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West Nile virus versus the host cell: Identification of factors that modulate infection

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West Nile virus versus the host cell: Identification of host factors that modulate infection

by

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Dedication

To my family and friends who have helped me and encouraged me throughout my graduate career and life. A special ‘shout-out’ goes to Bridget, who has been a constant friend since we first started school. You helped me open my shell and I will never forget your part in me meeting Sergio!

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West Nile virus versus the host cell: Identification of host factors that modulate infection

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There are two competing aspects of virus-host interactions: (*i*) those that enhance virus transmission and (*ii*) those that reduce or prevent viral replication and transmission. Each of these types of interactions is critical and both must be investigated to fully understand viral pathogenesis. Cells encode a variety of molecules which are critical for recognizing viral infections. One such molecule, the dsRNA sensor PKR, has been shown to be important for IFN-β induction. Therefore, to determine whether PKR was involved in the recognition of WNV infection, cells lacking PKR were infected with WNV and assayed for IFN production. Interestingly, PKR-null cells demonstrated dramatically lower levels of WNV-induced IFN compared to wild type cells. Additionally, chemical inhibition of PKR activity or post-translational gene silencing of PKR expression severely impaired WNV-induced IFN production, suggesting that PKR is critical for the induction of IFN following WNV infection. Further analysis suggested that PKR may be important for the activation of NFκB, suggesting a possible mechanism of IFN-β

induction. Consistent with cell line data, PKR was shown to be critical for WNV-induced IFN production in primary mouse bone marrow-derived dendritic cells.

The recognition of WNV is an important aspect of controlling infection; however, it is only one side of the story. Host factors which WNV utilizes to facilitate its infection and replication are also key to understanding viral pathogenesis. The presence or absence of specific factors may control the level of viral replication, host tropisms and, ultimately, viral pathogenesis. To identify host co-factors that are essential for WNV infection and/or replication, small interfering RNAs (siRNAs) were used to systematically knockdown host gene products and levels of WNV infection and/or replication was assayed in the absence of these factors. A siRNA library screen identified ten cellular proteins which are essential for WNV infection and/or replication. Two of these genes encoded subunits of the proteasome. Chemical inhibitors of proteasome activity confirmed that the proteasome is critical for efficient WNV replication.

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CHAPTER 1: INTRODUCTION

Viruses within the family Flaviviridae are responsible for significant morbidity and mortality worldwide. The diseases caused by a few of these viruses, such as yellow fever virus (YFV) and Japanese encephalitis virus (JEV), have been controlled with varying success through the application of vaccines. For the majority of viruses in the family Flaviviridae, however, there are still limited vaccines available and there is only a single approved antiviral therapy for one virus within the family. Because of the lack of antiviral treatments and effective vaccines, the viruses within this family are likely to be the subject of intense scrutiny for years to come.

Family Flaviviridae

The family Flaviviridae consists of three genera classified by their antigenic specificity and genome and nucleotide similarity: *Flavivirus*, *Pestivirus*, and *Hepacivirus*. These three genera contain more than 70 different viruses which cause a broad range of diseases and are able to infect a wide variety of both invertebrates and vertebrates. Although the members of the different genera are quite distinct, they do share many common characteristics, such as a similar gene order and conserved nonstructural protein motifs (324). All of the viruses within the family contain a single-stranded, positive-sense RNA genome encoded in a single open reading frame (ORF). They are all spherical in nature, approximately 30-80nm in diameter and encased in a lipid bilayer that originates from the host cell. Although they are within the same family and share some conserved genotypic and morphologic features, the viruses within the three genera are quite different in disease pathology, transmission and host range.

GENUS *HEPACIVIRUS*

Hepatitis C virus (HCV) is the prototype member of the genus *Hepacivirus* (324). The virus, first identified in 1975 (133), is the causative agent of a variety of disease states ranging from acute liver dysfunction to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (324, 480). It is estimated that nearly 2.2% of the world's population have been infected with HCV with more than 170 million chronic carriers (9, 514). Although treatment exists for those infected with HCV—sustained combination therapy of pegylated IFN-α and ribavirin—the treatments are only effective in controlling infection in approximately 50% of infected individuals (141). Thus, HCV remains a significant public health problem worldwide.

The HCV genome is approximately 9.6 kilobases (kb) in length and flanked by 5' and 3' untranslated regions (UTR). The HCV genome lacks a 5' cap; instead it contains an internal ribosomal entry site (IRES) that permits cap-independent translation (324). The genomic RNA is translated as a single polyprotein and cleaved by viral and cellular proteases co- and post-translationally to form ten viral proteins. Three structural proteins, C, E1 and E2, are responsible for the formation of the virion; while seven nonstructural proteins, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B, are responsible for the formation of the replication complex (324). Following the identification of HCV, studying its replication was difficult due to a lack of any real cell culture systems. In 1999, however, the development of subgenomic HCV RNAs capable of replication within cell lines opened the door for a plethora of fresh molecular research (333). Recently, full-length HCV has been adapted to grow in cell culture (321, 572, 623), allowing study of the complete replication cycle of this important human pathogen.

GENUS *PESTIVIRUS*

Viruses within the genus *Pestivirus* cause acute or persistent infections of wild and domestic animals, such as cattle and sheep. Members of this genus include the type-member bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV) and border disease viruses (BDV). These viruses are of particular economic importance as they cause major diseases (including wasting disease, hemorrhagic syndromes or fatal disease) in livestock. In fact, it is estimated that 0.5-2% of livestock are persistently infected with BVDV alone and 60-85% are antibody positive, resulting in an estimated loss of up to $40 million per million calvings due to fetal death, birth defects or other acute syndromes (228). Persistently infected cattle can shed large amounts of virus [more than 10^7 tissue culture infectious dose^{50} ($TCID^{50}$)], which is the major source of transmission within herds. There is a vaccine available for BVDV; however, animals which have a prior persistent infection can develop mucosal disease from vaccination (324).

The genome structure and replication strategies of pestiviruses are more closely related to hepaciviruses than to flaviviruses. There are, however, distinct differences between pestiviruses and the other two genera in the family. The pestivirus genome is approximately 12 kb in length and, like HCV, viral translation is initiated via an IRES present on the 5' end of the genome. As with other members of the family *Flaviviridae*, the pestivirus genome is translated as a single polyprotein and cleaved co- and post-translationally to form four structural proteins, C, E^{rns}, E1 and E2, and seven nonstructural proteins, N^{pro}, NS2, NS3, NS4A, NS4B, NS5A and NS5B. An additional protein, p7, is located between E2 and NS2; however, it is not known whether this protein serves as a structural or nonstructural gene (324). One unique feature of the pestivirus

genome is that the first protein expressed in the viral polyprotein is a nonstructural protein, N^{pro}. This protein is critical for cleaving the N^{pro}/C site (324). Three of the structural proteins, C, E1 and E2 compose the virus particle; however, the function of E^{rns} has not been fully characterized. The nonstructural proteins make up the replication complex (324). Of particular interested is the NS2-3 complex, which serves as the viral protease. In BVDV, the protein can exist as a cleaved or uncleaved moiety. Interestingly, there is a direct link between the cleaved status of NS2-3 and cytopathology, uncleaved protein is present in noncytopathic strains while the cleaved form is present in cytopathic strains (324).

GENUS *FLAVIVIRUS*

Viruses within the genus *Flavivirus* are distinct from other members of the Flaviviridae family in that the majority of the viruses are transmitted primarily via arthropod vectors and, hence, fit into the large group of viruses known as arboviruses. Several flaviviruses have world-wide distribution and multiple flaviviruses can co-exist in the same geographic location. In fact, one or more flaviviruses can be detected on every continent on Earth, barring Antarctica (183). The genus includes several viruses of major public health concern, including dengue virus (DENV), JEV and YFV. These viruses cause a range of diseases, from subclinical infections to severe hemorrhagic or encephalitic fevers. In recent years, likely due to a decrease in vector control and an increase in global transportation and urbanization, several flaviviruses, including DENV and West Nile virus (WNV), have reemerged to become a serious problem (16, 247, 302, 394).

There are approximately 53 recognized virus species within the *Flavivirus* genus: 27 classified as mosquito-borne, 12 classified as tick-borne and 14 classified as zoonotic,

non-vectored viruses. The further classification of the genus into subtypes/subspecies has lead to estimations of nearly 80 distinct viruses. The mosquito-borne viruses within the genus can be further classified into two groups, based primarily on clinical presentations and ecology (184). One group, the encephalitic group, contains viruses which utilize birds as the natural host and the *Culex* species of mosquitoes the primary vector. These viruses include JEV, WNV, Murray valley encephalitis virus (MVEV) and Saint Louis encephalitis virus (SLEV). The second group includes YFV and DENV, which are more viscerotreopic and are capable of causing hemorrhagic fever. These viruses primarily utilize a forest cycle consisting of primarily *Aedes* species of mosquitoes and lower primates (184). DENV, however, has also developed a successful urban cycle, allowing for direct transmission between humans and mosquitoes (182). At one time, YFV also had an urban cycle, however, it was essentially eliminated due to large scale mosquito control in the early 20^{th} century (527).

