATGTCATCACGATTC	ACA-GAC	Thr-Asp
T20G	GTAAATGCTACTGACGTC**GG**AGATGTCATCACGATTC	ACA-GGA	Thr-Gly
T20Q	GTAAATGCTACTGACGTC**CC**AGATGTCATCACGATTC	ACA-CCA	Thr-Gln
T24A	CGTCACAGATGTCATC**G**CGATTCCAACAGCTGC	ACG-GCG	Thr-Ala
T24L	CGTCACAGATGTCATC**CT**GATTCCAACAGCTGC	ACG-CTG	Thr-Leu
T24R	CGTCACAGATGTCATCA**G**GATTCCAACAGCTGC	ACG-AGG	Thr-Arg
K31A	GATTCCAACAGCTGCTGGA**GC**GAACCTATGCATTGTCAGA	AAG-GCG	Lys-Ala
K31V	GATTCCAACAGCTGCTGGA**GT**GAACCTATGCATTGTCAGA	AAG-GTG	Lys-Val
K31T	GATTCCAACAGCTGCTGGA**AC**GAACCTATGCATTGTCAGA	AAG-ACG	Lys-Thr
L33A	AACAGCTGCTGGAAAGAAC**GC**ATGCATTGTCAGAGCAATG	CTA-GCA	Leu-Ala
L33K	AACAGCTGCTGGAAAGAAC**AA**ATGCATTGTCAGAGCAATG	CTA-AAA	Leu-Lys
L33Q	AACAGCTGCTGGAAAGAAC**CA**ATGCATTGTCAGAGCAATG	CTA-CAA	Leu-Gln
L33T	AACAGCTGCTGGAAAGAAC**AC**ATGCATTGTCAGAGCAATG	CTA-ACA	Leu-Thr
T70A	ACTGTTGGTGC**GCA**AAGTCAGCAGTCTACGTCAGGTATGG	ACA-GCA	Thr-Ala
S72A	CTGTTGGTGCACAAAG**GCA**GCAGTCTACGTCAGGTATGG	TCA-GCA	Ser-Ala
K83A	CGTCAGGTATGGAAGATGCACC**GCG**ACACG	AAG-GCG	Lys-Ala

[a] The mismatched nucleotides causing the desired substitutions are shown in bold.

anti-flavivirus antibodies was used as detector antibody. Concentrations of VLP producing an absorbance of 1.0 (450 nm) were used in subsequent ELISAs to determine epitope specificity.

Results of epitope mapping are expressed as a fold-reduction in the hMAb end-point titer of the mutant VLP compared to the WT WNV VLP antigen. Since mutations were tested on individual transfectants, absolute endpoints were compared and fold differences were used to compare mutant and WT VLP reactivity. A 4-fold or greater reduction in the reactivity was considered significant (Table 3.3). Generally, the hMAbs were reactive with the mutant VLPs to some degree except for mutations made at V19 and L33. When the V19 in the pr peptide was mutated to N or T there was a loss of reactivity with all three hMAbs. Similarly, when L33 was mutated to any of 4 AA substitutions made (A, K, Q, or T) all reactivity with the hMAbs was lost. Seven mutations were made that did not result in a significant loss of reactivity with the hMAbs (V19R, T20G, T24L, T24R T70A, S72A and K83A). When A was substituted for the either T20 or T24 there was a reduction in reactivity with hMAbs 8G8 and 5G12. Additionally, when T20 was mutated to a Q there was a loss of reactivity only with 5G12. For several mutations there was some loss of reactivity with hMAbs (V19A with 5G12 and T24A with 2E8), but since the reduction was less than four-fold these were not considered significant.

When mutant VLPs were tested for secretion in an ELISA using rabbit hyper-immune serum as capture antibody and human polyclonal serum as detector antibody, four mutations (T20D, K31A, K31V, and K31T) abrogated VLP secretion. Since these mutant VLPs could not be tested for hMAb binding by ELISA, the mutant transfected COS-1 cells were fixed and used to test the

Table 3.3. Effect of hMAb binding to WN VLP antigen of amino acid substitutions in pr peptide

pr AA substitution[a]	Fold change in hMAb titer with pr mutants[b]		
	2E8	8G8	5G12
WT prM	1	1	1
V19A			2.5
V19N	**>256**	**>256**	**>256**
V19T	**9**	**24**	**64**
T20A		**4**	**4**
T20Q			**4.5**
T24A	2.5	**129**	**516**
L33A	**10.0**	**9.9**	**40.1**
L33K	**>102**	**> 102**	**>103**
L33Q	**412**	**128**	**206**
L33T	**13.6**	**25.6**	**103**

[a]amino acid substitution at specific residue in pr portion of the protein
[b] 4-fold reduction or more (bold) in MAb end-point titer of mutant VLPs compared to wild type VLP). Blank means two-fold or less reduction in titers. Actual end-point titration on wild-type (WT) prM containing VLP with these MAbs were: 2E8= 0.0161 μg/ml, 8G8= 0.0256 μg/ml, and 5G12= 0.0321 μg/ml.

hMAbs' ability to bind to mutant intracellular antigens by immunofluorescence. All of the mutant-transfected cells bound the anti-prM hMAbs, and no significant increase or decrease in reactivity of the hMAbs with these antigens was found.

Animal protection studies. Since the hMAbs were unable to neutralize virus in a PRNT it was unclear whether they would be able to protect mice from a lethal WNV infection. Therefore, the ability of these hMAbs to be used as a prophylactic treatment for WNV infection was tested in both Swiss Webster (SW) outbred mice and interferon deficient AG129 mice. Three groups (n=10) of AG129 or SW mice were treated with 500 μg of hMAb 8G8, 500 μg of hMAb 5G12, or 500 μg of a hMAb to tetanus toxoid (hMAb TT) as a negative control antibody (Table 3. 4). AG129 and SW mice inoculated with PBS (n=3 and n=6, respectively) served as an additional negative control group. Twenty-four hours after inoculation with hMAb mice were challenged with 100 pfu of WNV NY99, a challenge dose previously determined to be 100% lethal for AG129 mice and 83% lethal for SW mice (Calvert et al., 2006; Yamshchikov et al., 2004). Mice were monitored for signs of morbidity, including weight loss, a change in body temperature, and neurological signs of infection. While no AG129 mice survived WNV challenge, median survival times (MSTs) of the mice treated with hMAb 8G8 were significantly longer than those treated with the hMAb TT (P=0.0007). Relative to the control group treated with the hMAb TT, treatment with 8G8 increased mouse MST by 1 day following WNV challenge. The mouse group treated with 5G12 did not have an increase in MST (4 days) following WNV challenge compared to those mice treated with hMAb TT (P=0.6; 4 days) (Table 3.4). When SW mice were treated with 5G12, 3 out of 10 mice survived challenge compared to 1 out of 10 mice treated with 8G8, and none survived challenge when treated with hMAb TT. The MST for mice

treated with 5G12 was 9 days, a significant increase when compared to mice treated with TT (P=0.007, 7.5 days). Mice treated with 8G8 had a MST of 8.0 days which was not considered significant when compared to the MST of mice treated with TT (P=0.24).

Discussion

This is the first report of the isolation and characterization of fully hMAbs to WNV from PBLs from a person previously infected with the virus fused to MFP-2 cell lines Previously, MFP-2 cells have been used to develop fully hMAbs to breast cancer associated antigens (Kalantarov et al., 2002; Kirman et al., 2002; Rudchenko et al., 2008). While an abundance of information exists on the structure and function of the E protein and its importance in flavivirus immunity in mice and humans, little is known about prM and its role in immunity. This study is the first report of the development and characterization of hMAbs for the WNV prM protein, their use in analyzing the antigenic structure of the WNV prM, and its contribution to protective immunity.

These hMAbs were found to be WNV type-specific, and the epitopes they recognize mapped to the most variable region of the prM protein. Not surprisingly, evaluation of the high-resolution crystal structure of the DENV2 prM-E heterodimer at neutral pH, revealed that the region for binding of these pr-specific hMAbs (V19 and L33, highlighted in red in Fig. 3.3) is localized at the top of the prM protein (Li et al., 2008). The other residues that affected binding of hMAbs, T20 and T24 (highlighted in orange in Fig 3.3,) are in close proximity to V19 and L33. While

Table 3.4. Median survival times of AG129 and Swiss Webster (SW) mice treated with hMAbs and challenged with WNV 24 hours later

Group[a]	AG129 Survivors/total	AG129 MST	SW Survivors/total	SW MST
8G8	0/10	5[b] (*P*=0.0007)	1/10	8 (*P*=0.24)
5G12	0/10	4 (*P*=0.6)	3/10	9 (*P*=0.007)
TT	0/10	4	0/10	7.5
PBS	0/3	3	1/6	7.5

[a] AG129 or Swiss Webster (SW) mice treated i.p. with 500 μg of hMAb or PBS.
[b] Median survival time in days.

these AA were important in the binding of hMAbs 8G8 and 5G12, their influence is less pronounced in mutations made in this region that change the epitope only slightly (*e.g.,* T20A or T24A changes). The T20Q mutation may sterically obstruct the ability of the hMAbs to bind to the key epitope at V19.

It is interesting that all three of these hMAbs were found to be specific for the prM protein of WNV, since the donor serum was most reactive with the E and C proteins. While much is known about the structure and composition of flaviviruses grown in cell culture, nothing is known about the structure and composition of the virus as it replicates in either vertebrate or mosquito hosts. We know that the prM→M cleavage is dependent on both the presence of prM→M cleavage motif and the cells in which the virus grows with mosquito cell lines producing larger amounts of prM containing virus than mammalian cells (Fischl et al., 2008; Keelapang et al., 2004; Randolph, Winkler, and Stollar, 1990). It is conceivable that flaviviruses circulating in the vertebrate host might contain a significant amount of prM-containing immature particles. If this is the case, a robust anti-prM antibody response might be expected.

While little is known about the protective capacity of antibodies to prM, some studies have shown that prM is able to induce protective antibody responses in mice. Kaufman *et al*. (1989) isolated 5 anti-prM mMAbs to DENV3 and DENV4. These were shown to passively protect mice against homologous as well as heterologous DENV challenge. Two out of five of the MAbs were found to have some neutralizing activity while four out of five were also able to fix complement (Kaufman et al., 1989). By constructing recombinant vaccinia viruses expressing

prM, M or pr proteins of DENV4, Bray and Lai (1991) found that mice immunized with the recombinant prM or M were protected against homologous virus challenge. However, vaccinating with the recombinant virus expressing pr alone was not protective (Bray and Lai, 1991). Synthetic peptides of the DENV2 prM protein were found to elicit neutralizing antibody that was protective against virus challenge. A synthetic peptide of the DENV2 prM protein containing residues 1-33 (B19-6) was found to elicit neutralizing antibody in mice that allowed for a protective response against DENV2 challenge (Vazquez et al., 2002). It is interesting that this peptide contained all of the AAs that we have defined as critical to the binding of our pr-specific hMAbs.

These hMAbs were found to be minimally protective in outbred mice as well as interferon deficient mice. These anti-prM hMAbs also did not neutralize WNV infectivity *in vitro*. The non-neutralizing capacity of the hMAbs may be due to a reduced number of epitopes available on the virion to which the antibody can bind after prM/M cleavage. The cleavage of the prM protein by furin in the secretory pathway results in the M protein being present in the viral envelope, while the pr peptide remains associated until the virion reaches a neutral pH. Once the virion has reached a neutral pH the pr peptide dissociates from the virion, resulting in a fusion competent particle (Yu et al., 2009). As stated previously, since this cleavage by furin can be inefficient, not all virions released from cells may contain solely M, and in fact, there might be a mixed population of virions containing only M, only prM or a mixture of prM and M on the virion surface (Cherrier et al., 2009). Consequently, the availability of prM to pr-reactive antibody likely varies from virus to virus, and also from cell type to cell type in which the virus grows.

It has been shown that complete maturation of virions results in a reduction of neutralization with E protein-specific antibodies reactive with epitopes that may be more accessible in immature forms of the virus (Nelson et al., 2008). It is also necessary for antibodies that recognize epitopes that are not as exposed to have more sites bound in order to neutralize the virus (Pierson et al., 2007). It is possible that the virus used in this study to challenge mice contained more M than prM, and therefore, there was a lack of epitopes on the surface of the virion for antibodies to bind. This lack of saturation in our study by the hMAbs may have resulted in an inability of the antibodies to neutralize virus effectively, thereby not protecting mice from a lethal virus infection.

Four mutations made in the WN VLP (T20D, K31A, K31V, K31T) resulted in failure of transfected cells to release particles. Although VLPs with these mutations were not secreted, prM was identified in the virus-infected cells. These and other mutations made in the pVAXWN plasmid were near the N-terminal of the protein. It seems unlikely that these mutations would have an effect on cleavage of prM→M, and their influence on antigen secretion must be due to another mechanism. Little has been reported on the requirements in the prM protein for effective viral maturation and secretion. Tan *et al*. (2009) found that the highly conserved Y78 residue in the ectodomain of the prM was essential for virus assembly and secretion from cells *in vitro*, while mutations made at V76, R77 or G79 had no effect (Tan et al., 2009). H99 was also found to be critical for the formation of stable prM-E heterodimers (Lin and Wu, 2005). In analyzing the structure of the prM-E heterodimer, Li *et al*. (2008) found three areas in the pr (R6; E46, D47; and D63, D65) that may be involved in electrostatic bonds with the E protein, therefore mutations in this region may have an effect on prM-E formation and particle secretion (Li et al.,

2008). All of the residues reported previously to have an effect on viral particle maturation and secretion are in the interior regions of the protein. All mutations introduced at residue K31 resulted in lack of WN VLP secretion. This residue is on the surface of the pr peptide and does not appear to interact with the E protein in the heterodimer, or on the neighboring E protein in the particle assembly, but in fact, its placement is at the top of prM, which sits on top of the E protein in the immature particle. The investigation into the mutations at K31 along with the mutation T20D and their involvement in WNV particle maturation and secretion are discussed in the next chapter.