Genome organization

The flavivirus genome is, for the most part, similar to those of HCV and the pestiviruses. The genome is approximately 11 kb in length and has no poly(A) tail. However, unlike the other two genera, flaviviruses do not contain an IRES; instead, translation is initiated via a 5' methylated cap ((m^7G5'ppp5'A) (581)). Since the virus is of positive polarity, the genomic RNA serves as a messenger for translation. Although early studies suggested that the viral genome was translated from multiple initiation sites (582), the sequencing of the YFV genome (and subsequent sequencing of other flaviviruses) indicated that the RNA consists of a single ORF translated as a polyprotein (190, 366, 372, 464).

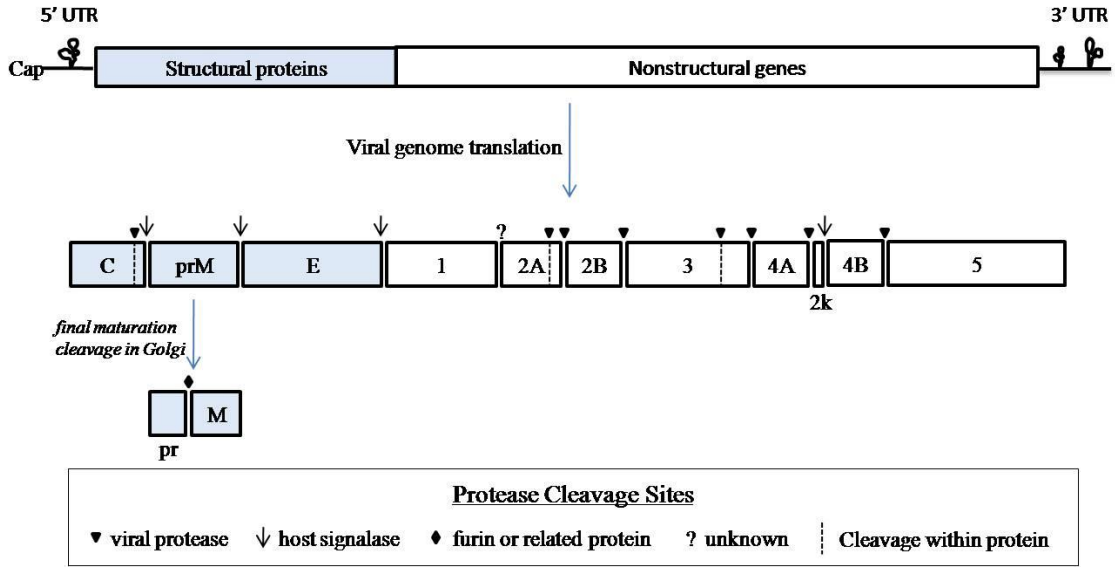


FIGURE 1.1: GENOME ORGANIZATION AND POLYPROTEIN PROCESSING SITES OF THE FLAVIVIRUS GENOME. Structural proteins are depicted in light blue. Image was inspired by Field's Virology (Lindenbach, Thiel and Rice, 2007).

The ORF encodes a total of ten proteins: three structural proteins encoded on the first 1/3 of the genome, and seven nonstructural proteins encoded on the remaining 2/3 of the genome. The three structural proteins—capsid (C), membrane (prM/M) and envelope (E)—are required for packaging the genomic RNA and producing progeny virions. The seven nonstructural proteins (NS)—NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 make up the replication complex and play various roles in the lifecycle of the virus. As mentioned, these proteins are translated as a single entity and cleaved co- and post-translationally by viral and host proteases (Figure 1.1) (324). Specific details on the functions of the viral proteins and viral translation and replication will be discussed in later sections.

The ORF of the viral genome is flanked by a 5' and 3' UTR of approximately 100 nucleotides (nt) and 400 to 700 nt, respectively. These regions contain sequences rich in predicted RNA secondary structures thought to serve as *cis*-acting elements during viral

replication, translation or packaging (324). Although the 5' UTR sequences are poorly conserved between flaviviruses, they contain common secondary structures (53, 59). The primary function of the 5' UTR is through its reverse complement, the minus-sense 3' UTR, which serves as an initiation site for positive-strand synthesis (324). Studies of the 5' UTR of DENV indicated that most deletions within the long stem region resulted in a lethal phenotype. However, deletions in the short stem or loop structure were usually viable, with variable levels of attenuation (59). Several host proteins have been shown to interact with regions of the stem loop of the minus-strand 3' UTR (52, 313).

The 3' UTR of the flavivirus genome serves as a promoter for minus-strand synthesis (324). Although the sequence and size of the 3' UTR is divergent between the different flaviviruses, there are some conserved regions among all flaviviruses as well as some similarities among specific taxonomic groups, particularly at the 3' terminal region of the genome. Biochemical analysis has demonstrated that the 90-120 nt present at the terminal end of the 3' UTR contain a thermodynamically stable stem loop structure common to all flaviviruses (54, 66, 189, 573). This region is thought to be critical for flavivirus replication, as several mutations introduced into the 3' stem loop structure of DENV-4 resulted in a reduction or complete loss in viral production (468). Additionally, both viral (72) and host proteins (38, 39, 106, 154) have been shown to interact directly with the 3' stem loop structure.

All mosquito-borne flaviviruses contain conserved cyclization sequences (CS) in the 3' UTR, which are capable of base pairing with CS present on the 5' end of the ORF (at the beginning of C) resulting in the cyclization of the genome. This cyclization is thought to be required, as deletions in the CS region of DENV are lethal (378). Sequences of conserved RNA structure resembling cyclization sequences have also been

observed in the 5' UTR of tick-borne flaviviruses, as well (355, 356, 358). The viral polymerase, NS5, has also been shown to bind to the 5' UTR, promoting negative RNA synthesis (136).

Flavivirus proteins

Structural proteins

The first protein encoded by the ORF is the capsid protein (C). C is an 11 kilodalton (kd), highly basic protein that forms the ribonucleoprotein complex with the genomic RNA (324). The complex formation is thought to be mediated by the positively-charged, basic residues that cluster along the amino- and carboxy-terminals of C, which can presumably interact with the negative charges present within the genomic RNA. C is translated as an immature protein with a small, hydrophobic domain on its C-terminal end (anch-C). This segment encodes a signal sequence responsible for targeting and anchoring C and prM to the endoplasmic reticulum (ER) (412). Cleavage between C and this hydrophobic domain by the viral serine protease (NS2B/NS3; see below) generates the mature C protein capable of forming the ribonucleocapsid (10, 330, 412, 604).

The second of the three structural proteins is prM (26kd) which is the glycoprotein precursor of the mature M protein present in the lipid bilayer of the mature virion. As mentioned above, a short, hydrophobic sequence following the mature C protein is the signal sequence that targets prM across the ER membrane into the lumen. The cleavage of this hydrophobic domain from C is essential for the viral serine protease (NS2A-NS3)-mediated cleavage of the C-prM junction (10, 330, 604). In fact, if the cleavage of anch-C does not occur prior to the cleavage of the C-prM junction, there is an increase in the production of nucleocapsid-free particles and a decrease (or complete abolishment) of viral infectivity (306, 331).

The prM protein contains two C-terminal transmembrane domains which have multiple functions. They anchor prM to the ER membrane, serve as the signal peptidase for E and appear to aid in the formation of virions (319, 418, 419). There appear to be two primary functions for prM: aiding in the proper folding of envelope (E) protein and chaperoning E through the secretory pathway. In the absence of prM, E cannot be folded correctly or be incorporated into virions (289, 335). Additionally, the absence of prM made E sensitive to low pH (185, 335), suggesting that prM may serve as a protective chaperone through its potentially hazardous (low pH conditions) treck through exocytic vesicles. Following the heterodimerization of prM and E, the complexes are shuttled through the secretory pathway where prM is cleaved by furin, or another related protease (529). This cleavage, which allows for the production of mature virions (324), is essential for the production of infectious virions, as blocking it results in the release of noninfectious, immature particles (123, 185, 618).

The final structural protein translated from the ORF is the envelope glycoprotein, E (53kd). Although both E and M are present in the mature virion at equimolar concentrations, E is predominantly present on the virion surface (399). E primarily serves as a mediator of receptor binding and membrane fusion and is also the major target for flavivirus neutralizing antibodies (324). As with prM, E is anchored to the ER membrane via two transmembrane domains. The protein contains 12 conserved cysteine residues that form disulfide bonds, which are thought to be critical to the structure of E (413). The E proteins of most flaviviruses contain one, or multiple, N-glycosylation sites (590). The virulence of WNV, in fact, appears to be determined, in part, by presence or absence of a single N-linked glycosylation site on E (28, 518).