Recent studies have explored the possibility of prM antibodies facilitating the infection of DENV *in vitro*. Antibodies to the prM protein can enhance DENV infection with non-Fc receptor bearing cells, suggesting a mechanism different from that of anti-E antibodies (Huang et al., 2008; Huang et al., 2006). Specifically, human anti-prM antibodies to the M3 epitope (AA 52-67) cross-reacted with epithelial, kidney, endothelial cells and T lymphocytes. They were also found to cross-react with heat shock protein 60 (HP60) on the surface of BHK and A549 cells. This interaction may cause apoptosis in endothelial cells or may indirectly cause complement mediated cytotoxicity or antibody dependent cellular cytotoxicity (Huang et al., 2008). It is likely that anti-prM antibodies to DENV assist immature particles in cell entry, where they are able to undergo the conformational change and cleavage of the prM protein by furin in the acidic endosomal environment, making an otherwise non-infectious virus particle infectious. Therefore, pre-existing heterotypic anti-prM antibodies may be an important component of antibody dependent enhancement along with immature virus particles in secondary infections, assisting in increasing the viral load, and increasing the burden of disease (Dejnirattisai et al., 2010;

Rodenhuis-Zybert et al., 2010). In fact, antibodies to WNV prM were shown to cause fully immature virus particles to become infectious *in vivo,* increasing the number of infectious particles present during infection and thereby increasing the severity of disease in mice (Colpitts et al., 2011b). This is quite possibly another reason the prM hMAbs in this study were not protective in mice.

The hMAbs described here offer a new tool in understanding the human immune response to WNV. While most studies of prM have been in the context of creating an immunogenic virus particle with the E protein for vaccinations, it should not be assumed that the anti-prM antibody response in humans is not important. Although our results indicate that these anti-prM antibodies alone did not offer significant protection in mice, the inclusion of anti-prM antibodies in the human antibody repertoire may be more important than previously considered. Further study into the immunogenic capacity of prM in humans or humanized animal models is necessary in order to fully understand the importance of prM in a WNV infection.

Materials and Methods

Cells and viruses. C6/36 cells and COS-1 cells were cultured as described previously (Davis et al., 2001; Huang et al., 2000). Cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 110 mg/L sodium pyruvate, 0.1 mM nonessential amino acids, 20 ml/L 7.5% sodium bicarbonate and penicillin (100 U/ml)/streptomycin (100 μg/ml). COS-1 cells were grown at 37^{o}C with 5% CO_2 while C6/36

cells were grown at 28°C with 5% CO_2. WNV strain NY99 35262, originally isolated from a Chilean flamingo at the Bronx Zoo (NY, USA) in 1999, was obtained from the Arbovirus Diseases Branch, Diagnostic and Reference Activity, CDC. The virus was passaged once in suckling mouse brain and twice in C6/36 cells. Stock virus was grown in C6/36 cells to a titer of 1.5×10^9 pfu/ml.

Production of hMAbs to WNV. Hybridoma cell lines secreting fully hMAbs 2E8, 8G8 and 5G12 were produced as a result of PEG-fusion between the MFP-2 fusion partner cell line and human peripheral blood lymphocytes from a donor diagnosed with WN fever (Kalantarov et.al, 2002). All three cell lines were grown at 37°C with 5% CO_2 and maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, L-glutamine, non-essential amino acids, Na pyruvate, and vitamins. Positive hybridomas were selected in ELISA, using mMAb D1-4G2-4-15 as a capturing antibody (ATCC cat# HB-112). Plates were coated overnight with 4G2, then washed and blocked with 1% BSA in PBS. Plates were then incubated with WNV-VLP (1:20) and COS-1 (1:20) negative control (Hennessy Research, cat# P120-1) for 2 hours, washed, and incubated with hybridoma supernatants, as well as positive and negative human sera for 1 hour. Goat-anti-human IgG-HRP (mouse adsorbed, Invitrogen cat# H10507) was used as the detector antibody. Hybridomas were also adapted for growth in serum-free medium (HyQ-CCM1, HyClone, cat#SH30043.03). Hollow fiber cartridges 4300-C2011 (Fiber Cell Systems) were used for large-scale production of all three hMAbs.

Purification of virus. WNV NY99 was grown in C6/36 cells and purified by ultracentrifugation on glycerol-potassium tartrate gradients as described by Obijeski *et al*. (1976). Briefly, 20 T150 flasks of C6/36 cells were infected with WNV NY99 at MOI 1.0. After 5 days virus supernatant was harvested and centrifuged in a Beckman JLA 16.250 rotor for 30 minutes at 10,000 rpm/4^{o}C. Virus was precipitated in 8% polyethylene glycol-8000 with constant stirring for at least 2 hours at 4^{o}C, after which the precipitate was collected by centrifugation at 10,000 rpm/4^{o}C for 30 minutes. Precipitated virus was resuspended in TNE buffer (100mM Tris, 2.0M NaCl, 10mM EDTA, pH 7.4) and applied to a 30% glycerol/45% potassium tartrate continuous gradient. The gradient was centrifuged in a SW41 rotor at 26,000 RPM overnight at 4^{o}C. The banded virus was pulled off the gradient and pelleted at 39,000 rpm for 4 hours at 4^{o}C. The pelleted virus was resuspended in 1.0 ml of 1mM Tris buffer (pH 8.0) and stored at -70^{o}C. (Obijeski et al., 1976). The purified stock was used in the immunoblot.

Purification of hMAbs. For animal studies, hMAbs 2E8, 8G8 and 5G12 were purified from highly concentrated culture medium using protein A/G agarose (Pierce, cat# 20423) according to the manufacturer's instructions. hMAbs were further dialyzed against PBS and the concentration was measured in ELISA using purified human IgG (GenScript) as the calibrator.

Immunoblotting. WNV proteins from purified virus were separated by SDS-PAGE on a reduced or non-reduced 4-12% Bis/Tris polyacrylamide gel (Invitrogen). All procedures were performed at room temperature. Proteins were blotted electrophoretically from the gels onto nitrocellulose membranes and washed for 15 minutes in PBS/0.1% Tween wash buffer. Non-

specific binding sites were blocked with 1% BSA/PBS for one hour while rocking. Undiluted hybridoma supernatants were incubated with the membrane for 1 hour with gentle rocking. Membranes were washed again in PBS/0.1% Tween wash buffer three times for 5 minutes each. Goat anti-human antibody conjugated to horseradish peroxidase (Invitrogen) was diluted 1:500 and incubated on the membrane for 1 hour with gentle rocking. Membranes were washed and Novex HRP chromogenic (TMB) substrate (Invitrogen) was added to the membrane until a color change appeared. The reaction was stopped by the addition of water.

Plaque-reduction neutralization test (PRNT). Human serum was heat-inactivated at 56^{o}C for 30 minutes. One hundred plaque-forming units (pfu) of WNV NY99 were incubated with equal volumes of serial two-fold dilutions of hybridoma supernatant or human serum for one hour at 37^{o}C. Six-well plates of Vero cells were then inoculated with the virus-antibody mixtures and incubated at 37^{o}C with 5% CO_2 for one hour after which cells were overlayed with 3 ml of medium containing 1% SeaKem LE agarose (FMC BioProducts) in nutrient medium (0.165% lactalbumin hydrolysate, 0.033% yeast extract [Difco], Earle's balanced salt solution and 2% FBS). Following incubation at 37^{o}C for two days, a second overlay containing 80 μg of neutral red vital stain (GIBCO-BRL) per ml was added. Plaques were counted on day 3 and endpoint titers were expressed as the concentration of antibody (mg/ml) that yielded 90% reduction in the number of plaques ($PRNT_{90}$).

Site-directed mutagenesis. Site-specific mutations were introduced into the prM gene using the QuikChange Site-Directed Mutagenesis kit (Stratagene), with pVAXWN plasmid previously

described as a DNA template (Davis et al., 2001). Mutagenic primer sequences used for all constructs are listed in Table 3.2. After transformation with mutagenized PCR products, colonies were grown in Luria-Bertani broth (5 ml), plasmid was purified and sequenced to determine the correct mutation was present, and then the structural genes and regulatory elements of all purified plasmids were sequenced. Automated DNA sequencing was performed with a 3130X Genetic Analyzer (Applied Biosystems) and sequences were analyzed with Lasergene software (DNAStar). Molecular models of the mutation sites were constructed using Pymol software (v0.99) and the PDB image (3C6E) (Li et al., 2008).

Transient expression of WN VLP in COS-1 cells by electroporation. COS-1 cells were electroporated with the pVAXWN plasmid as previously described (Davis et al., 2001). Electroporated cells were recovered in 6 ml of DMEM supplemented with 10% FBS. Cells were seeded into 25-cm^2 culture flasks for VLP expression, and incubated at 37^{o}C with 5% CO_2. Six hours after electroporation, the growth medium in flasks was replaced with DMEM containing 2% FCS, and flasks were incubated at 28^{o}C with 5% CO_2. Tissue culture medium and cells were harvested 5 days after electroporation for antigen characterization by ELISA and immunofluorescence.

ELISAs. All ELISAs were performed in 96-well plates (Maxisorp plates, Nunc). Starting concentrations of hMAbs from serum-free hybridoma supernatant in ELISAs were as follows: 2E8, 6.7 μg/ml, 8G8, 6.0 μg/ml, 5G12, 5.5 μg/ml. Starting concentrations of hMAbs to test for cross-reactivity with WN and SLE VLP were as follows: 2E8, 3.0 μg/ml, 8G8, 5.5 μg/ml, 5G12,

7.5 μg/ml. All flaviviral VLP antigens for ELISAs to determine cross-reactivity were obtained from the Reference Collection of the Diagnostic Lab, DVBID, CDC. Plates were coated with a capture antibody, flavivirus group-reactive mMAb 4G2 ascitic fluid diluted 1:1000 in carbonate/bicarbonate buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6) and incubated overnight at 4^{o}C. Plates were washed five times with PBS/0.1% Tween wash buffer with an automatic plate washer. Non-specific binding sites were blocked with Pierce Starting Block (PBS) blocking buffer (100μl/well). Blocking buffer was removed immediately before flavivirus antigen diluted in PBS was added to the wells (50μl/well). VLP antigen was incubated on the plates for two hours at 37^{o}C, after which the plates were washed as previously described. Hybridoma supernatant was added in 3-fold dilutions (50μl/well) and incubated for one hour at 37^{o}C. Plates were washed five times before the addition of goat anti-human antibody conjugated to horseradish peroxidase (50μl/well), diluted 1:3000 in 5% skim milk/PBS. After an incubation period of one hour at 37^{o}C, plates were washed again ten times. Enhanced K-blue TMB substrate (Neogen) was added to each well of the plate (100μl/well) and incubated in the dark at room temperature for 10 minutes. The reaction was stopped with the addition of 1N H_2SO_4 (50 μl/well), and the plates were read at 450 nm.

For the standardization of mutant WN VLPs the protocol remained the same with some exceptions. The capture antibody, 4G2, was replaced with rabbit hyper-immune sera to WN prM and E proteins diluted 1:1000. The antigens produced from transfections of COS-1 cells with the mutated pVAXWN plasmids were diluted two-fold in PBS. Human serum positive for reactivity with all flaviviruses was obtained from the Reference Collection of the Diagnostic Laboratory,

ADB, DVBID, CDC, and used as the primary antibody at a dilution of 1:1000 in PBS. To test the reactivity of the mutant VLPs with the hMAbs, VLPs were diluted appropriately to obtain a standardized amount of antigen for all preparations. Hybridoma supernatant was used as the primary antibody and added to the wells in four-fold dilutions (50μl/well), and the ELISA was carried out as described above.

Immunofluorescence. In order to determine the reactivity of hMAbs with mutated VLP plasmid-derived prM that was unable to be secreted in particles, COS-1 cells were harvested 5 days after transfection and fixed to 12-well glass slides. Cells were fixed in 70% acetone in PBS at -20^{o}C for 20 minutes. Hybridoma supernatant was added in 2-fold dilutions (20μl/well) to the wells of the slide and incubated at 37^{o}C for 30 minutes. Slides were washed three times in PBS and allowed to dry before goat anti-human IgG conjugated to FITC (Invitrogen) diluted 1:200 in PBS was added (20μl/well) and incubated at 37^{o}C for 30 minutes. Slides were washed, dried and mounted with Dabco mounting medium and examined on a Zeiss epifluorescence microscope.

Mouse experiments. AG129 mice deficient in IFN-α/β and –γ receptors (van den Broek et al., 1995) and SW mice were bred in-house. The mice were handled as specified by institutional guidelines for care and use in accordance with the Institutional Animal Care and Use Committee recommendations. HMAbs 8G8 and 5G12 were evaluated for their ability to inhibit WNV infection in 5-8 week old AG129 mice, and 3-4 week old SW mice. For each breed two groups of mice (*n*=10) were inoculated intraperitoneally (i.p.) with 500 μg of hMAbs 8G8 or 5G12. One

group of mice (*n*=10) was inoculated i.p. with a hMAb of the same isotype (IgG1) to tetanus toxoid (TT) as a control (hMAb TT). Another group of mice (n=3, AG129 mice and n=6, SW mice) was inoculated i.p. with 100 μl of PBS. Challenge was performed by i.p. inoculation with 100 pfu of WNV NY99 diluted in medium 24 hours after the hMAb inoculation (Calvert et al., 2006; Yamshchikov et al., 2004). Challenged mice were monitored for changes in normal temperature as measured by implantable temperature transponders (BMDS), weight loss and other signs of morbidity (hunched posture, neurological signs). Mice that showed signs of morbidity were euthanized immediately.

Chapter 4: Mutations in the West Nile prM Protein Affect VLP and Virion Secretion *In Vitro*

Introduction

West Nile virus (WNV) is an emerging global pathogen causing WN fever and meningoencephalitis. Since its introduction into the Western Hemisphere in 1999 it has spread throughout North and Central America and the Caribbean and currently is the leading cause of mosquito-borne human encephalitis in the region (Mackenzie, Gubler, and Petersen, 2004). WNV is a member of the family *Flaviviridae*, genus *Flavivirus*. It is maintained in an enzootic cycle between mosquitoes and birds with humans and other mammals as incidental hosts (Mackenzie, Gubler, and Petersen, 2004). Other medically important flaviviruses include Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), yellow fever virus, and the four serotypes of dengue viruses (DENV).

WNV has a single-stranded, positive sense 11kb RNA genome that encodes 3 structural proteins at its 5'-end. The envelope (E) protein is the major virion glycoprotein responsible for virus membrane attachment and fusion. The capsid (C) protein binds the genomic RNA to form the nucleocapsid. The pre-membrane (prM) protein is a chaperone that assists in the maturation of the E protein, and occurs as a prM-E heterodimer. The prM-E heterodimers form 60 trimeric spikes on the surface of the immature DEN2 virion that measures 600 Å in diameter (Li et al., 2008). The pr peptide is the amino terminal part of the prM protein that is cleaved from prM

during virion maturation by the host multibasic amino acid recognition protease furin. The pr remains associated with the virion to protect the fusion loop of the E protein from premature fusion until reaching an extracellular neutral pH environment. Once the virion reaches a neutral pH, the pr peptide dissociates, resulting in the formation of a fusion-competent particle (Yu et al., 2009). Since this cleavage by furin can be inefficient, and appears to be host-cell dependent, not all virions released from cells contain mature M and in fact, there are likely populations of virions containing M, prM or a mixture of prM and M on the virion surface as demonstrated by cryo-electron microscopy (Cherrier et al., 2009; Junjhon et al., 2010; Pokidysheva et al., 2006; Zhang et al., 2003). The existence of prM in infectious flavivirus particles has also been established with experiments measuring pH sensitivity, viral tropism and neutralization capacity of antibodies with less accessible binding sites on E when prM is present (Davis et al., 2006; Guirakhoo, Bolin, and Roehrig, 1992; Nelson et al., 2008).

The polyprotein encoded by the flavivirus RNA genome is translated in association with the rough endoplasmic reticulum (ER). Several transmembrane domains of the polyprotein traverse the ER membrane and co- and post-translational cleavages to produce functional products are carried out by the host enzyme signalase and the virally-encoded protease NS2B-3 (Lindenbach and Rice, 2003). The viral structural proteins prM and E form dimers on the ER membrane, producing an icosahedral scaffold that may or may not enclose the viral RNA genome packaged in the nucleocapsid (Konishi et al., 1992; Schalich et al., 1996). The virus particles acquire their lipid bilayer envelopes as they bud into the lumen of the ER and are transported through the trans-Golgi network (TGN) for further envelope protein modification, including glycosylation of the structural proteins and cleavage of the pr peptide from the prM protein by host furin protease.

Virus particles contained in vesicles are released from the cell through the exocytic pathway (Mackenzie and Westaway, 2001).

Recent studies have shown the importance of the prM protein in the maturation and secretion of virions and VLPs. Tan *et al*. (2009) showed that the highly conserved tyrosine at amino acid (AA) residue 78 in the ectodomain of the prM protein of WNV is essential for virus assembly and secretion from cells (Tan et al., 2009). The H99 residue in the prM protein of JEV, positioned opposite from hydrophobic surfaces on the E protein, was shown to be critical in the formation of prM-E heterodimers. The transition to neutral pH may change interactions between amino acids at this prM-E interface, leading to release of pr and formation of mature virions (Li et al., 2008). Yoshii *et al*. (2004) found that mutating the prM P63 to a serine greatly reduced the production of VLPs and virions of TBEV (Yoshii et al., 2004). This AA lies in the pr region of the prM protein that is conserved in flaviviruses and was shown to be crucial in the formation of prM-E heterodimers (Yoshii et al., 2012). The loss of the N-linked glycosylation site in the pr portion of JEV prM protein results in a 20-fold decrease in infectious virion production and decreased virulence in mice inoculated peripherally with mutant virus (Kim et al., 2008).

In order to map the epitope binding of 3 fully human monoclonal antibodies (hMAbs), four mutations (T20D, K31A, K31V, K31T) were made in the prM protein of the WN VLP that resulted in failure to secrete VLP antigen from transfected cells (Calvert et al., 2011). Although VLPs were not secreted as a result of these mutations, prM antigen was detected in the transfected COS-1 cells. In this chapter the effects of these prM mutations on WN VLP

assembly and secretion are examined as well as the effects on infectious virus replication and protein expression.

Results

Mutations in WNV prM protein affect secretion of VLP. In the previous chapter WN VLP mutants were constructed to identify the epitopes on the prM protein recognized by 3 virus-specific hMAbs (Calvert et al., 2011). All three of the mutations made at K31 in the prM protein (K31A, K31T, K31V) resulted in significantly reduced secretion of VLP. Another mutation, T20D had the same effect on VLP secretion. These mutations occurred in AA residues that are located on the surface-exposed face of pr and did not appear to be involved in interactions with the E protein in prM-E heterodimers or disulfide bonds in the prM protein (Li et al., 2008).

To investigate the effects of these mutations on VLP secretion, COS-1 cells were transfected with WT and mutant (T20D, K31A, K31T, and K31V) pVAXWN plasmids, cell culture supernatant was harvested on days 2 and 7 post-transfection, and detection of viral proteins was performed by antigen-capture ELISA, using polyclonal rabbit serum to WNV prM and E proteins as capture antibody and mouse hyperimmune ascitic fluid (MHIAF) to WNV as detector antibody. Transfected cells were incubated at 37^{o}C up to 7 days. Detection of secreted prM and E proteins was reduced significantly for all prM mutants at day 2 and day 7 compared to WT using Dunnett's method of multiple comparisons with an overall type I error of 0.05 (Fig. 4.1).

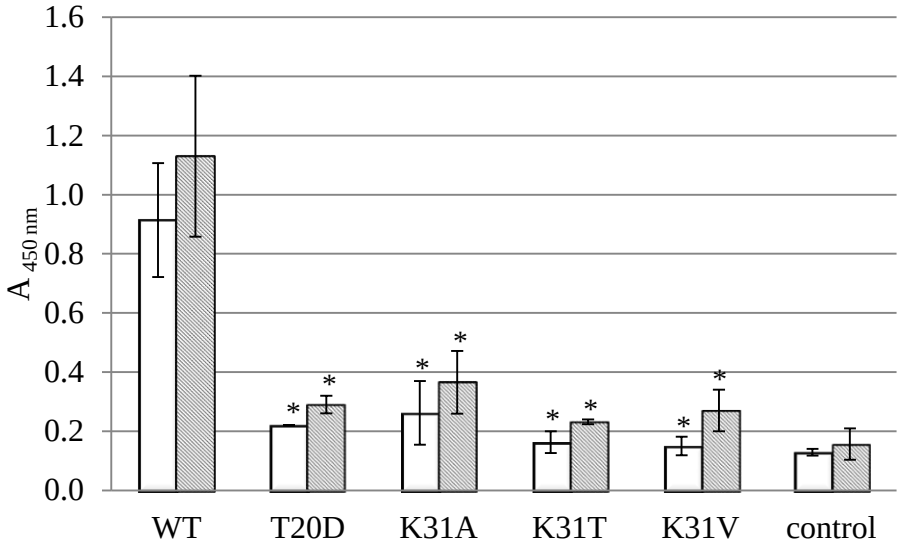

Figure 4.1. Mutations in the prM protein reduce secretion of VLPs. COS-1 cells were transfected with wild type (WT) and mutant pVAXWN plasmids. Cell culture supernatant was harvested 2 (open bars) and 7 (cross-hatched bars) days post-transfection, and prM and E proteins were detected by ELISA. Mean absorbance values (± SE) were calculated from two independent experiments performed in duplicate. Asterisks indicate statistically significant differences compared to WT plasmid using Dunnett's method of multiple comparisons with an overall type I error of 0.05. Control, non-transfected cell control.

These data indicate that K31 and to some extent T20 may play important roles in WN VLP secretion.

Effect of mutations in VLP prM on prM-E heterodimerization. During flavivirus assembly prM and E proteins form heterodimers that associate with the nucleocapsid and bud through the ER membrane to form immature virus particles. Co-expression of prM with E is necessary for correct folding of the E protein; however, prM is able to form its native structure in the absence of E protein (Konishi and Mason, 1993; Lorenz et al., 2002). To determine if the mutations in prM disrupted prM-E heterodimerization during formation of VLPs, COS-1 cells transfected with WT and prM mutant pVAXWN plasmids were lysed, prM and E proteins were immunoprecipitated with either anti-E or anti-prM MAb, and precipitated viral proteins were detected by immunoblot (Fig. 4.2). When VLPs were immunoprecipitated with the anti-prM hMAb 8G8, the E protein was co-precipitated and detected in all cell lysates transfected with WT and mutant plasmids. (Fig 4.2B). Interestingly, prM protein from the T20D mutant was detected as a doublet corresponding to the glycosylated and non-glycosylated forms of the protein. When VLPs were immunoprecipitated with the anti-E mMAb 3.91D, prM protein was detected in co-precipitate from lysates of cells transfected with WT and K31A, K31T and K31V mutant plasmids but apparently not with T20D mutant plasmid. When the concentration of lysate from cells transfected with T20D pVAXWN plasmid was increased 2-fold in the immunoprecipitation reaction a very small amount of non-glycosylated prM was detected (Fig. 4.2B). These results suggest that heterodimerization of the E and prM protein is not affected by mutations at K31, but is affected by the T20D mutation on the prM protein. The mutation at T20

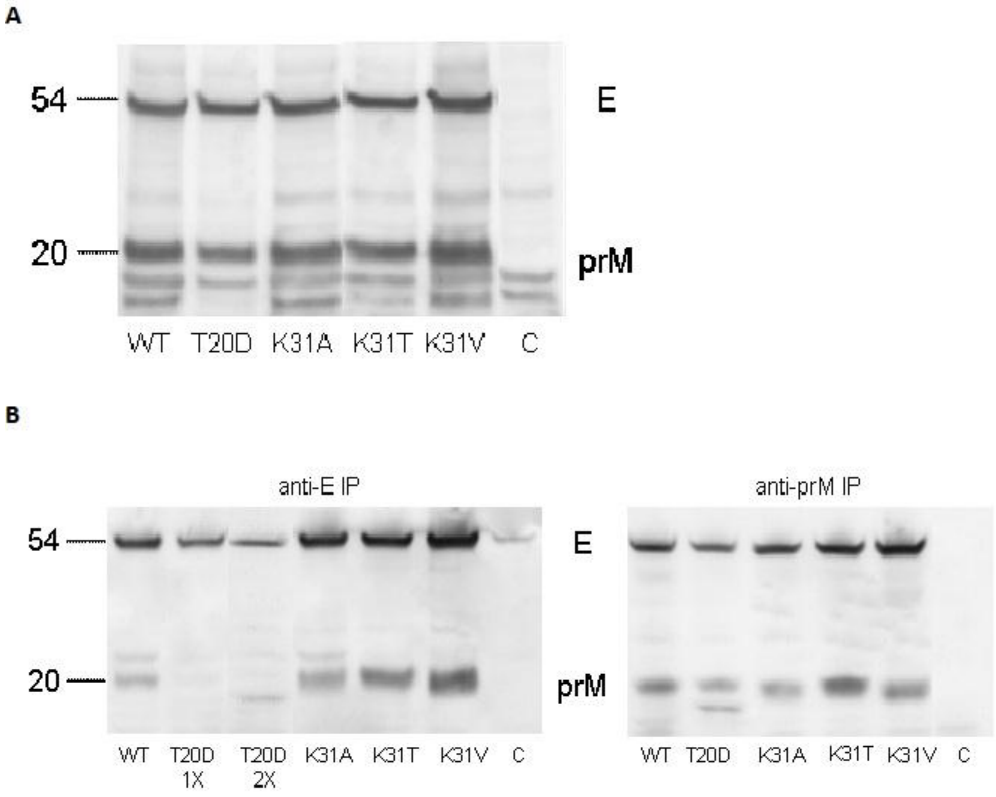

Figure 4.2. Immunoprecipitation of prM and E proteins in COS-1 cells transfected with mutant VLP. COS-1 cells were transfected with WT and mutant pVAXWN plasmids. At 2 days post-transfection, cells were lysed. Whole cell lysates (A) and lysates immunoprecipitated with an anti-E mMAb (3.91D) or an anti-prM hMAb (8G8) (B) were separated by SDS-PAGE on a reduced 4-12% Bis-Tris gel and transferred to nitrocellulose membranes. Proteins bands were detected using anti-E mMAb (3.67G) and anti-prM rabbit polyclonal sera. Non-transfected COS-1 cell lysate was used as a control (C).

permits production of both glycosylated and non-glycosylated forms of prM, and only the non-glycosylated form of the protein appears to associate with the E protein.