In the mature virion, the E protein forms a 'head-to-tail' homodimer complex that lies parallel to the virion membrane (462). The structure of E from TBEV revealed that the E tertiary structure folds into three distinct domains: domain I (DI), domain II (DII) and domain III (DIII) (462). DII forms the central dimerization region of the protein and contains the putative fusion peptide at the tip of the region (distal to the transmembrane domain) and DIII is an immunoglobulin-like domain, thought to be responsible for receptor binding and serves as a major target for neutralizing antibodies (8, 324). The presence of a hinge region at the base of DII appears to aid in conformation changes required during transitions from heterodimers to homodimers, and then to homotrimers that participate in fusion (399, 462).

Cryoelectron microscopy and image reconstruction technology have been used to reveal the structures for multiple flaviviruses. The flavivirus virion itself is small, only 50nm in diameter containing a small, dense nucleocapsid core (30nm). This nucleocapsid is composed of the genomic RNA encapsidated by a relatively unstructured array of C molecules surrounded by a lipid bilayer obtained from the host's ER membrane (297). Surprisingly, there appears to be an electron lucid region between the nucleocapsid and the membrane arguing against a direct structural connection. Prior to release, the virion is present in its immature form. This form contains 60 trimeric spikes composed of prM-E heterodimers with icosahedral symmetry (621). This immature form, which contains the uncleaved prM, hides the fusion peptide and the furin cleavage site, which is only exposed when the pH drops (123, 185). The surface of the mature form of the flavivirus virion, on the other hand, is relatively smooth. Instead of forming spikes, the E proteins on the mature virion form 90 head-to-tail homodimers that cover the entire surface of the virion in a 'herringbone' pattern (297, 399, 462, 621). The three-dimensional image of

WNV is shown in Figure 1.2 (399). Interestingly, the prM and E proteins can also self-assemble without C or viral RNA into sub-viral particles (SVPs) (336, 367, 493, 525).

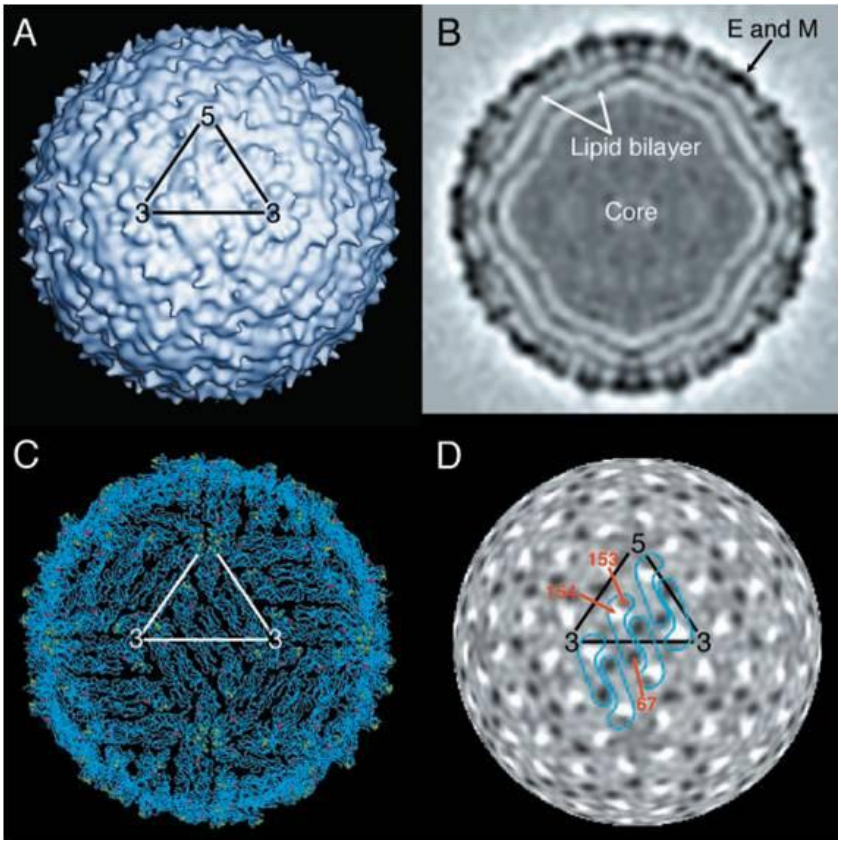


FIGURE 1.2: STRUCTURE OF WNV PARTICLE. The structure of the WNV virion was determined by cryoelectron microscopy. Panel A shows the relatively smooth surface of the WNV particle and panel B is a cross-section of a particle highlighting the different electron-dense layers within the virion. Panel C shows the arrangement of E on the surface of the virus and panel D highlights density differences between WNV (white) and DENV (black). The locations of 3 E dimers in the icosahedral synthetic unit are outlined in blue. Image was reprinted with permission from AAAS from Mukhopadhyay, Suchet et al., 2003. Structure of West Nile virus. Science 302:248.

Nonstructural proteins

As mentioned above, the flavivirus ORF encodes seven nonstructural proteins translated as a single polyprotein. All seven proteins are involved in the replication of the viral genome, but several other distinct roles have been described for many of them. The

first of the seven proteins is the NS1 glycoprotein (46kd). NS1, which was once called soluble complement fixing antigen (49, 129), is translated relatively early—detected after approximately 20-30 minutes—and translocated into the lumen of the ER. Here it is cleaved from E by a host signal peptidase and from NS2A (at its C-terminal end) by an, as yet, unknown host protease (126, 127). Mutation studMutagenesis indicated that only eight residues present in the C-terminal region of NS1 are required for partial cleavage (224); however, a much larger portion of the NS2A protein, up to 70%, is required for complete cleavage of NS1 from NS2A (126, 127, 224). NS1 contains multiple N-linked glycosylation sites (41, 42, 365) and 12 highly conserved cysteines which are able to form disulfide bonds (42).

One unique feature of the translated NS1 protein is the fact that it exists as multiple entities: truncated or elongated, and intracellular, extracellular or cell surface associated (41, 323, 365). The expression of the truncated or elongated form of NS1, at least in the case of JEV and Murray valley encephalitis virus (MVEV), is thought to be due to different C-terminal cleavage sites (41, 365). The intracellular, extracellular and cell surface-associated NS1 moieties appear to have different roles during infection and will be discussed below. Immediately after synthesis, NS1 has hydrophilic characteristics and, unlike the structural proteins described previously, lacks a transmembrane domain (591). However, NS1 quickly forms stable homodimers, which demonstrate increased hydrophobicity. The dimers are shuttled through the secretory pathway where they either associate with the cell surface or are secreted from the infected cell (130, 591, 592).

Although it is known that the intracellular moiety is involved in genome replication, the exact nature of NS1's role is still unclear. The protein is thought to be part of the replication complex, as several studies have indicated that NS1 colocalizes with

double-stranded RNA, a presumed intermediate of flavivirus genomic replication (349, 350). Interference with either the dimerization (191) or the glycosylation (98, 402, 403) of NS1 affect both viral replication and virulence in mice. Further study of the role of NS1 and viral replication indicated that NS1 functions at early stages of replication and an interaction between NS1 and NS4A is involved in the replicase function of NS1 (268, 269, 322).

As mentioned, NS1 can also be secreted from the cell. The dimers formed within the cell are expelled as multimeric complexes, presumably in a glycosylation-dependent fashion (99, 140). These secreted complexes of NS1 are able to elicit strong humoral responses in animals (129). The flavivirus NS1 protein has even been suggested as a target for diagnostics for WNV and DENV, as NS1 appears during the acute phase of infection and can be detected even when viral RNA is undetectable (6, 347). Antibodies against NS1 have also been shown to be useful diagnostic reagents (307). Several lines of evidence suggest that NS1 may play a role in viral pathogenesis. The secreted form of NS1 is preferentially taken up by liver cells and hepatocytes treated with NS1 generate higher viral titers following infection compared to untreated hepatocytes (5). Additionally, the NS1-specific antibodies produced during DENV infection may bind to endothelial cells (125), triggering apoptosis. This could account for the vascular leakage observed during dengue hemorrhagic fever. Additionally, WNV NS1 may be able to reduce complement-mediated recognition of infected cells by binding to the regulatory protein factor H (85). Recently, NS1 has also been shown to block TLR3-mediated signaling (587). This may allow WNV to evade the immune system.

However, NS1 has been implicated as a potential factor in viral immunity. Immunization with NS1 or passive transfer of NS1-specific antibodies has been shown to

be protective (86, 96, 320, 500, 596). In fact, multiple groups have attempted to utilize this protective response by generating DNA-based NS1-expressing vaccines against several flaviviruses, including JEV (320) and DENV-2 (96, 97, 596). This protection is thought to be mediated, in part, by NS1-specific antibodies binding to the NS1 present on the surface of the infected cell, mediating Fc-γ receptor-mediated phagocytosis, clearing the virus-infected cells and, thus, reducing viral yield (86).

The NS2A protein is a small (22kd), hydrophobic, membrane-bound protein. As mentioned, the N-terminal end is cleaved by an unidentified signal peptidase present in the ER (127). However, cleavage of the C-terminal NS2A/NS2B junction takes place in the cytosol of the cell by the viral serine protease (discussed below) (67), suggesting the protein has a transmembrane topology. For YFV, cleavage of NS2A at the C-terminal region of NS2A occurs at two different sites: one producing a full-length NS2A protein and one producing a truncated from, resulting from partial cleavage from NS2B (67).

NS2A, as with NS1, is thought to be involved in viral genome replication, as studies have indicated that NS2A colocalizes with dsRNA and binds strongly to the 3' UTR, NS3, NS4A and NS5, all of which are thought to be components of the replication complex (350). Interestingly, NS2A also appears to be involved in RNA packaging. Mutations in NS2A of KUNV (326) or YFV (300) dramatically reduce virus production without affecting viral replication. For YFV, the ability to form SVP (particles containing only prM/M and E—no ribonucleocapsid; see above) was also unimpaired by NS2A mutations (300), suggesting that NS2A may be particularly important for packing viral RNA into the virions. This is not entirely unexpected, as RNA packaging and RNA replication have been shown to be closely related (271). Additionally, mutations in WNV NS2A associated with adaptation of replicons to be maintained in cells in culture that

coincided with reduced genome copy number (474, 475), suggested a role for NS2A in WNV genome replication. NS2A has also been shown to have an immune-evasion function in addition to its role in replication and packaging. NS2A of DENV was shown to inhibit IFN-mediated signaling (401) and NS2A of KUNV appears to inhibit IFN-β induction (327, 328). Interestingly, several other flaviviruses, including TBEV (35) and JEV (316), NS5, not NS2A, is responsible for impairing IFN-mediated signaling.

NS2B is a small (14kd) membrane-associated protein containing a central hydrophilic domain surrounded by two hydrophobic domains at the N-terminus and one at the C-terminus (92, 128). NS2B serves as a cofactor for the viral protease, NS3. Analysis of the different domains demonstrated a critical requirement for a small stretch of amino acids present at the central hydrophilic domain for complete protease activity (128). However, additional studies have suggested that the membrane-bound, hydrophobic regions may be important for enhancement of protease activity (92).

NS3 is a large (70kd), multifunctional, hydrophilic protein which displays several activities required for RNA polyprotein processing and RNA replication. NS3, along with NS2B as a cofactor, serves as the viral serine protease responsible for the cleavage of the majority of the viral polyprotein, including NS2A/2B, NS2B/3, NS3A/4A, NS4B/5, and specific sites within NS4A, NS2A and NS3 (see Figure 1.1 for summarization of cleavage sites) (324). The NS2B/NS3 catalytic triad (Histidine-53, Aspartic acid-77 and Serine-138 for YFV) that is responsible for the serine proteases' activity is located at the amino-terminal end of NS3 (27, 68, 175).

In addition to its protease activity, NS3 has many other functions critical for RNA replication. The NS3 protein of JEV directly interacts with the 3' UTR and NS5, which may serve as the basis for the replication complex (72). In fact, an interaction between

DENV NS3 and NS5 appears to enhance the activity of NS3, further supporting NS3's proposed role in genome replication (100). Additionally, the carboxy-terminal end of NS3 encodes RNA helicase domains possessing nucleotide triphosphatase activity (NTPase) (176, 577, 578). The structure of the helicase-NTPase domain NS3, solved for YFV, indicates the presence of 3 subdomains: 2 domains that are conserved among RNA helicases and one domain containing virus-specific RNA and protein recognition domains (594, 600). The helicase-NTPase activity is required for the proper unwinding of RNA secondary structures on the viral genome as well as the unwinding of the growing negative-sense RNA strand from the genomic RNA. Interestingly, NS3 appears to also have RNA triphosphatase activity (RTPase), suggesting that it aids in the methyltransferase activity of NS5 (discussed below) (580). For several flaviviruses, an alternative, truncated form of NS3 has been identified, although the exact function of this truncated from is unknown (14, 449). NS3 may also play a role in flavivirus pathogenesis, as the introduction of plasmids expressing NS3 derived from WNV (454), LGT (448) or DENV-2 (513) into cells leads to apoptosis, presumably via caspase 8 activation.

NS4A and NS4B are both small, hydrophobic, membrane-bound proteins of approximately 16kd and 27kd, respectively. The two proteins are expressed side-by-side in the polyprotein, but are separated by a 2kd protein thought to be a signal peptide that helps target the polyprotein to the ER. As mentioned, the junctions between NS3 and NS4A as well as NS4B and NS5 are cleaved by the viral serine protease. However, the junction between NS4A and NS4B is slightly more complicated. Cleavage of the 2kd signal peptide from NS4B, generating the mature form of the protein, first requires viral serine protease-medicated cleavage at the C-terminus of NS4A, a site referred to as the

4A/2K site (315, 447, 469). The precise regulation of the cleavage of the NS4A-2K-NS4B sites is critical for the induction of membrane rearrangements necessary for replication (469). As with the other proteins discussed, both NS4A and NS4B are thought to be involved in flavivirus replication. NS4A has been shown to colocalize with dsRNA (350) and it appears as though an interaction between NS4A and NS1 is an important mediator of replication (322). NS4B colocalizes with NS3 and dsRNA (388); in some cases, NS4B may even be found in the nucleus (583), although the function of this localization is not known. Similar to NS2A, both NS4A and NS4B have demonstrated an ability to block IFN-mediated signaling *in vitro*, although NS4B appears to have greater effectiveness compared to NS4A (400, 401).

NS5, the final protein in the polyprotein, is a large (105kd), highly conserved, hydrophilic protein which appears to be composed of two functional domains. The N-terminus of the protein encodes a region containing methyltransferase (MTase) activity and a putative S-adenesyl-methionine (SAM)-binding site (290). NS5 exhibits nucleoside-2'-O-methyltransferase activity thought to be mediated, in part, by a guanosine triphosphate (GTP)-binding site (119) which recognizes the mRNA cap structure. The recognition of GTP by NS5 is thought to be important for replication, as mutations in the GTP-binding site in DENV-4 attenuate the virus *in vitro* and *in vivo* (193). Additionally, ribavirin 5'-triphosphate inhibits DENV NS5 methyltransferase activity, presumably by competing for the GTP-binding site (29). The C-terminal region of NS5 serves as the RNA-dependent RNA polymerase (187, 547). Mutational analysis of the KUNV NS5 confirmed that it is required for virus replication, although replication can be restored by trans-complementation with wild type NS5 (266, 270). Due to the

nature of its functions, it is likely NS5 localizes to sites of RNA replication; however, this localization has yet to be demonstrated.

As mentioned, NS5 forms a complex with both NS3 and the 3' SL of the viral RNA (72, 100). The interaction between NS3 and NS5 is thought to enhance NTPase and RTPase activities of NS3 (614). Studies have indicated that NS5 can be post-translationally modified, particularly by the addition of phosphate moieties at multiple serine residues within the protein (259). As with a few other flavivirus proteins, one report has suggested that a portion of NS5 can be found within the host cell nucleus (57). The region of NS5 responsible for binding NS3 contains a putative nuclear localization sequence, which could be recognized by importin-β1 and importin-α/β (55, 251). It is thought that the phosphorylation status of NS5 may be a factor determining its cellular localization (259). NS5 also appears to have some immune-modulation functions, as DENV NS5 has been shown to induce interleukin-8 expression (376). Additionally, as mentioned above, the NS5 protein in TBEV (35) and JEV (316) is responsible for disrupting IFN-mediated signaling.

Flavivirus lifecycle

Virus binding and entry

The flavivirus lifecycle, depicted in Figure 1.3, is similar to the lifecycle of other positive-stranded RNA viruses. As with most viral infections, the first step is binding and entry into the target cell. Flaviviruses enter the host cell via receptor-mediated endocytosis. Due to their ability to infect a wide range of vertebrate and invertebrate animals and cell lines, several scenarios are possible: flaviviruses (*i*) utilize a receptor ubiquitously expressed on many cell types, (*ii*) utilize many different receptors or, (*iii*) utilize a combination of these two scenarios. Although DENV receptor utilization has

been studied in the greatest detail (76, 249, 408, 463, 481, 482, 551), putative receptors have been identified for many different flaviviruses, including YFV (158), JEV (305, 461, 541), TBEV (291) and WNV (83, 103). Both DENV (408, 551) and WNV (103) are thought to utilize DC-SIGN—a lectin molecule expressed primarily on dendritic cells (DCs)—for virus attachment. However, other molecules must be able to serve as receptors for virus entry, as infection of DCs by DENV was independent of the internalization of DC-SIGN (340), and many cells and cell lines that lack this molecule are readily infected by flaviviruses. Several other proteins besides DC-SIGN have been suggested as putative receptors for flaviviruses as well, including heat shock protein (HSP) 90 and HSP 70 (461, 463), glucose regulated protein 78 (GRP78) (249), αvβ3 integrin (83), and CD14-related molecules (76). Opsinization of flavivirus particles with immunoglobulins can also result in enhanced binding in an Fc-receptor dependent fashion (429, 499). This is thought to be particularly important for antibody-dependent enhancement of DENV infections. Furthermore, highly sulfated glycosaminoglycans, such as heparan-sulfate (HS), appear to play an important role in the initial attachment of DENV (74, 158), TBEV (357), MVEV (305) and JEV (541). In fact, introducing mutations affecting HS binding in E can result in alterations in flavivirus attachment and entry *in vitro* and viral attenuation *in vivo* (304, 305, 357).