To confirm that the T20D mutation resulted in production of both glycosylated and non-glycosylated forms of prM, and that glycosylation of the E and prM proteins was not affected by the K31 mutations, prM and E proteins were immunoprecipitated from cell lysates and digested with the endoglycosidase PNGaseF, which cleaves all types of N-linked glycans. The migration patterns of treated and non-treated samples were compared (Fig. 4.3A). The E protein from WT and prM mutants was shown to be glycosylated as indicated by an apparent shift in mobility from 54 KDa to 50 KDa. The prM proteins expressed from WT and K31 mutant plasmids were also shown to be glycosylated as indicated by a shift in mobility from 20 KDa to 17 KDa. PNGaseF digestion also confirmed that the prM doublet seen in the lysate from cells expressing the T20D mutant was due to expression of both glycosylated and non-glycosylated forms of the protein since only the smaller band (17KDa) was visible after digestion. To determine the form of the carbohydrates on prM and E, immunoprecipitated proteins were digested with endoglycosidase H (EndoH), an enzyme that cleaves the chitobiosyl unit, a dimer of β-1,4-linked glucosamine units, of the high mannose form of the carbohydrate on the glycoprotein. Migration patterns of EndoH treated and untreated samples were compared (Fig. 4.3B). The E and prM glycoproteins of WT and mutants were shown to be sensitive to EndoH digestion, indicating that in cells the glycoprotein glyans were predominately in high mannose form. Processing of high mannose oligosaccharides to complex forms takes place in the TGN; therefore, since these proteins still contained the high mannose form of the oligosaccharides, they must have been localized to the ER. The overall decrease in prM and E protein concentrations from cells

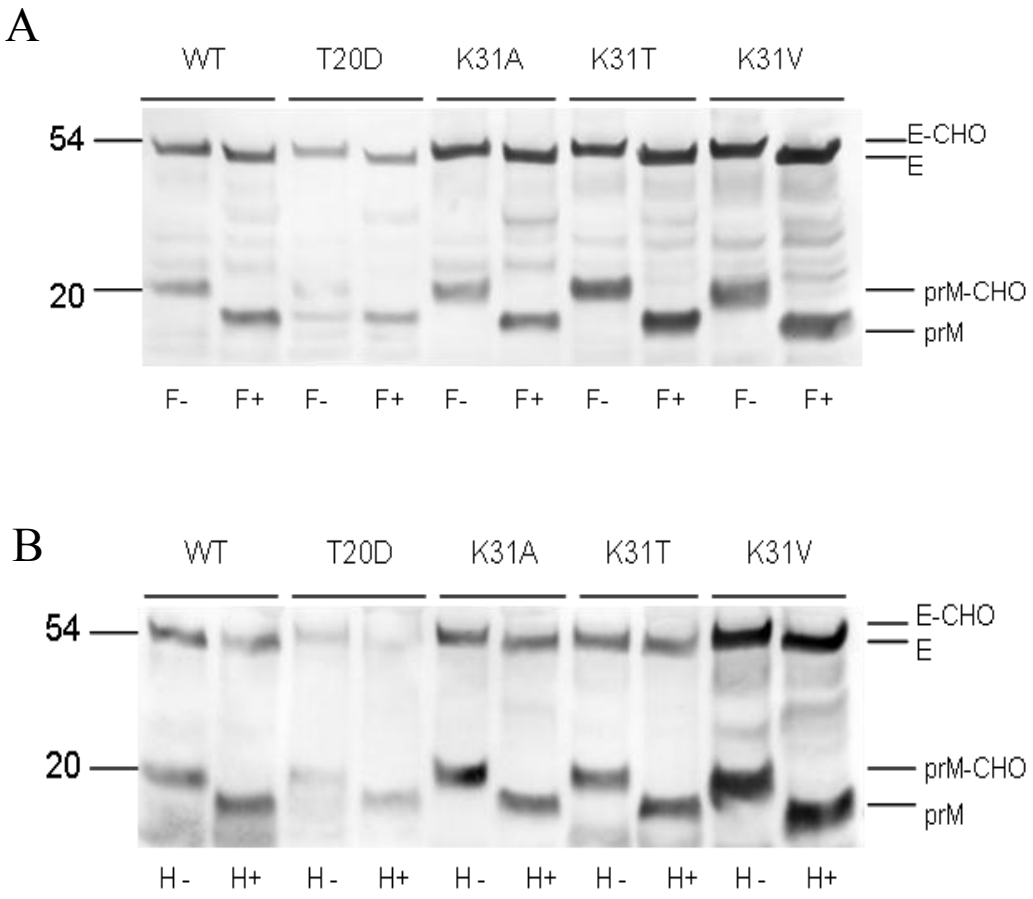

Figure 4.3. Analysis of glycosylation of prM and E proteins in COS-1 cells transfected with mutant pVAXWN plasmids. COS-1 cells were transfected with WT and mutant pVAXWN plasmids. At 48 hours post-transfection, cells were lysed. Lysates were immunoprecipitated with an anti-prM hMAb (8G8). Immunoprecipitated proteins were digested with (A) PNGaseF (F+) or without PNGaseF (F-), and with (B) EndoH (H+) or without EndoH (H-). Proteins were separated by SDS-PAGE on a reduced 4-12% Bis-Tris gel and transferred to nitrocellulose membranes. Proteins bands were detected using anti-E mMAb 3.67G, and anti-prM rabbit polyclonal sera.

transfected with T20D compared to WT may reflect the inability of this construct to assemble heterodimers. Since the K31 mutants expressed amounts of prM and E proteins that were equivalend to those of WT in transfected cells, these contructs may be able to form stable prM-E heterodimers, but unable to form stable particles that can interact with cellular factors for efficient release from cell.

Mutations in WNV prM protein affect cellular localization of viral proteins. The immunoprecipitation results indicated that intracellular prM-E heterodimers are formed with the K31 prM mutants and to a much lesser extent with the T20D prM mutant; however, as shown previously, the VLPs are not secreted. Therefore, the block in secretion must occur in a later step in replication. During assembly, flaviviruses bud into the ER lumen of virus-infected cells and are transported to the TGN for subsequent processing (Lindenbach and Rice, 2003). To determine whether the prM mutations had an impact on the distrubution of viral proteins inside the cell, COS-1 cells transfected with WT and prM mutant pVAXWN plasmids were fixed at 6, 12, 24 and 48 hours post-transfection, permeabilized and double stained with antibodies to detect E protein (mMAb 3.91D) as well as one of the following markers: ER (anti-calreticulin), coatomer (anti-βCOP I), ER-Golgi intermediate compartment (anti-ERGIC53) or TGN (anti-GRASP65). At 6 hours post-transfection, E protein expressed by WT and prM mutant plasmids coincided with ER and ERGIC markers, with some coincidence with βCOPI and little coincidence with TGN (Fig. 4.4). In WT transfected cells at 12 hours post-transfection, the distribution of the E protein completely coincided with the ER marker, ERGIC and to some extent the TGN (Fig. 4.5). At 24 hours post-transfection WT E protein completely coincided with ER and ERGIC markers while moderate coincidence of E protein with the TGN marker was

observed (Fig. 4.6). Staining of cells transfected with T20D mutant had similar staining to WT. At 12 hours post-transfection distribution of the E protein completely coincided with the ER and ERGIC markers, with moderate coincidence with TGN marker; however, unlike WT, T20D E protein also conincided with βCOPI marker (Fig. 4.5). At 24 hours postransfection distribution of the E protein expressed from the T20D mutant moderately coincided with ER and ERGIC markers with less coincidence with βCOPI marker and no coincidence with TGN marker (Fig. 4.6).

Distribution of the E protein expressed by the K31 mutants in transfected cells appeared to be different from WT. Moderate coincidence of E protein with βCOPI and TGN markers and complete coincidence with ER and ERGIC markers were observed in cells transfected with K31 mutant plasmids at 12 hours post-transfection (Fig. 4.5). At 24 hours post-transfection the E protein expressed by K31 mutants coincided with ER, ERGIC and somewhat with βCOPI markers. Staining of E protein expressed by K31 mutants and TGN marker was not present at 24 hours (Fig. 4.6). At 48 hours E protein expressed by WT and prM mutant plasmids coincided with ER, βCOPI, and ERGIC markers (Fig. 4.7). Unlike WT, E protein expressed from K31 mutants coincided somewhat with TGN marker (Fig 4.7).

The most striking result was the extremely concentrated and localized distribution of the E protein in cells expressing mutants compared to the staining of the E protein in cells transfected with WT plasmid. These observations suggest that mutations at K31 in the prM protein result in an accumulation of E protein in the ER, ER-golgi intermediary compartments and to some extent

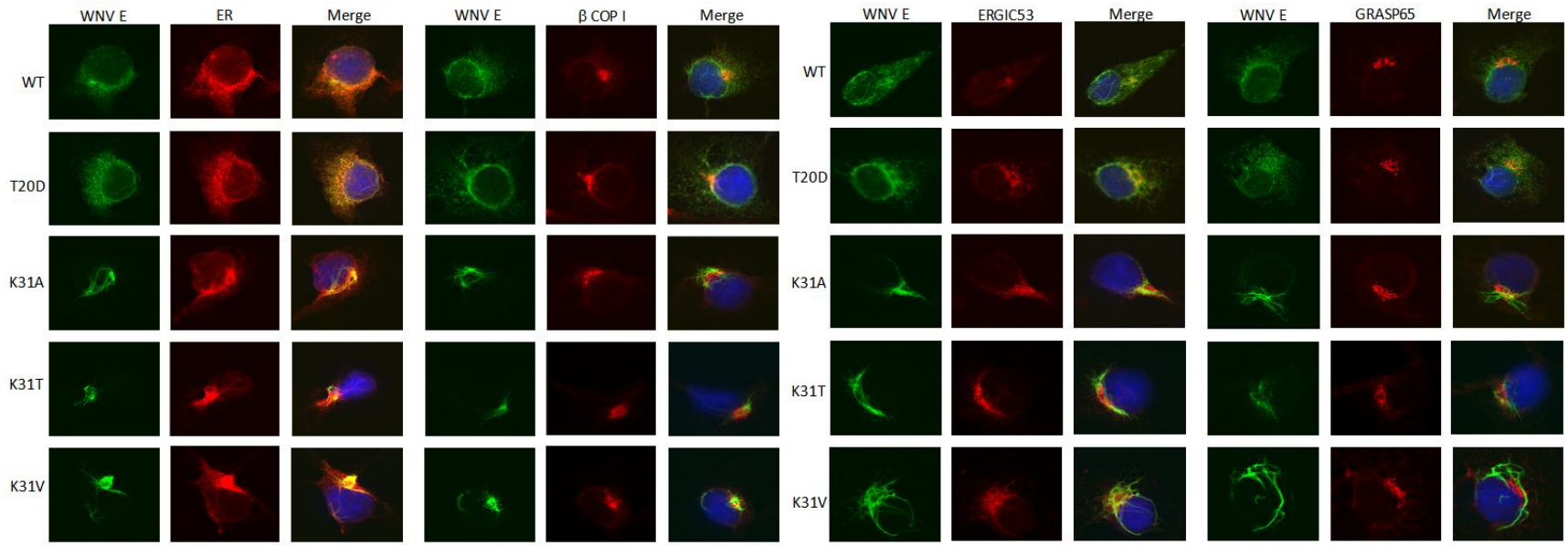

Figure 4.4. Intracellular localization of expressed E proteins in COS-1 cells transfected with prM mutant pVAXWN plasmids 6 hours post-transfection. Transfected COS-1 cells expressing pVAXWN WT and prM mutant plasmids were fixed 6 hours post-transfection and double-stained with antibodies against WNV E protein (3.91D) and calreticulin (an ER marker), ER-Golgi intermediary complexes (βCOP1, ERGIC53) or GRASP65 (TGN marker). Co-localization of the E protein with these marker proteins are depicted in the merged images. Nuclei of cells were stained with DAPI and shown in the merged images.

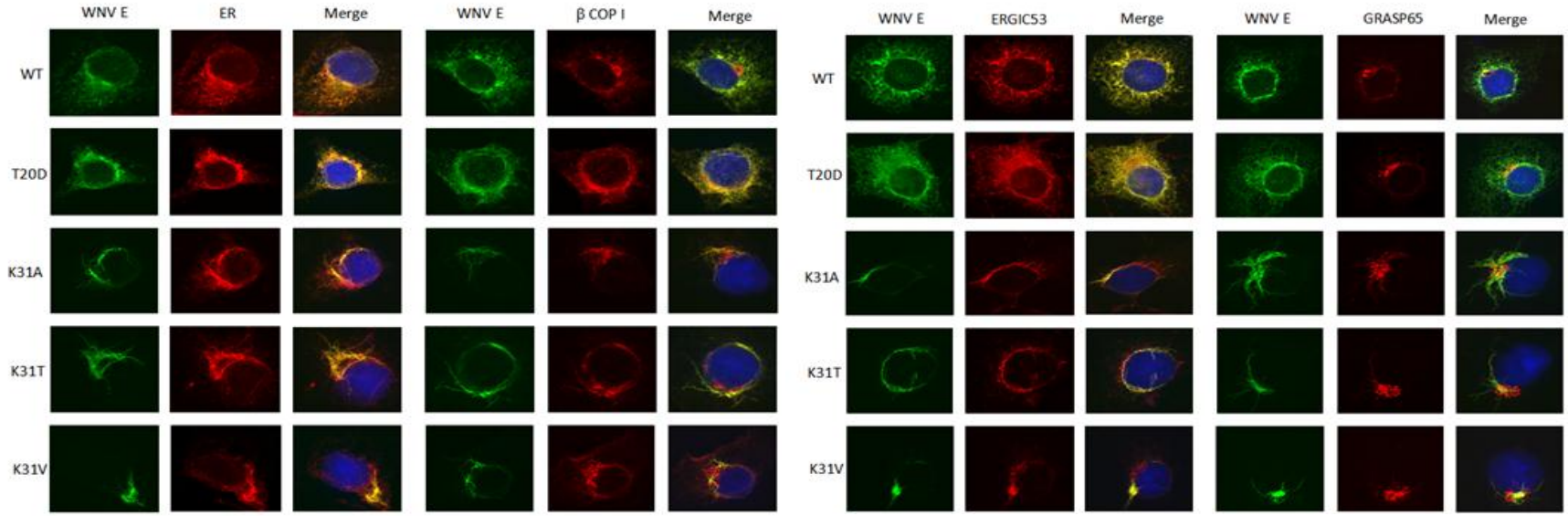

Figure 4.5. Intracellular localization of expressed E proteins in COS-1 cells transfected with prM mutant pVAXWN plasmids 12 hours post-transfection. Transfected COS-1 cells expressing pVAXWN WT and prM mutant plasmids were fixed 12 hours post-transfection and double-stained with antibodies against WNV E protein (3.91D) and calreticulin (an ER marker), ER-Golgi intermediary complexes (βCOP1, ERGIC53) or GRASP65 (TGN marker). Co-localization of the E protein with these marker proteins are depicted in the merged images. Nuclei of cells were stained with DAPI and shown in the merged images.