Upon interaction with cellular receptor(s), flaviviruses are internalized into endosomes via clathrin-coated pits (82) and trafficked to pre-lysosomal endocytic compartments (172). Fusion between the virus lipid envelope and the pre-lysosomal compartment is triggered by, and requires, a pH-dependent (173, 174, 532, 533) conformational change in E. The E homodimers covering the surface of the virion disassociate into monomers and then quickly reassemble into homotrimers, exposing a

fusion loop. This reassembly is thought to be the trigger for membrane fusion between the viral and endosomal membranes and subsequent release of the nucleocapsid into the cell cytoplasm (534).

Translation and replication

As mentioned, flaviviruses encode a positive-sense RNA genome which can serve as a template for translation. Thus, once the nucleocapsid is released from the virus particle into the cell cytoplasm, RNA translation can occur immediately. Using the host cell's translational machinery, as well as specialized secondary structures/sequences within the 5' and 3'UTR, the viral genome is translated in a cap-dependent manner (324). Interestingly, one group has suggested that DENV is able to initiate cap-independent translation when conditions for cap-dependent translation are unfavorable (118), although this observation has not been confirmed. The viral genome is targeted to ER membrane where it is translated as a single polyprotein which is co- and post-translationally cleaved to form the individual proteins.

Since replication occurs on ER membranes, it is not surprising that flavivirus infection induces ultrastructural changes in the host cell membranes. These changes result in the formation (or expansion) of multiple membrane structures including convoluted membranes, paracystalline arrays and vesicle packets (VP) (324). Interestingly, these different membrane structures contain a distinct set of both viral and cellular proteins, suggesting they may be involved in different aspects of the flavivirus lifecycle (348). The VPs, for example, are thought to be the site of the flavivirus genome replication complex (349).

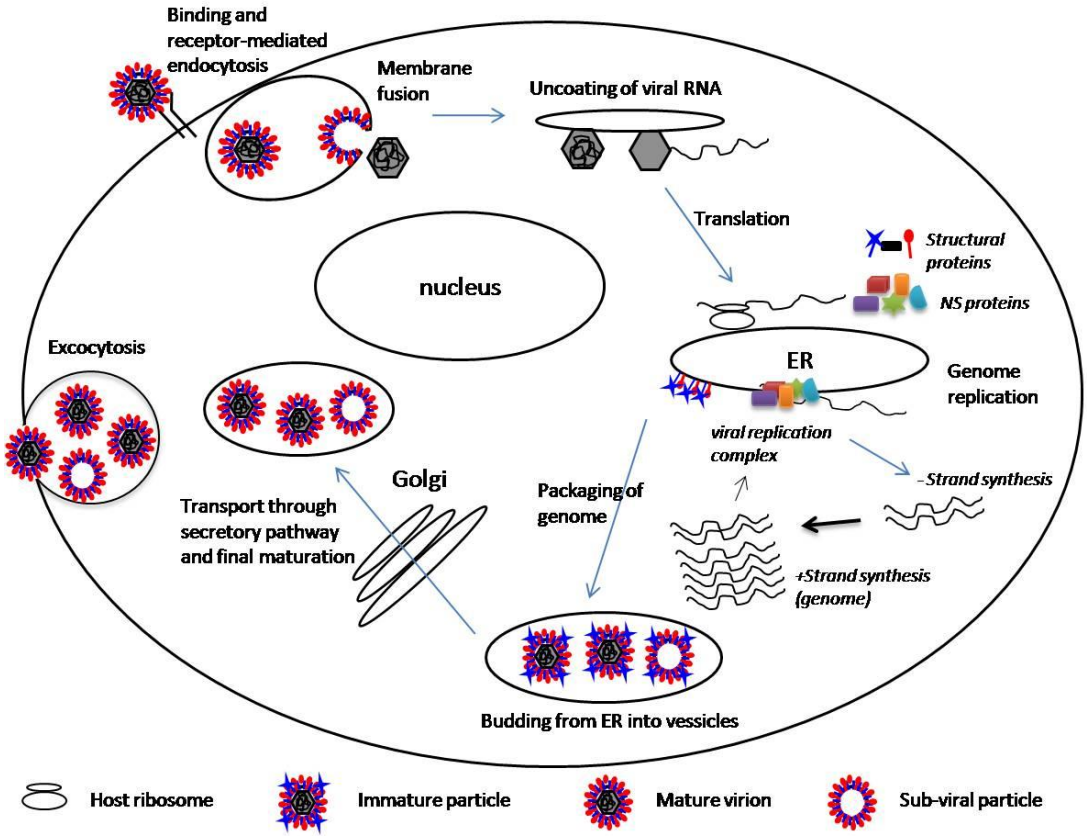


FIGURE 1.3: FLAVIVIRUS LIFECYCLE. The intracellular lifecycle of flaviviruses begins with receptor-mediated endocytosis followed by translation, genome replication and virion packaging. Following final maturation, the virions are released by exocytosis. Image was inspired by Field's Virology (Lindenbach et al., 2007).

Three major species of RNA can be identified during flavivirus replication: the genomic form, a double-stranded replicative form (RF) and a heterogeneous population of replicative intermediates (RI) (324). The first step in flaviviral RNA replication is the generation of the negative-sense RNA using the genomic RNA as a template (semiconservative RNA synthesis). This synthesized negative-sense RNA molecule serves as a template for the generation of positive-sense RNA molecules via RF and RI forms of the viral RNA (90, 136, 324). Flavivirus RNA replication is asymmetric, as multiple positive-stranded RNA molecules are generated from a single negative-stranded

RNA molecule. In fact, RNA molecules of positive polarity are generated at levels up to 10 times higher than RNA molecules of negative polarity (90).

Assembly and particle release

Once the genomic RNA is replicated, it must be packaged into mature virions and released from the cell. Stretches of basic residues present within the C protein are thought to interact with the viral genomic RNA, neutralizing the nucleic acids' charge and allowing it to form the nucleocapsid (274, 324). The nucleocapsid acquires an envelope by budding into the lumen of the ER, producing fully formed, immature virus particles. The assembly of these particles is thought to be relatively fast, as no budding intermediates or clearly distinguishable cytoplasmic nucleocapids have been observed (324). The immature virion particle is formed from multiple copies of the prM and E proteins. These two proteins, which are both anchored into the ER membrane via C-terminal transmembrane domains (419), form stable heterodimers (206, 579). In fact, as mentioned previously, E cannot be properly folded in the absence of prM (289, 335). The immature particles composed of the nucleocapsid, prM and E are shuttled through the secretory pathway/trans-Golgi network (336, 351) where any glycans on E are modified (67, 365). Prior to release from the cell, a change in pH allows the furin cleavage of prM to M, forming the mature, infectious particle (529). Since intracellular M is not detected, it is thought that prM cleavage occurs just prior to particle release (324). Mature particles are released from the cell via fusion between the trafficking vesicles and the plasma membrane—cell lysis is not required for the release of flavivirus particles.

West Nile Virus

WNV has been infecting humans, horses and birds for the better part of the past seventy yearsto the best of our knowledge; although it seems likely the virus was circulating prior to its initial isolation. However, until recently, diseases caused by WNV were relatively mild and the virus was not considered an important human or animal pathogen (181). This changed in the late 1990's with several large outbreaks associated with more severe neurological complications and the introduction of WNV into the United States in 1999. These outbreaks promoted large numbers of studies to better understand the epidemiology, ecology and molecular virology of WNV.

HISTORY OF WNV AND RECENT OUTBREAKS

West Nile virus was first isolated in 1937 from a woman in Uganda by researchers who were conducting a field study monitoring YFV infections (526). Unfortunately, no information about the disease itself was available at that time, as the patient denied being sick and would not provide additional information. However, serum from the patient inoculated into mice resulted in severe mortality and subsequent passages of the virus into mice resulted in an isolate that was uniformly lethal.

After this first isolation, WNV disappeared from the attention of public health officials for more than a decade. In the 1950's the perseverance of WNV wasmore apparent following several outbreaks of West Nile fever in Israel, one in 1951 and one in 1957. In 1951, there were 123 reported cases from 303 inhabitants of the small community (31). The majority of cases were in children under the age of 3 years and there were no reports of severe neurological disease or mortality. Another large outbreak in 1957 produced the first indication that WNV produced more severe symptoms in the

elderly. In this outbreak, soldiers living on a nearby base and adults and children living in the town presented primarily with mild febrile illness, with only a few cases of meningoencephalitis. On the other hand, nearly 27% of the elderly patients confirmed to have WNV by serology presented with more severe meningoencephalitis and four other patients died from encephalitis, although these cases were not confirmed by serology so it is unknown whether these deaths were directly related to WNV infection (199). It is thought that these cases may represent the first human deaths associated with natural WNV infection.

From the time WNV was first isolated in 1937 until mid-1990, it was infrequently associated with severe disease in man or horses. In fact, it was primarily considered a disease of birds, which sometimes infected humans and equines, causing only minor disease. The lone exception was an outbreak in France in 1964 in which at least 80 horses presented with signs of disease with a 25-30% mortality rate among the horses that were sick (64). Other epidemics had been reported during this period, although none were associated with severe disease in man or equine.

Things appeared to change in the 1990s, however, as the number and severity of outbreaks appeared to be increasing. Small outbreaks associated with human and equine mortality were reported in Algeria (1994), Morocco (1996), Tunisia (1997), Italy (1998) and France (2000). These were followed by several large outbreaks that thrust WNV into view as a major health problem. From July-October in 1996, Romania was hit with a large epidemic with nearly 400 confirmed cases of severe, neurologic WNV infections. Seventeen of these patients over the age of 50 succumbed to the infection, confirming the suggestion that WNV is more severe in elderly individuals. Serosurveys performed following the epidemic suggested that tens-of-thousands of individuals were likely

infected with mild or sub-clinical infections throughout the course of the four-month epidemic (561). Studies investigating mosquito and avian populations indicated that a high percentage of domesticated fowl were seropositive, but the virus was isolated from only one mosquito (491).

Interestingly, WNV induced high morbidity and mortality in white storks and geese in Israel in 1998. Although WNV has been detected in birds previously, it had not been associated with high mortality. The high mortality observed in this case would be repeated a year later following an outbreak in the United States (to be discussed later). The discovery of WNV in these bird populations was followed by an epidemic in Israel in August-October of 2000. This epidemic resulted in approximately 439 cases and 29 fatalities (37). In the summer of 1999, an epidemic occurred in Volgograd Region, Russia, resulting in a large number of confirmed and suspected cases of WNV infection (444). More than 800 patients were admitted with fevers or neurological disorders in a period of four months (July-October). Although only 183 were serologically confirmed for WNV, the total number of suspected cases of WNV-induced disease was nearly 500. Forty patients succumbed to lethal WNV infection (444).

Also in 1999, WNV was introduced into the United States, which thrust WNV into the public spotlight in this country. The epidemic began in the Queens Borough of New York City and spread to surrounding areas. Although first serological evidence suggested that the agent was St. Louis encephalitis virus (SLEV) (466), the etiologic cause of the epidemic was later confirmed to be WNV, a relative of SLEV (302). The epidemic began when residents living in the area noticed an unusually high number of dead birds. WNV was later isolated from dead crows as well as many other bird species (11, 120, 285, 302, 531) and surveys of mosquito populations late in the summer of 1999

identified several WNV-positive pools of mosquitoes, primarily of the *Culex* species (406). Horses were also affected during this outbreak, with 20 horses in the area presenting with neurologic disease that were confirmed WNV infections (560).

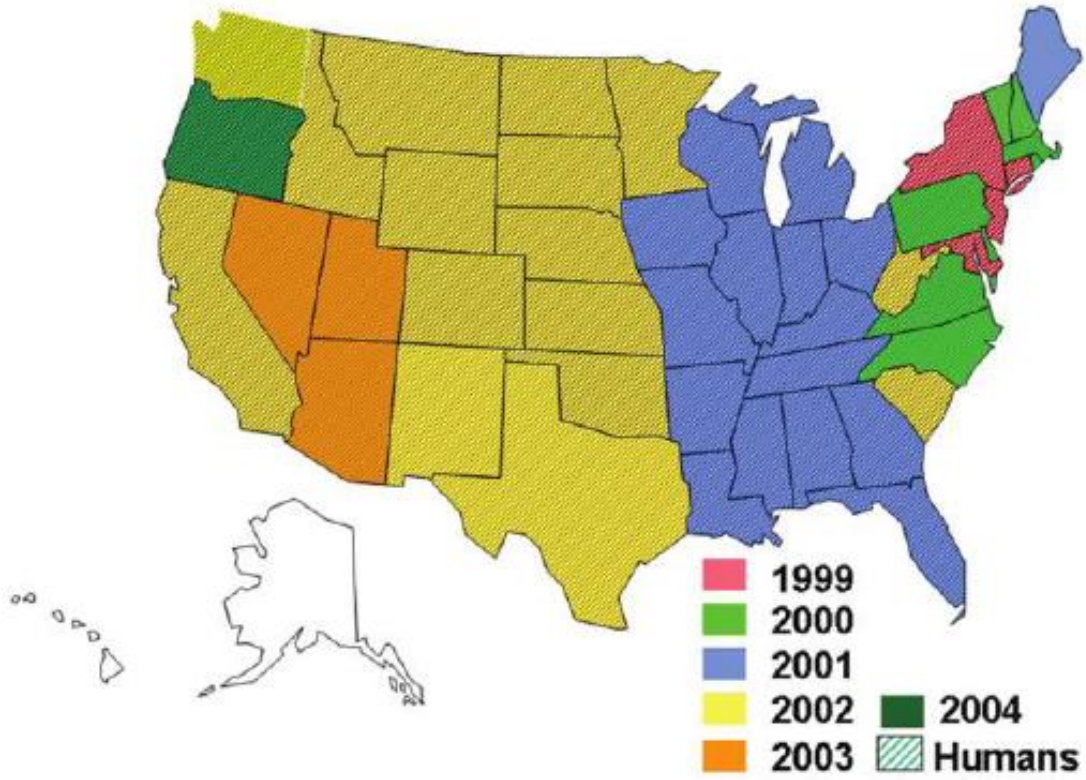


FIGURE 1.4: SPREAD OF WNV ACROSS UNITED STATES. Following the introduction of WNV into the USA in 1999, WNV has steadily spread across the continent. By 2005, WNV was detected in mosquitoes, birds, humans or all three in all 48 contiguous states. As of mid-2008, WNV has not yet reached Alaska or Hawaii. Figure was reprinted courtesy of the Centers for Disease Control and Prevention.

A cluster of patients presenting with neurological complications were admitted into the Flushing hospital in New York in mid-August. It is thought that these were the first documented cases of WNV infection in humans in the Western hemisphere (16). By the end of the summer, 62 confirmed cases of WNV infection were identified, with 7 resulting in death; however, subsequent studies suggested that thousands of asymptomatic infections had also occurred (181). At the end of the year, there were many

questions as to whether WNV would survive over the winter, however, WNV was detected in overwintering adult *Culex* mosquitoes (405) as well as in a hawk found dead in New York (156). Another outbreak, albeit smaller, occurred in the same area in 2000 and, in the next few years, WNV continued to spread across the continental United States upwards into Canada (430) and Southwards into Mexico (40, 338). The summer of 2003 met with the largest epidemic of WNV in the United States, with nearly 10,000 human cases resulting in over 250 fatalities (181). Over the next years, WNV would spread steadily across the United States (Figure 1.4), reaching every contiguous state and continuing to spread downwards into the Caribbean (282) and into some areas of South America (395). It is now thought that WNV is endemic to the United States, with low, but consistent levels of incidences each year. Figure 1.4 demonstrates the progression of WNV across the United States.

TAXONOMY

Within the genus *Flavivirus*, WNV is most closely related to members of the Japanese encephalitis antigenic complex. This complex includes viruses found on multiple continents: JEV, found in Asia; Murray Valley encephalitis virus and Alfuy virus, found in Australia; Kortango and Usutu viruses found in Africa; and SLEV, Rocia virus, Ilheus virus and Cacipacore virus in the Americas (61). Many different WNV isolates have been collected since 1937, separated by both time and space. Early nucleotide sequence analyses of various isolates indicate that WNV stains can be subdivided into at least two different lineages (Figure 1.5) (34, 58, 302, 491, 498). Lineage I contains isolates primarily from Europe, Middle East, South Asia, Australia and North America. This lineage is subdivided into three clades which are associated with different geographic locations (Figure 1.5). The Australia strain, known as Kunjin

virus (KUNV), was originally thought to be a separate virus within the genus *Flavivirus*; however, recent taxonomic classification has put KUNV as a subtype of WNV. A recent report, however, suggests that KUNV could be considered, instead, as an additional lineage within the WNV subgroup (498). Lineage II viruses, which includes the original 1937 isolate, are almost exclusively African isolates (281). Lineage I viruses are most often associated with pathogenesis in humans and equines, while Lineage II viruses are more often considered nonpathogenic.