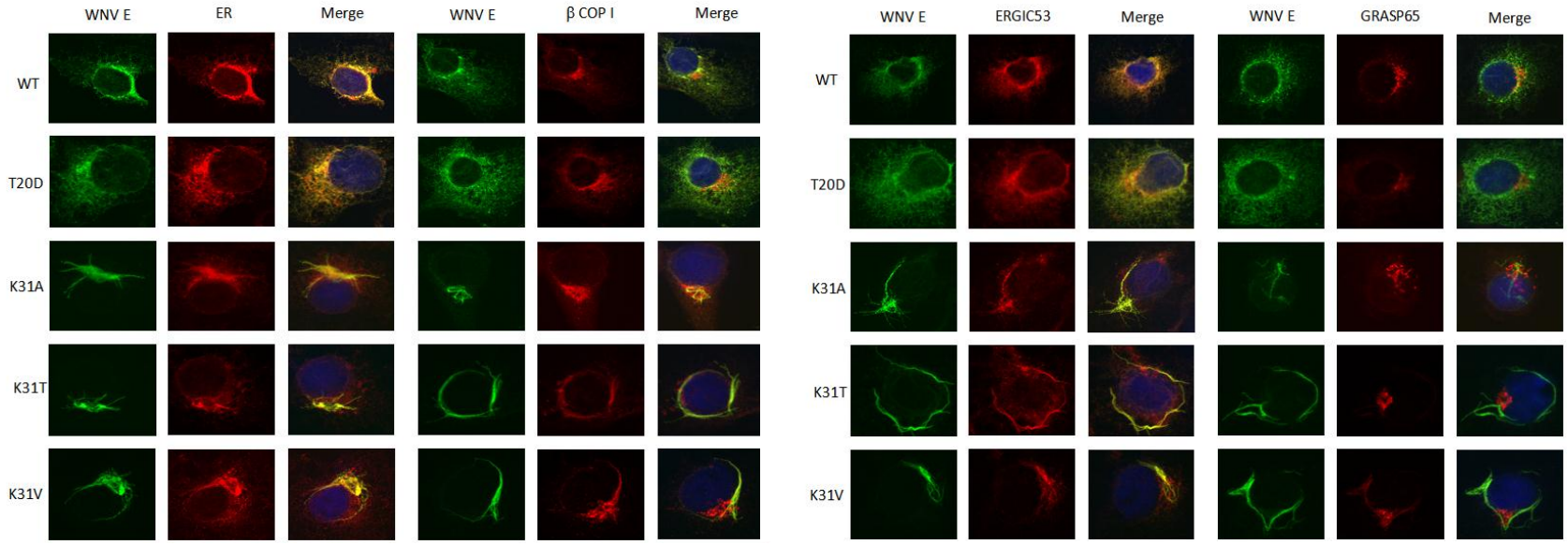

Figure 4.6. Intracellular localization of expressed E proteins in COS-1 cells transfected with prM mutant pVAXWN plasmids 24 hours post-transfection. Transfected COS-1 cells expressing pVAXWN WT and prM mutant plasmids were fixed 24 hours post-transfection and double-stained with antibodies against WNV E protein (3.91D) and calreticulin (ER marker), ER-Golgi intermediary complexes (βCOP1, ERGIC53) or GRASP65 (TGN marker). Co-localization of the E protein with these marker proteins are depicted in the merged images. Nuclei of cells were stained with DAPI and shown in the merged images.

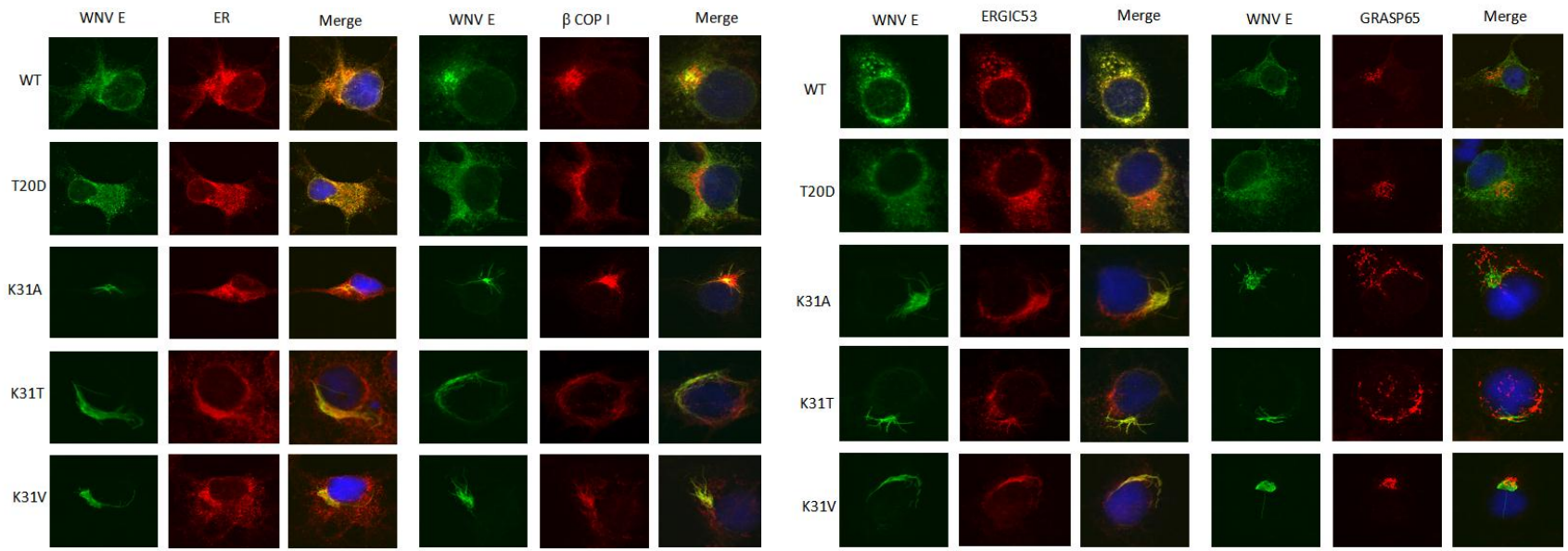

Figure 4.7. Intracellular localization of expressed E proteins in COS-1 cells transfected with prM mutant pVAXWN plasmids. Transfected COS-1 cells expressing pVAXWN WT and prM mutant plasmids were fixed 48 hours post-transfection and double-stained with antibodies against WNV E protein (3.91D) and calreticulin (ER marker), ER-Golgi intermediary complexes (βCOP1, ERGIC53) or GRASP65 (TGN marker). Co-localization of the E protein with these marker proteins are depicted in the merged images. Nuclei of cells were stained with DAPI and shown in the merged images.

the TGN, possibly due to the inability of these mutant prM-E heterodimers to effectively interact with unknown cellular factors in order to be secreted from the cell.

Effect of mutations in prM protein on virus replication. To determine if these prM VLP secretion-blocking mutations had an effect on virus maturation and release, the same mutations were introduced into an infectious cDNA clone of the WNV genome. C6/36 cells were transfected with *in vitro* transcribed, mutant WNV RNA and virus was harvested 7 days after transfection. Virus and viral RNA production were measured by plaque assay and quantitative RT-PCR (qRT-PCR). The prM and E genes of WT and mutant virus RNA were sequenced on day 0 and day 7, and the introduced mutations were found to be present with no compensatory mutations identified. Growth characteristics of viruses were determined in one mosquito cell line (C6/36) and two mammalian cell lines (Vero and COS-1) by infecting cells at a multiplicity of infection (MOI) of 0.1 and analyzing daily medium samples by plaque assay. All 4 prM mutant viruses grew similarly to WT in C6/36 cells except for K31T, which had a titer 10-fold lower than WT virus from day 1 to day 4 (Fig. 4.8A). The prM mutant viruses also grew similarly to WT virus in Vero cells after day 2; however, all mutant viruses grew more slowly than WT between days 0 and 2 (Fig. 4.8B). Growth of the prM mutant viruses as compared to WT were most different in COS-1 cells. While K31V grew similarly to WT virus, T20D, K31A and K31T virus titers were about 10 to 100-fold lower (Fig. 4.8C). Average plaque sizes in Vero cells of prM mutant viruses harvested from these growth studies were compared to WT virus. No significant difference between T20D and WT plaque sizes was seen in viruses grown in any cell type. Average plaque sizes for K31A and K31T were significantly smaller compared to WT for viruses grown in all three cell lines. The average plaque sizes were significantly less only for

the K31V mutant grown in C6/36 and Vero cells (Table 4.1). These results indicate that while virus growth was delayed, and plaque sizes were reduced in Vero cells compared to WT virus, the prM mutant viruses were able to overcome deleterious effects and replicate in all three cell types.

Since the prM mutants did not show significant differences from WT virus growth in all cell culture types, we determined the viral RNA-to-pfu ratio of the mutants to assess the presence of defective particles and infectious virus compared to WT. Infectious virus titers in cell culture medium were determined when peak WT titer was achieved (C6/36 and COS-1 cells, day 4; Vero cells, day 2) by plaque assay, and extracellular RNA in the same samples was measured by qRT-PCR. The ratio of RNA copies/ ml to PFU/ml for mutant viruses was compared to WT virus in each cell type and results are shown in Figure 4.9. In C6/36 cells there was only a significant difference in the ratio of RNA copies/ml to PFU/ml for the K31V mutant compared to WT. WT virus produced 1.4 times more RNA copies/ml than PFU/ml, while the T20D, K31A, K31T and K31V mutants produced 2.4, 6.3, 7.2 and 15.4 times more RNA copies/ml than PFU/ml, respectively (Fig. 4.9A). None of the mutants had significantly higher ratios of RNA copies/ml than PFU/ml when compared to WT in Vero cells. WT virus produced 22 times more RNA copies/ml than PFU/ml in Vero cells, while T20D, K31A, K31T and K31V mutants produced 26, 49, 57 and 32 times more RNA copies/ml than PFU/ml, respectively (Fig. 4.9B). In COS-1 cells the T20D, K31T and K31V mutants had significantly higher ratios of RNA copies/ml to PFU/ml compared to WT virus. WT virus produced 15 times more RNA copies/ml

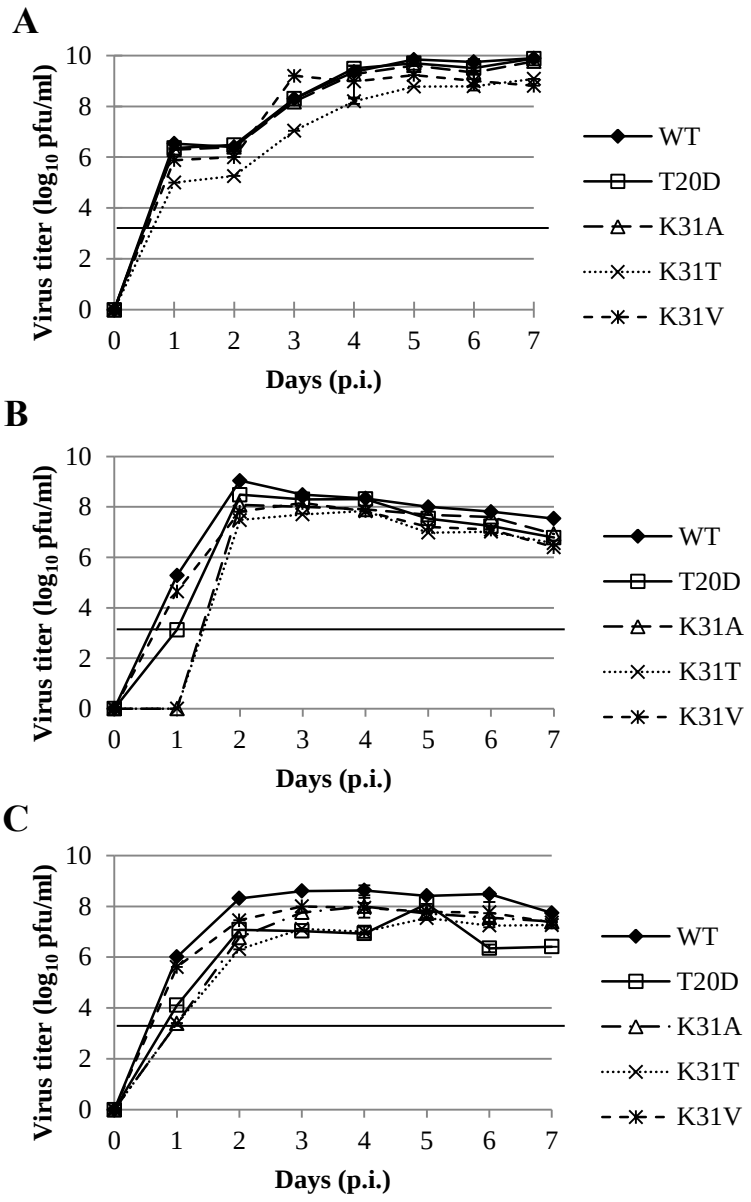

Figure 4.8. Virus growth kinetics in C636, Vero, and COS-1 cells. C636 (A), Vero (B) or COS-1 (C) cells were infected at a MOI of 0.1 and virus titers were measured by plaque assay every day for seven days. Bold line indicates assay limit of detection. Standard error bars are shown for days 4 and 6. The data shown are from two independent experiments.