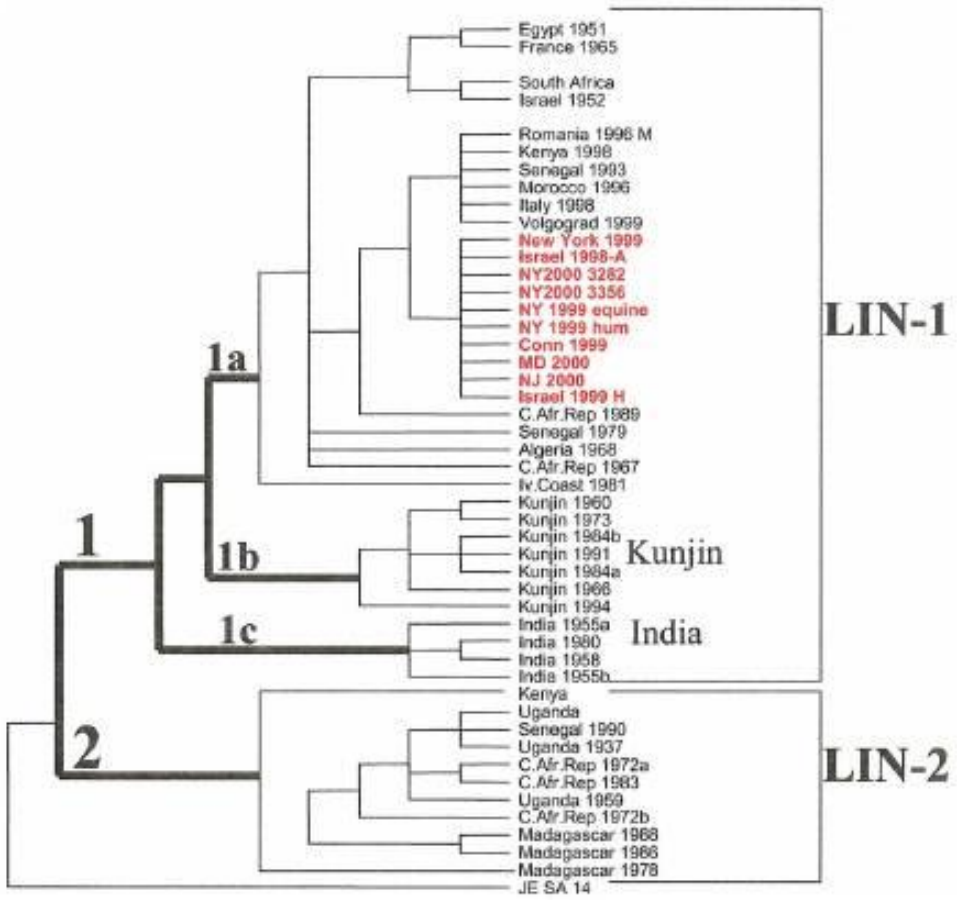


FIGURE 1.5: WNV LINEAGES. Phylogenetic tree generated by parsimony analysis using nucleotide sequences from 47 WNV strains. A JEV strain is used as an outgroup. The US/Israel clade is shown in red. Horizontal lengths are not related to sequence distances. Figure is reprinted with permission from Elsevier from Lanciotti, R et al. 2002. Complete genome sequences and phylogenetic analysis of West Nile virus strains isolated from the United States, Europe, and the Middle East. Virology 298:96-105.

Some recent studies have suggested that WNV strains should be subdivided into 5 distinct lineages, instead of just two (45). Under this system, researchers suggest dividing lineage I into two subclades (with KUNV isolates making up the second clade) and lineage II, similar to the previous division, would contain the African isolates. Lineage III and lineage IV both would contain a single isolate from the Czech Republic and Russia, respectively. Lineage V would comprise WNV isolates from India (45).

WNV TRANSMISSION

WNV transmission in nature occurs via an avian-mosquito cycle. In order to transmit the virus to a feeding mosquito, a reservoir host must be able to maintain a sufficient viremia. Laboratory studies have demonstrated that consuming a bloodmeal containing approximately 10^7 plaque forming units (pfu) of virus resulted in the infection of mosquitoes (*Cx tarsalis*); however, few mosquitoes were able to be infected following a bloodmeal containing less than 10^5 pfu of virus (201). Humans appear to only develop a maximum viremia of approximately 10^3 pfu (201), which supports the supposition that humans are not involved in the natural transmission cycle. However, although it varies from species to species, birds generally develop a viremia titer of up to 10^{10} pfu, levels more than sufficient to infect a feeding mosquito (283). This implicates birds as key for the stable maintenance of the virus in nature.

In contrast to many other arboviruses, WNV infection is not restricted to a single species or group of vertebrates, presumably due, in part, to the promiscuity of the feeding behavior of many mosquitoes able to transmit the virus. Although most infections do not result in viremia levels sufficient for further transmission of the virus (201), experimental infections of some reptiles, amphibians, well as several rodent species (200, 445, 471, 557) have implicated them as competent amplifying hosts for WNV infection. The exact

role of these infections in the natural cycle of WNV, however, is still unknown. It is interesting to note that WNV transmission between co-feeding mosquitoes has been observed in laboratory settings. In these cases, uninfected mosquitoes became infected by co-feeding on an uninfected mouse with an infected mosquito, although infection rates were low (212).

Mosquito transmission

Mosquitoes serve as the primary vector for the natural transmission of WNV and, to date, numerous studies have been performed to examine their vector competence, that is, their ability to be infected by and transmit WNV. These studies have indicated that WNV is primarily transmitted via mosquitoes within the *Culex* genus. Within the United States, more than fifty species of mosquitoes have been shown to be competent vectors in laboratory settings (201); however, only a few are thought to serve as principle vectors. Although all *Cx.* species tested are competent vectors in the laboratory, members of the *Cx. pipiens* complex have been shown to be the primary vectors for WNV in the North American transmission cycle, specifically *Cx. pipiens pipiens*, *Cx. pipiens quinquefasciatus* and *Cx. tarsalis* (201). These mosquitoes have the highest infection rate in nature (as measured in trapping studies) and are the most competent vectors in laboratory studies. Other species of mosquitoes, such as *Aedes* and *Ochlerotatus*, are also competent vectors of WNV (562, 563). However, these mosquitoes are not expected to be important for maintenance of WNV in nature due to their feeding preferences. Instead, these mosquitoes may serve as bridge vectors, linking mammalian hosts to the natural avian-mosquito cycle. Interestingly, a few species of ticks have also been shown to be susceptible to WNV infection (201), although they are not thought to be important for the natural transmission cycle.

As mentioned, the majority of studies performed on mosquitoes have centered on their permissiveness to WNV and their ability to transmit WNV. Some more recent studies, however, have begun to examine how WNV disseminates within the infected mosquito and whether WNV infection produces pathology in its insect vector. One such study by Girard et al. followed the path of WNV through *Cx pipiens quinquefasciatus*, mosquitoes which are thought to be important vectors for WNV in the Southwest United States (392). WNV appears to escape from the mosquito midgut following replication within epithelial cells (164). WNV antigen was first detected within the posterior midgut by day 2 post infection followed by spread into the anterior midgut, presumably through cell-to-cell spread by day 3-5. Viral antigen was subsequently found in the cardia epithelium, thorax, head and abdominal fat body cells at around day 6. By day 8, WNV was readily detectable within the salivary glands, suggesting that, at this point, WNV could be transmitted. WNV was persistent in the salivary glands throughout the remaining 21 day observation period (164). On day 14, weak antigen staining was detected in the ovaries of infected mosquitoes; however, this was transient in nature and was gone or nearly gone by 21 days. Other groups have indicated that vertical, transovarial transmission of WNV is possible (21, 387, 554), but the role of this type of transmission during the natural cycle of WNV is unclear.

When a mosquito takes a bloodmeal, she probes throughout the dermis and capillaries until she finds a suitable location. During this time, she is depositing both saliva and virus into the host dermis layer as well as directly into the blood (540). Experimental evidence suggests that, during this probing, the mosquito deposits anywhere from 10^1-10^6 pfu of virus (540, 567), with up to 10^2 pfu entering directly into the bloodstream (540). Mosquito saliva has been shown to be an important aspect of

mosquito feeding and viral infection. Several groups have demonstrated that mosquito saliva contains a variety of molecules designed to counteract host hemostatic responses, reducing inflammation and altering host immunity (501). WNV infection was shown to be enhanced in chickens (539) and mice (502) following mosquito bite compared to needle inoculation.

The effect of WNV infection on mosquitoes is relatively unclear. Although WNV infection does not appear to have detrimental effects on the mosquito as a whole, several recent reports have suggested that, at a cellular level, WNV infection does have an impact. The first cells to be infected appear to be the cells of the midgut (504) and, interestingly, WNV-induced apoptosis can be observed in the midgets of mosquitoes that are refractory to WNV infection (565). It is thought that this apoptosis may prevent WNV from spreading further. In susceptible mosquitoes, WNV infection led to membrane proliferation and structural changes in the ER (165), consistent with studies conducted in mammalian cells. However, at later times (14 days post infection and later) cytopathology (apoptosis) was evident in the infected mosquitoes salivary glands (165, 166). This appeared to correlate with a decrease in saliva virus titers as well as a decrease in WNV-positive saliva samples (166), suggesting that the ability of mosquitoes to transmit WNV decreases with age.