Table 4.1. Average plaque sizes of WT and prM mutants viruses.

Cells[a]	Virus plaque size (mm)[b]				
	WT	T20D	K31A	K31T	K31V
C636	5.75 ± 1.0	5.35 ± 1.2	3.5 ± 0.9*[c]	2.75 ± 0.7*	4.4 ± 1.2*
Vero	5.6 ± 1.6	5.2 ± 1.2	3.1 ± 0.8*	3.0 ± 1.1*	4.15 ± 1.0*
COS-1	5.85 ± 1.3	5.9 ± 1.0	4.4 ± 1.2*	2.65 ± 0.7*	5.2 ± 1.4

[a] virus was grown in three different cell types prior to plaque assay in Vero cells.
[b] average plaque sizes (mean diameters ± standard deviations) were estimated by counting 20 representative plaques.
[c] significant difference in plaque size compared to WT virus calculated by Dunnett's method of multiple comparisons with an overall type I error of 0.05.

than PFU/ml and T20D, K31A, K31T and K31V produced 236, 29, 45 and 51 times more RNA copies/ml than PFU/ml, respectively (Fig. 4.9C). These results indicate that in COS-1 cells, the T20D, K31T and K31V prM mutations resulted in less efficient production of infectious virus, and growth of the K31V mutant in C6/36 cells was less efficient in infectious virus production than WT virus.

Effect of prM mutations on hMAb binding to virions. Previously, the binding of hMAbs to WN VLP epitopes was characterized, showing that V19 and L33 were key amino acid residues in the VLP epitope recognized by all three hMAbs (Calvert et al., 2011). Since the prM mutations (T20D, K31A, K31T and K31V) prevented secretion of VLP, binding could not be tested in ELISA. Therefore, these mutations, along with 9 other mutations (V19A, V19N, V19T, T20A, T20Q, T24A, L33A, L33K, L33Q) in the pr peptide found to have significant effects on hMAb binding, were incorporated into the WNV infectious cDNA clone (Calvert et al., 2011). *In vitro*- transcribed RNA was transfected into C6/36 cells, and virus was harvested 7 days later. Introduced mutations were confirmed by sequencing of the prM and E genes, and virus titer was determined by plaque assay. COS-1 cells were infected with all prM mutant WNVs at an MOI of 0.1 to produce virus for antigen-capture ELISA to test hMAb binding. COS-1 derived virus was used in the assay in order to directly compare hMAb binding to previous results with VLPs secreted from transfected COS-1 cells. Human sera positive for anti-flavivirus antibody was used as a control in the ELISA to validate that the concentrations of viral proteins were uniform for all mutant viruses tested. Fold changes in reactivity with the control sera range from 0 to 2 and indicate that virus preparations used in the ELISA contained comparative concentrations of viral proteins.

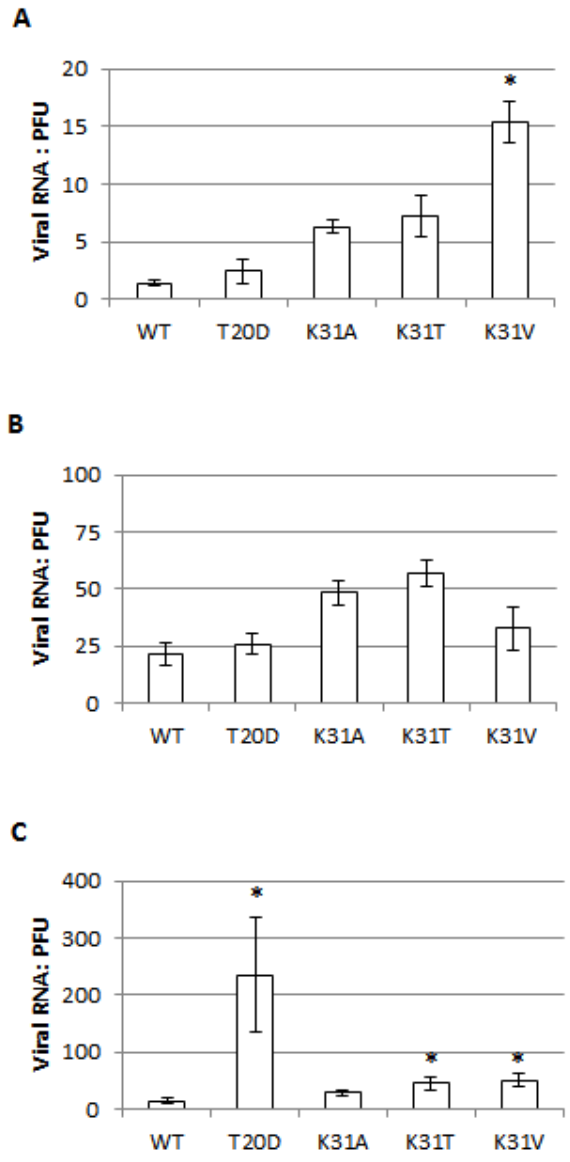

Figure 4.9. Viral RNA: PFU Ratio of prM mutants. Virus was grown in C6/36 (A), Vero (B), or COS-1 (C) cells and virus supernatant was harvested on days of peak virus titer. RNA was extracted and RNA copies/ml was determined by qRT-PCR. Virus titer was determined by plaque assay. The number of RNA copies/ml was divided by the virus titer to obtain the ratio of RNA copies/ml to PFU/ml. Asterisks indicate statistically significant differences compared to WT virus using Dunnett's method of multiple comparisons with an overall type I error of 0.05. The data shown are from three independent tests. Plaque assays to determine virus titer were performed in duplicate, while qRT-PCR experiments were performed in triplicate for each independent test.

The only mutations that resulted in a significant reduction in reactivity with hMAb 8G8 were K31A and K31V, with 5- and 4-fold reductions of end-point titers, respectively (Table 4.2). The T20D mutation resulted in a 10-fold increase in reactivity with hMAb 8G8. All other mutations in the pr peptide of WNV resulted in 3-fold or less reduction in hMAb end-point titers, which was not considered significant (Table 4.2). These results suggest that the K31 amino acid residue in virion prM may be important in the binding of hMAb 8G8.

Discussion

Recent research on the functional importance of the prM protein in flavivirus infections has revealed that the protein is not only important for viral structure and assembly, but also plays an important role in infection and immunity. New evidence shows that non-neutralizing antibodies to prM can enhance DENV infection of Fc receptor-bearing cells *in vitro* as well as enhance WNV infection *in vivo* (Colpitts et al., 2011a; Huang et al., 2008; Huang et al., 2006). These anti-prM antibodies may assist immature virus particles in cell entry, thereby increasing the number of virus-producing cells in secondary infections (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010). The prM protein of flaviviruses also plays an important role as a chaperone to the major envelope glycoprotein E. Not only is the prM protein required for proper folding and maturation of the E protein during virus assembly, it also protects the fusion loop on the E protein from premature fusion before exiting the cell (Konishi and Mason, 1993; Lorenz et al., 2002; Yu et al., 2009). Recently, the prM protein of DENV has been shown to associate with members of the ADP-ribosylation factor (Arf) family of proteins

Table 4.2. Effect of hMAb binding to WNV IC mutant viruses with amino acid substitutions in pr.

pr AA substitution in WNV-IC[a]	Fold change in hMAb titer with pr mutants[b]		
	8G8	5G12	human sera[c]
WT prM	1.0	1.0	1.0
V19A	2.5	1.7	2.0
V19N	1.5	1.0	1.5
V19T	2.0	1.7	1.5
T20A	3.0	2.0	2.0
T20D	0.1	1.7	1.0
T20Q	1.5	1.3	1.0
T24A	1.3	1.5	1.3
K31A	**5.0**	2.7	1.2
K31T	2.7	2.0	2.0
K31V	**4.0**	2.0	2.0
L33A	2.0	2.0	1.3
L33K	2.0	2.0	1.3
L33Q	2.0	1.5	1.3

[a] Amino acid substitution at specific residue in the pr portion. Mutations in bold were previously determined to have a significant effect on VLP-hMAb reactivity.
[b] Four fold reduction or more (bold) in MAb endpoint titer of mutant viruses compared to WT was considered significant.
[c] Human sera positive for anti-flavivirus antibody was used as control in ELISA to verify normalized viral protein concentrations.

(Kudelko et al., 2012; Wang et al., 2009). These host cellular factors are known to play a critical role in intracellular trafficking as well as modulating membrane curvature, a function critical to the assembly and budding of flaviviruses from the ER membrane (Beck et al., 2008; Garoff, Hewson, and Opstelten, 1998). The prM protein of DENV was also shown to interact with vacuolar-ATPases (V-ATPases) in infected cells. These enzymes acidify intracellular organelles, including those in the secretory pathway, and pump protons across the plasma membrane of the cell. This interaction could be significant in both virus entry and membrane fusion via acidified endosomes and secretion of newly assembled virus particles from the cell (Duan et al., 2008).

In this chapter, the effects of 4 specific mutations in the WNV prM protein on WN VLP secretion are studied. Mutations made in prM K31and T20 in the prM expressed from the pVAXWN plasmid resulted in a loss of VLP secretion from transfected COS-1 cells. While K31 mutants were shown to efficiently form prM-E heterodimers, VLP particle secretion was blocked, resulting in accumulation of viral proteins in the ER, ER-Golgi intermediary compartments and TGN of transfected cells.

Assembly and maturation of viral particles begins at the membrane of the ER. The prM and E proteins dimerize and then form an icosahedral scaffold that leads to budding of the enveloped particles into the lumen of the ER . These virus particles are transported through the TGN and released from the cell via the exocytic pathway; however, the exact details of this process are poorly characterized (Mackenzie and Westaway, 2001; Welsch et al., 2009). Flaviviruses have been shown to associate with calnexin and calreticulin, lectins found in the ER that assist in the

proper folding of glycoproteins, chaperoning them through the ER and retaining incompletely folded proteins (Courageot et al., 2000; Lorenz et al., 2003; Mackenzie and Westaway, 2001; Wu et al., 2002). Arf proteins have been implicated in DENV VLP trafficking from the ER to the TGN since depletion of Arf1, Arf4 and Arf5 resulted in blocking secretion of VLPs and virions (Kudelko et al., 2012; Wang et al., 2009). In fact, the prM protein was found to interact with these cellular factors, which may be crucial in the trafficking and secretion of virus particles through the cell. While all four DENVs and YFV were shown to produce less virus when these proteins were down-regulated in the infected cell, this had no effect on WNV infection and secretion (Kudelko et al., 2012). This descrepancy may be due to differences in the two viral particle systems studied, or it may be due to an inconsistency of the role of these cellular proteins in flavivirus maturation and secretion.

Interestingly, the epitope containing K31 in the prM protein is positioned at the top of the prM-E trimer spike in immature virus particles (Fig. 4.10A). These mutations had no effect on prM-E heterodimer formation and protein glycosylation, and appear to play no role in stabilization of trimer spike formation. Since this role of K31 in virus assembly does not appear to be structurally-related, we can hypothesize that it must play some role in interacting with host cellular factors in trafficking the particles through the ER, TGN and ultimately out of the cell. L33 was previously mutated in the WN VLP to alanine, lysine, glutamine or threonine, none of which had an effect on VLP secretion (Calvert et al., 2011). This epitope is not positioned as prominently on the top of the prM-E trimer spike as K31(Fig. 4.10A and 4.10B). It is also interesting that the K31 AA residue is sandwiched between two highly conserved residues

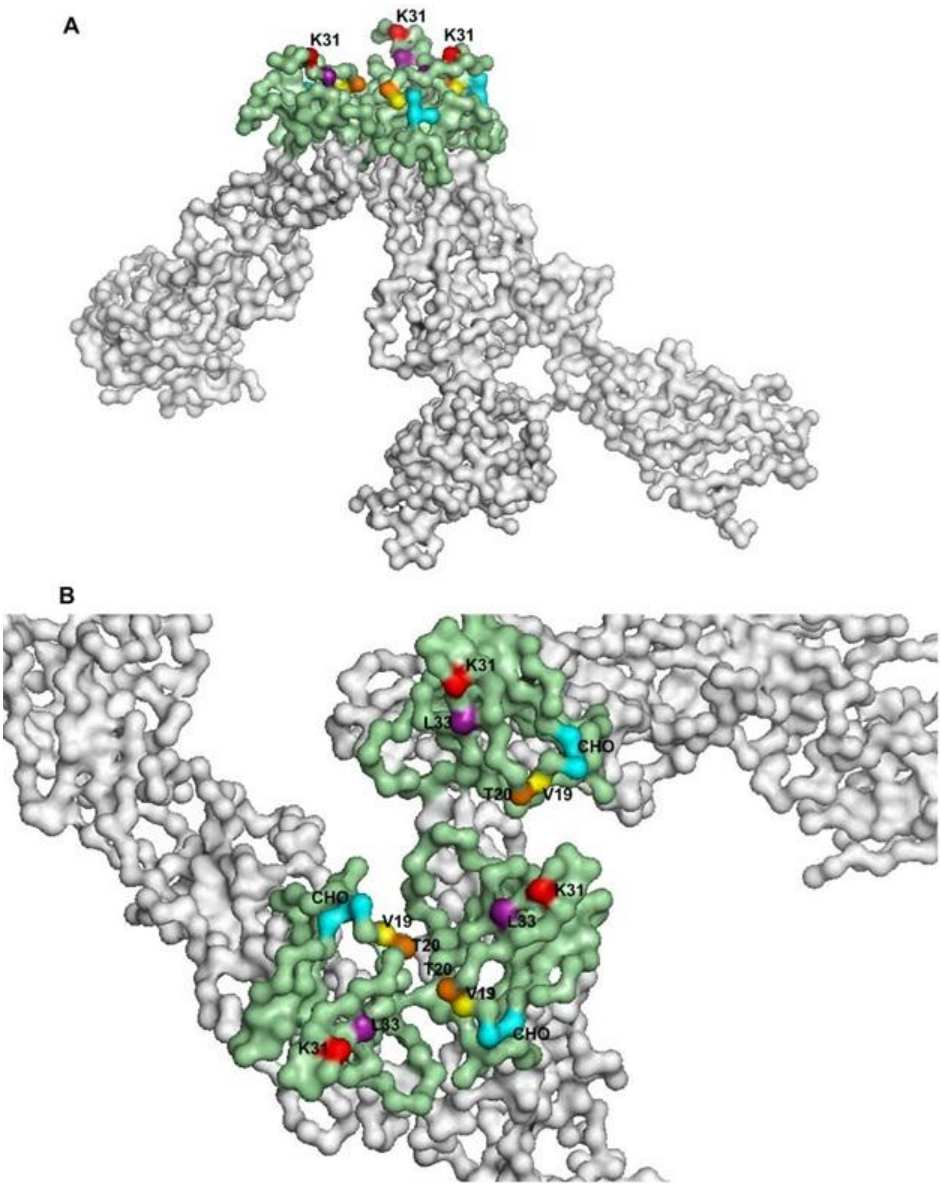

Figure 4.10. DEN2 prM-E trimer spike at neutral pH (PDB ID: 3C6D). A) Side view of the viral surface of the DEN2 prM-E trimer spike. B) Top view toward the viral surface. The E protein is shown in white, while the prM protein is shown in green. V19 is highlighted in yellow, T20 is highlighted in orange, K31 is highlighted in red, L33 is highlighted in purple and the glycosylation site (labeled CHO) is highlighted in cyan.

among flaviviruses, G30 and N32, which may indicate that this region of prM plays some critical role for prM structure and function.

The T20D mutation in the prM protein resulted in failure to form the prM-E heterodimer, as shown by immunoprecipitation studies of lysates from cells transfected with the mutated plasmid. This may be due to inefficient glycosylation of the prM protein, since only small amounts of non-glycosylated prM were shown to associate with the E protein (Fig. 4.2B). Glycosylation of the E protein of flaviviruses has been studied extensively and shown to be a critical factor in flavivirus replication, maturation and neuroinvasiveness in mice (Beasley et al., 2005b; Lad et al., 2000; Li et al., 2006; Scherret et al., 2001). Glycosylation of the prM protein has also been shown to be important for heterodimer formation and secretion of viral proteins from the cell. In JEV, glycosylation on the prM protein was shown to be an important factor in virion assembly and release as well as viral pathogenicity in mice (Kim et al., 2008). Deletion of the prM glycosylation site resulted in a reduction of secreted TBE and WN VLPs (Goto et al., 2005; Hanna et al., 2005). The T20 AA residue lies in close proximity to the glycosylation site on the prM protein in WNV at N15-X16-T17 (Fig 4.10B). While the mutation does not directly affect the sequence of the glycosylation site, mutating the small hydrophilic AA residue threonine to a large negatively charged aspartic acid could affect the local biochemistry of the region, which in turn could prevent glycosylation of the protein and destabilize heterodimer formation. Likewise, each of the prM-E heterodimers may be affected by the T20 residues in the other two prM proteins in close proximity in the prM-E trimer spike (Fig. 4.10B). In the previous chapter it was shown that mutations made at this AA residue (A, G, Q) and those made at AA residue V19 (A, N, R, T) had no effect on VLP secretion (Calvert et al., 2011).

While these mutations had significant effects on VLP secretion, their effect on virus replication was measurable, but not as dramatic. K31 mutants replicated reasonably well in the three cell types tested (C6/36, COS-1 and Vero cells), with a few exceptions. The K31T mutant replicated more slowly in C6/36 cells than WT or the other prM mutant viruses, while in Vero cells all the mutants replicated more slowly than WT before day 2 post-infection. In contrast, K31V was the only mutant to replicate similarly to WT virus in COS-1 cells. Comparisons of plaque morphology revealed that all K31 mutants grown in any of the three cell types (except for K31V in COS-1 cells) had significantly smaller plaques than WT viruses, indicating a replication defect. In comparisons of the amount of extracellular virus RNA (presumably packaged in defective particles) to infectious virus particles, the K31V mutant produced significantly higher ratios of RNA to infectious virus than WT virus in C6/36 and COS-1 cells, while the K31T mutant only produced significantly higher ratios of RNA to infectious virus than WT virus in COS-1 cells. The T20D mutant also produced higher ratios of RNA to virus than WT virus in COS-1 cells. While the differences in production of VLP and virus particles with these mutations are surprising, it is not unprecedented. TBE VLP secretion was inhibited by a mutation at prM AA residue 63 from a proline to a serine; however, when this same mutation was incorporated into the virus genome, only a 10-fold decrease in titer over the first 48 hours of replication was documented (Yoshii et al., 2004). Likewise, when mutations in JEV ablated glycosylation of the prM protein, virus particles continued to be produced, although at a 20-fold decreased titer from WT (Kim et al., 2008). Taken together, these studies demonstrate that mutations that affect VLP formation and secretion may not completely have the same effects on virions. This observation is important to consider when using VLPs to study virus structure and function, and significant findings should be confirmed with replicating virus.

Epitope mapping with hMAbs to the WNV WT and mutant prM proteins revealed a large disparity between VLP and virion presentation of the prM protein. This may be due to the relative concentrations of prM present in the two systems, or to the presentation of the protein itself and the accessibility of the epitopes recognized by these hMAbs. Concentrations of VLPs were standardized by optical density in an ELISA designed to detect all prM and E proteins in the supernatant. Virions were standardized by PFU/ml titers, and viral protein concentrations were validated with the inclusion of polyclonal anti-flavivirus human sera. TBE VLP, which only contains M, is the only VLP structure to be solved of the flaviviruses, therefore it is difficult to compare the unknown structure of the WN VLP with the resolved structure of the mature and immature flavivirus particle (Ferlenghi et al., 2001; Kuhn et al., 2002; Zhang et al., 2003). While differences between virus- and VLP-expressed E protein do not appear to affect epitope expression, differences in protein arrangements between VLP, mature and immature virions could account for differences in epitope recognition on the prM protein by the hMAbs in our study. co-crystallization studies are currently being conducted by Dr. Richard Kuhn at Purdue University to elucidate binding of virions by these hMAbs.

This chapter reiterates the importance of the prM protein in maturation and assembly of VLP and virions and highlights a structural region that has not been previously shown to be important for particle maturation and secretion. The K31 AA residue in the prM protein of WNV has a significant impact on WNV particle assembly and secretion processes. Further investigation into the specific role of the prM protein and the critical epitopes involved will be needed to understand the process of intracellular trafficking of virus.

Materials and Methods

Cells. C6/36 (*Aedes albopictus* mosquito), Vero (African green monkey kidney) and COS-1 (African green monkey kidney) cells were cultured as described previously (Davis et al., 2001; Huang et al., 2000). Cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 110 mg/L sodium pyruvate, 0.1 mM nonessential amino acids, 20 ml/L 7.5% sodium bicarbonate and penicillin (100 U/ml)/streptomycin (100 μg/ml). Vero and COS-1 cells were grown at 37^{o}C with 5% CO_2 while C6/36 cells were grown at 28^{o}C with 5% CO_2.

Antibodies. The anti-prM MAbs 8G8 and 5G12 are WNV specific hMAbs reacting to AA residues V19 and L33 in the prM protein of the VLP (Calvert *et al.* 2011). Anti-E MAbs 3.91D and 3.67G used in the immunoprecipitation studies are WNV specific mMAbs reacting to AA residues S306, K307 and T332 in DIII of the E protein (David C. Clark, unpublished data).

Site-directed mutagenesis and production of mutant infectious WNV and WN VLP. The WNV two-plasmid infectious clone (WN-IC) and pVAXWN plasmid were utilized to produce WNV and VLP respectively, as previously described (Beasley et al., 2005a; Davis et al., 2001; Kinney et al., 2006). Site-specific mutations were introduced into the prM gene of both systems using the QuikChange site-directed mutagenesis kit (Stratagene). Mutagenic primer sequences used for all constructs and transient expression of WN VLP in COS-1 cells by pVAXWN electroporation were described in Chapter 3. Electroporated COS-1 cells were recovered in 6 ml

of DMEM supplemented with 10% FCS. Cells were seeded into 25-cm^2 culture flasks for VLP expression and incubated at 37 °C with 5% CO_2. Six hours after electroporation, the growth medium in flasks was replaced with DMEM containing 2% FCS. Tissue culture medium was harvested 2 and 7 days after electroporation for determination of VLP secretion by ELISA.

Once full length WN-IC cDNA was obtained, *in vitro* transcription of RNA was carried out as previously described (Kinney et al., 2006). C6/36 cells were transfected with transcribed RNA by electroporation. Transfected cells were incubated at 28^{o}C/5% CO_2 for 7 days at which point supernatant was harvested and infectious virus titers were determined by plaque assay. The prM and E genes of WT and mutant viruses were sequenced, and the correct mutations were found to be present in all mutant viruses with no compensatory mutations present.

Characterization of WN-IC mutant viruses in culture. Viral growth curves were performed in triplicate in 6 well plates of Vero, COS-1 or C6/36 cells at a MOI of 0.1. After adsorption for 1 hour, maintenance medium containing 2% FCS and penicillin/streptomycin was added and cultures were incubated in 5%CO_2 at 37^{o}C (Vero and COS-1 cells) or 28^{o}C (C6/36 cells). Aliquots of culture medium were harvested every day for 7 days, FCS concentration was adjusted to 20% and aliquots were stored at -80^{o}C prior to titration and RNA extraction. Viral RNA was extracted from virus supernatant with a QIAmp Viral RNA kit (Qiagen). WNV-specific primers to the structural genes used were based on published data of WNV, strain NY99, and sequencing was carried out as previously described (Kinney et al., 2006). Viral RNA was also quantitated at peak viral titers on either day 2 for Vero or day 4 for COS-1 and C6/36 cells.

A 5 μl aliquot of each sample was added to master mix from iScript One-step RT-PCR kit (Bio-Rad) containing 200 nM of probe, WN1186F (5'-FAM-TGC CCG ACC ATG GGA GAA GCT C-3'), 200 nM of forward primer, WN1160 (5'-TCA GCG ATC TCT CCA CCA AAG-3') and 200 nM of reverse primer, cWN1229 (5'-GGG TCA GCA CGT TTG TCA TTG-3'). WNV-specific RNA was tested in triplicate and measured by qRT-PCR using homologous RNA standards on a Bio-Rad IQ5 Real-time PCR detection system under the following conditions: 50°C for 30 minutes, 95°C for 15 minutes, followed by 45 cycles of 94°C for 15 sec, and 57°C for 1 min with continuous fluorescence data collection. Virus plaque titrations were performed under double agarose overlay in six-well plates of confluent Vero cells (Huang et al., 2000). The second agarose overlay containing neutral red vital stain was added 4 days after infection, and plaques were counted on day 5 after infection.

Analysis of intracellular mutant VLP proteins. Two days after transfection of COS-1 cells with pVAXWN WT and mutant plasmids, cells were harvested and cell lysate was produced as previously described (Blitvich et al., 2003). Similarly, control antigen was produced with non-transfected COS-1 cells. Viral proteins were immunoprecipitated from lysates using the Pierce Immunoprecipitation kit (Thermo Scientific) with resin coupled to 75 μg of WNV murine anti-E 3.91D antibody or 75 μg of WNV human anti-prM 8G8 antibody following the manufacturer's protocol. Glycosylation patterns of viral proteins in lysates were analyzed by digestion after immunoprecipitation using *N*-glycosidase F (PNGaseF) or Endoglycosidase H (EndoH) (New England Biolabs) following the manufacturer's protocol, before analysis by SDS-PAGE.

SDS-PAGE and Immunoblotting. WN viral proteins from immunoprecipitated lysates were separated by SDS-PAGE on a reduced 4-12% Bis/Tris gel (Invitrogen). All procedures were performed at room temperature. Proteins were blotted electrophoretically from the gels onto nitrocellulose membranes and washed for 5 minutes in PBS/0.1% Tween wash buffer. Non-specific binding sites were blocked with 1% BSA/PBS for one hour while rocking. WNV murine anti-E MAb, 3.67G (10 μg), and purified rabbit polyclonal sera to WNV prM (10 μg) (Meridian LifeScience) were incubated with the membrane for 1 hour with gentle rocking. Membranes were washed again in PBS/0.1% Tween wash buffer three times for 5 minutes each. Goat anti-mouse antibody and goat anti-rabbit antibody conjugated to alkaline phosphatase (Jackson Immunoresearch) were diluted 1:200 and incubated on the membrane for 1 hour with gentle rocking. Membranes were washed as described previously and BCIP/NBT phosphatase substrate (KPL) was added to the membrane until a color change appeared. The reaction was stopped by the addition of water.

ELISAs. All ELISAs were performed in 96-well plates (Maxisorp plates, Nunc). To test for secretion of mutant VLP antigen, rabbit hyper-immune sera to WNV prM and E proteins was diluted 1:1000 in carbonate/bicarbonate buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6) added to the wells and incubated overnight at 4^{o}C. Plates were washed five times with PBS/0.1% Tween wash buffer with an automatic plate washer. Non-specific binding sites were blocked with 3% rabbit serum in PBS (100 μl/well) and incubated at 37^{o}C for 1 hour. Blocking buffer was removed before supernatant from transfected COS-1 cells was added to the plate in doubling dilutions in 3% rabbit serum/PBS (50 μl/well). Antigen was incubated at 37^{o}C for 2 hours, after which the plates were washed as previously described. MHIAF to WNV was

obtained from the Reference Collection of the Diagnostic Laboratory, ADB, DVBID, CDC, and used as the primary antibody at a dilution of 1:1600 in 3% rabbit serum/PBS and incubated at 37^{o}C for 1 hour. Plates were washed 5 times before the addition of rabbit anti-mouse antibody conjugated to horseradish peroxidase (50 μl/well), diluted 1:5000 in 3% rabbit serum/PBS. After an incubation period of one hour at 37^{o}C, plates were washed again ten times. Enhanced K-blue TMB substrate (Neogen) was added to each well of the plate (100 μl/well) and incubated in the dark at room temperature for 10 minutes. The reaction was stopped with the addition of 1N H_2SO_4 (50 μl/well), and the plates were read at 450 nm on an automatic plate reader.

To test the reactivity of the mutant viruses with the hMAbs the protocol remained the same with some exceptions. Viruses were diluted to a concentration of 10^6 PFU/ml in 3% rabbit serum/PBS. Purified hMAbs were used as the primary antibody and added to the wells in two-fold dilutions in 3% rabbit serum/PBS (50 μl/well) starting at 50 μg/ml. The secondary MAb, rabbit anti-human conjugated to horseradish peroxidase (50 μl/well) was diluted 1:5000 in 3% rabbit serum/PBS. The rest of the ELISA was carried out as described above.

Immunofluorescence and confocal microscopy. Transfected COS-1 cells were grown on 8-well chamber slides (Labtek) and incubated in 5%CO_2 at 37^{o}C. At 48 hours after transfection, medium was removed and cells were washed 3 times in PBS. Cells were fixed to the slide with 4% (w/v) paraformaldehyde in PBS for 10 minutes and permeabilized with 0.1% (v/v) Triton-X 100 in 0.2% (w/v) BSA/PBS for 10 minutes. Non-specific sites were blocked with 10% (v/v) goat serum in PBS for 30 minutes at 37^{o}C. Cells were incubated with WNV murine anti-E

antibody 3.91D (5 μg/ml) and either rabbit anti-calreticulin (1:500), rabbit anti-βCOP1 (1:100), rabbit anti-ERGIC53 (1:100) or rabbit anti-GRASP65 (1:500) diluted in PBS for 30 minutes at 37^{o}C before being washed 3 times in PBS. Secondary antibodies, goat anti-mouse conjugated to FITC and goat anti-rabbit conjugated to Texas Red, were diluted 1:100 in PBS and incubated on the cells for 30 minutes at 37^{o}C, and cells were washed again as previously described. Slides were dried before the addition of Prolong Gold anti-fade reagent with DAPI (Invitrogen). Coverslips were mounted on the slides and allowed to cure at room temperature overnight. Images were viewed and collected with a Carl Zeiss LSM-Pascal confocal microscope.

Chapter 5: Conclusion

hMAbs to a variety of disorders and agents of infectious illnesses have become important tools as therapeutic agents, and they may also have a promising future in the prophylaxis and treatment of flaviviruses infections. Anti-flavivirus hMAbs can also aid in our understanding of the complex human antibody response to these viruses during infection. hMAbs isolated from patients with DENV infections have shown the human antibody response to be highly focused on non-neutralizing cross-reactive epitopes of DI/DII of the E protein, and to some extent the prM protein (Beltramello et al. 2010; Dejnirattisai et al., 2010; Smith et al. 2012) . These are important findings to consider in DENV vaccine development, since incorporation of these highly immunogenic and cross-reactive sites in a vaccine may make development of highly effective vaccines with limited cross-reactivity difficult. Anti-flavivirus hMAbs also have an important role in their use to study the complex antigenic structures of these viruses from the perspective of the human immune system. This is an invaluable tool in understanding important epitopes during virus infection.

In this dissertation, an attempt to develop hMAbs to flaviviruses was made using a competent human fusion partner cell line, MFP-2, fused with several different sources of B cells. hMAbs secreted from hybridomas made from PBLs from persons with previous flavivirus vaccinations or infections working at the CDC were able to secrete IgG and IgM antibodies; however none was specific for anti-flavivirus antibody. While serum antibody concentrations and circulating memory B cells can be long-lived, the total number of cells needed to screen for specific B cells

is high (Bernasconi, Traggiai, and Lanzavecchia, 2002). Long-lived circulating memory B cells have been shown to occur for many vaccines including measles, mumps and rubella, tetanus and diphtheria, and influenza and DENV infections (Amanna, Carlson, and Slika, 2007; Beltramello et al., 2010, Dejnirattisai et al., 2010; Schieffelin et al., 2010; Smith et al., 2012; Wrammert et al., 2008; Yu et al., 2008). Isolating cells soon after vaccination or infection would increase the chances of isolating B cells producing antibodies reactive to flaviviruses. Collaborations with the Dengue Branch, CDC, San Juan, Puerto Rico have been established to find patients with recent DENV infections through the SEDSS at local area hospitals. Patients identified as having recent DENV infections will be enrolled in an IRB-approved study to collect PBLs within 7 to 10 days after onset of symptoms. Isolating B cells quickly after infection will increase the chances of finding those producing antibody specific for DENV. In addition, these cells will be immortalized with EBV in order to efficiently screen cells for positive anti-flavivirus antibody before fusion with MFP-2 cells.

The use of RAGHu mice vaccinated or infected with DENV as a source of B cells proved to be an impractical method to obtain suitable cells for fusions. Results reported here were consistent with several reports in which infected RAGHu mice and other humanized mouse models were unable to mount a robust humoral immune response (Baenziger et al., 2006; Becker et al., 2010; Biswas et al., 2011; Jaiswal et al., 2012a; Rajesh et al., 2010; Tonomura et al., 2008; Traggiai et al., 2004b; Watanabe et al., 2009). These animals produced very low and variable levels of virus specific antibody that did not class switch from IgM to IgG even after repeated booster immunizations or secondary infections, a feature consistent with a T-cell independent response. The inability of RAGHu mice and other humanized mice to develop a robust immune response

may be due to incomplete maturation of human T cells in the murine environment, including inadequate stimulation by murine cytokines (Biswas et al., 2011; Jaiswal et al., 2012a; Rajesh et al., 2010).

Three hMAbs were developed by Drs. Kalantarov and Trakht, Columbia University, using PBLs from a person previously infected with WNV and MFP-2 cells. This provides proof of concept that MFP-2 cells can be fused with human PBLs to produce hMAbs to infectious agents. Previously, MFP-2 cells had only been used to produce hMAbs to breast cancer specific antigens (Kalantarov et al., 2002; Kirman et al., 2002; Rudchenko et al., 2008). These hMAbs were found to be specific for a conformational epitope on the prM protein of WNV. The specific epitope recognized by these hMAbs map to the most variable region of prM at V19 and L33 located at the very top of the protein. Mutations made in the VLP to map the epitopes of hMAbs were also incorporated into the virion. Surprisingly, the epitope mapping with virions revealed a large disparity between the presentation of prM on VLP and virions. This may be due to the accessibility of the epitopes recognized by these hMAbs, or the concentration of prM in the two systems. Since only the crystal structure of TBE VLP has been solved, it will be necessary to solve the WNV VLP crystal structure to to explain this descrepancy.

These hMAbs were found to have no neutralization capacity *in vitro,* and offered minimal protection in outbred and interferon deficient mice receiving prophylactic doses before lethal WNV challenge. This result may not be surprising since recent reports have shown that anti-prM antibodies to DENV and WNV are able to enhance infection *in vitro* and *in vivo* (Colpitts et al.,

2011; Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010). These results add to the growing knowledge of antibodies to flavivirus prM in the literature and highlight their importance in the human humoral response.

Four mutations made in the WN-VLP to map the epitope recognition site of the hMAbs described did not result in VLP secretion. This was an interesting and unexpected result that led to a tangential research project using the hMAbs developed in this study to explore the significance of these mutations not only on VLP assembly and secretion, but their effect on virion processing as well. Heterodimer formation, glycosylation and intracellular localization were analyzed to understand the effect these mutations had on VLP secretion. K31 mutants were shown to efficiently form prM-E heterodimers, with no effect on glycosylation, and this AA residue appears to play no role in stabilization of the trimer spike. However, localization of E proteins in transfected cells appeared to be blocked at the ER, ER-Golgi intermediary compartments, and TGN. This AA residue in the prM, which sits at the very top of the prM-E trimer spike, may have a significant impact on VLP interaction with host cellular factors important in trafficking VLP particles through the secretory pathway. Interestingly, the T20D mutation did have an impact on prM-E heterodimer formation, possibly by affecting glycosylation of prM. Even though this mutation did not directly affect the glycosylation site itself, it may have affected the local biochemistry of the site, preventing glycosylation and destabilizing prM-E heterodimer formation.

When the mutations were introduced into the virion their effect was measurable, but not as dramatic as those seen in the VLP system. WNV with incorporated K31 mutations replicated reasonably well in all three cell types tested *in vitro*; however, comparisons of plaque morphologies revealed significantly smaller plaques, indicating a replication defect. In order to determine if more defective particles were being produced by these mutants, total WNV RNA released and infectious virus titers were directly compared. Three of the mutants (T20D, K31T, K31V) produced significantly higher ratios of RNA to infectious virus in COS-1 cell,s indicating a defect in replication. These results demonstrate that mutations that affect VLP formation and secretion may not have the same effect on virions. This is an important consideration since many researchers use the VLP system to study virus structure and function. The use of hMAbs characterized in this dissertation highlight their use as a tool in understanding the structural importance of the WNV prM protein in human infection, as well as in the virus life cycle.

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LIST OF ABBREVIATIONS

A	adenine
A	alanine
Å	Angstrom
AA	amino acid
ADB	Arbovirus Diseases Branch
ADE	antibody dependent enhancement
Arf	ADP ribosylation factor
ASC	antibody secreting cell
bCOPI	beta coatomer 1
BSA	bovine serum albumin
C	capsid
C	cytosine
CDC	Centers for Disease Control
cDNA	complimentary deoxyribonucleic acid
CDR	complimentarity determining region
CHO	carbohydrate
cIgG	chimeric Immunoglobulin G
CPE	cytopathic effect
C-terminal	carboxyl terminal
D	aspartic acid
DAPI	4',6-diamidino-2-phenylindole
DC-SIGN	dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin
DENV	dengue virus
DENV1	dengue virus 1
DENV2	dengue virus 2
DENV3	dengue virus 3
DENV4	dengue virus 4
DHF	dengue hemorrhagic fever
DI	domain I
DII	domain II
DIII	domain III
DNA	deoxyribonucleic acid
DSS	dengue shock syndrome
E	envelope
EBV	Epstein Barr virus
ELISA	enzyme linked immunosorbant assay
EndoH	endoglycosidase H
ER	endoplasmic reticulum
ERGIC	endoplasmic reticulum-Golgi intermediate compartment
FBS	fetal calf serum

FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
G	guanine
H	histidine
HBV	hepatitis B virus
HCV	hepatitis C virus
HIS	human immune system
hMAb	human monoclonal antibody
HSC	hematopoietic stem cells
i.p.	intraperitoneally
IFN	interferon
Ig	immunoglobulin
IV	intravenous
JEV	Japanese encephalitis virus
K	lysine
Kb	kilo base
Kda	kilo Dalton
kg	kilograms
L	leucine
L	liter
LGTV	Langat virus
LIV	Louping ill virus
M	membrane
M	molar
mg	milligrams
mg	micrograms
MHC	major histocompatability class
MHIAF	mouse hyper-immune ascitic fluid
ml	milliliters
mM	milli molar
mMAb	murine monoclonal antibody
moi	multiplicity of infection
MST	median survival time
MVEV	Murray Valley encephalitis virus
N	asparagine
N	normal
nM	nano molar
nm	nanometer
NS	nonstructural protein
N-terminal	amino terminal
NTR	non-translated region
^{o}C	degrees Celsius
OD	optical density
ORF	open reading frame

PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
PEG	polyethylene glycol
pfu	plaque forming unit
pi	post-infection
PNGaseF	N-Glycosidase F
POWV	Powassan virus
prM	pre-membrane
PRNT	plaque reduction neutralization test
Q	glutamine
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
R	arginine
RagHu	Humanized Rag2(-/-)gammac(-/-) mice
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
S	serine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEDSS	Sentinel Enhanced Dengue Surveillance System
SLEV	St. Louis encephalitis virus
SW	Swiss-Webster
T	threonine
T	thymine
TBEV	tick-borne encephalitis virus
TGN	trans-Golgi network
TT	tetanus toxoid
U	unit
v/v	volume per volume
VATPase	vacuolar ATPase
VEEV	Venezuelan equine encephalitis virus
VLP	virus-like particle
w/v	weight per volume
WN-IC	West Nile - infectious clone
WNV	West Nile virus
WT	wild type
x *g*	gravitational force
YFV	yellow fever virus