Non-vectored transmission

Transmission of WNV in the absence of mosquito vectors has also been observed. Human infections are considered dead end. As mentioned above, humans do not develop a sufficient viremia to pass the virus onto another mosquito. However, vertical transmission of WNV between humans has been documented. In 2002, WNV transmission from a mother to her unborn child was first documented. The child was born

with neurological complications. Although there have been other documented exposures of pregnant women to WNV during their pregnancies, no other child has been reported to have been infected with WNV *in utero* (202). One suspected case of transmission through breast milk was also reported in 2002 (202), however, no other cases have been documented. Transmissions through blood transfusions and organ transplantations have also been reported. In fact, during the outbreaks in the United States in 2003 and 2004, more than 1,000 blood donations were positive for WNV (201). Since then, strict testing of the blood supplies has been in place to help remove infected blood donations from the donor pool.

Non-vectored transmission in birds has also been noted. In February 2003, WNV was isolated from the brain of a hawk found dead in New York. This was significant since the hawk died in the middle of winter, when mosquitoes were not likely present to transmit the virus (156). Although there are several possible explanations for this death, it is thought that the hawk was exposed to WNV after preying on an infected animal. Experimental studies in multiple bird species also suggest that WNV can spread bird-to-bird through oral as well as contact transmission. Multiple bird species that were fed on either WNV-infected dead animals or mosquitoes became infected (283, 374), demonstrating viremia and disease profiles similar to those infected from the bite of an infected mosquito (albeit sometimes slightly delayed) (283). Interestingly, it appears as though WNV can spread between birds that are in close contact with each other. Several species of birds, including American crows, blue jays, black-billed magpies, ring-billed gulls and domestic geese (2, 283, 303, 374, 544), were shown to become infected with WNV simply by being in close contact with infected birds. Although some of these birds showed evidence of shedding WNV though the cloacae and the oral cavity (283), the

exact nature of this "cage mate transmission" is unclear. Direct WNV transmission between mammals has not been demonstrated; however, there is evidence of viral shedding. Viral RNA was present in urine, fecal and oral samples from experimentally infected fox squirrels (470) and WNV was isolated from similar samples from infected Eastern chipmunks (445). Additionally, WNV was detected in the urine of infected hamsters weeks after the initial infection (555, 559, 598). Despite this, however, the cage mate transmission phenomenon detected in birds has yet to be reported for mammals.

WNV DISEASE AND PATHOLOGY

The majority of data obtained about the pathogenesis and progression of WNV diseases has been derived from experimental infections. The primary model for WNV infection is the rodent, particularly mice and hamsters. These animals are generally infected intraperitoneal (ip), although many recent studies have began to examine WNV infection following subcutaneous (sc) infection via the mouse's footpad. This is thought to be the route of infection most closely resembling the bite of a mosquito, although sc injection still inoculates WNV much deeper than a mosquito bite. A few studies, particularly those looking at neuropathogenesis, inoculate with WNV intracranially (ic). This allows researchers to study neuropathology without the added affect of viral replication in the periphery and requirement of entry into the central nervous system (CNS).

WNV infection has been studied in multiple other systems, as well. Although accepted as the primary known vector for WNV, only a few studies on the effects of WNV infection on mosquitoes have been performed to date. Additionally, since an apparent increase in virulence following WNV introduction into the avian population in 1999 (United States), studies on the effect of WNV infection on birds, the amplifying

host/reservoir, have been performed more rigorously. In terms of human disease, most information has been derived from autopsies performed on individuals that succumbed to WNV infection. Although non-human primates are often used as a model for vaccine development, they are not entirely useful as models for human WNV infection (at least the severe forms of the disease), as, like most human infections, the majority of non-human primates do not develop clinically detectable disease following infection.

Avians

Birds are the primary vertebrate hosts for WNV. Serosurveys of birds and mammals in several areas of Africa, Europe and the Middle East demonstrated that birds can be infected with WNV (281). In addition, experimental infections of birds indicated that they develop high viremias, indicative of their ability to spread WNV to feeding mosquitoes. All vertebrates in these areas exposed to WNV were infected with WNV; however, unlike birds, none of the other vertebrates tested developed sufficient viremias for further spread of the virus. Some more recent data suggests that some vertebrates, specific species of amphibians, reptiles and rodents, can develop high viremias (201, 445, 470, 557); however, birds are considered the primary amplification hosts.

Around 1999, when WNV was first introduced into the United States, there appeared to be an increase in the pathogenicity of WNV in birds. This was first noted during an Israeli study investigating migrant storks, in which thirteen storks were found dead or dying. WNV was isolated from four of the dead birds and eleven were shown to be positive for WNV neutralizing antibodies (354). Experimental infection of young domestic geese with the New York 1999 strain of WNV caused weight loss, encephalitis and myocarditis, although the geese appeared to be more resistant to infection as they aged (544). The introduction of WNV into the United States resulted in an increase in

research to understand WNV infection in birds. A sampling of birds completed during the outbreak in New York in 1999 revealed that approximately 33% of the 430 birds (representing 18 different species) tested were positive for WNV neutralizing antibodies. The species with the highest seroprevalence were the domestic goose, the domestic chicken, the house sparrow, the Canada goose and the rock dove. At the same time, samples from domestic dogs, cats and horses showed low levels of WNV infection (as measured by serology). In fact, none of the cats (12 total), 5% of the dogs and 3% of the horses tested were positive for WNV neutralizing antibodies (284).

Experimental infections of birds have suggested that members of the order Passeriformes (often referred to as the 'perching birds') are the most competent reservoir hosts, as they developed high, and sustained, WNV viremias (281, 283). In a comprehensive study conducted by Komar et al. following the 1999 outbreak, species within this order (including the American crow and the blue jay) developed titers as high as 10^{10} pfu/ml of serum (283). Unsurprising due to their high viremias, these birds also tended to develop disease. The birds that became ill showed symptoms such as ruffles feathers, lethargy and ataxia which, more often than not, preceded death by approximately 24 hours (283). Necropsies performed on euthanized birds showed evidence of WNV in multiple organs, including brain, eye, kidney, heart, spleen, liver, lung, intestines, gonads, esophagus and skin (283). All of the American crows, ring-billed gulls, black-billed magpies and house finches infected with WNV in this study succumbed to the infection. In fact, WNV is so virulent in American crows, they have been used as sentinels to detect circulating WNV (120). A recent study examining the bloodmeals of mosquitoes in nature indicated that *Cx. pipiens* most often fed on the American robin (391), suggesting this bird is an important amplifying host.

Rodent models

Most of what is known about the pathogenesis of WNV has been discovered through experimental infections of mouse or hamster models. Mice are the most commonly used animal model and, thus, most of the information pertaining to WNV pathology is derived from experimental infections of mice. Mice inoculated ip show no signs of illness for the first 5 days following infection. However, within 24 to 48 hours post infection, infectious virus can be detected in the serum. This viremia peaks around day 3 to day 6. Also by 48 hours post infection, virus can be detected in the spleen, kidneys and heart. The viremia is usually undetectable by day 5 or 6, concurrent with a rise in anti-WNV IgM antibodies (110). WNV is first detected in the brain of mice by day 4, although the route of infection influences the exact timeline (intravenous inoculation or mosquito bite increases neuroinvasion—earlier and increased titers) (294, 502). After WNV enters the brain, the mice begin to show signs of ruffled fur and lethargy. The mice quickly progress to neurological disease, as defined by ataxia and paralysis, and between 7-10 days post infection, the mice succumb to infection. The presentation of neurological disease is often associated with a large number of infected neurons (520), which are subsequently destroyed by apoptosis (115, 485, 520). The killing of these nonrenewable cell types is what is thought to be the cause of the neurological disease.

One advantage of using a mouse model is the ability to create and utilize knockout mice. Knockout mice are particularly useful for determining specific host factors involved in WNV infection and pathogenesis. Infection of mice deficient in B cells had similar titers of WNV compared to WT mice in peripheral organs; however, the virus was not cleared as quickly from the organs and serum titers were higher and more sustained in the B cell deficient mice (110). WNV titers in the brain were also higher in

mice lacking B cells compared to WT mice. WNV infection in B cell-deficient mice was 100% lethal (compared to approximately 40% lethality in WT mice) (110), suggesting that B cells and/or antibody production is critical for controlling WNV infection. T cells and an effective T cell response have also been shown to be critical for controlling WNV infection (484, 521). In addition, many aspects of the host's innate immune response, such as IFN-α (111, 483) and IFN-γ (522), are critical for controlling WNV infection; however, these will be discussed in more detail in Chapter 2.

The progression of disease in hamsters is similar to that observed in mice. When inoculated ip with 10^4 $TCID^{50}$ units of WNV, golden Syrian hamsters did not show any signs of illness for the first five days post infection (598). At six days post infection the hamsters were beginning to show signs of illness and on days 7-10, they showed neurological symptoms, including hind limb paralysis, tremors and loss of balance. Most of the hamsters died between day 7 and 14 days post infection and, of those that survived, a few showed neurological sequela (598). Viremia in these animals was detected by day one and lasted through day 6. The peak viremia, which occurred around day 2 and day 3, was approximately 10^5 $TCID^{50}$/ml. Antibodies were detected in the sera of infected hamsters by day 5 and steadily increased through day 7. Interestingly, WNV titers in the brain, which were between 10^3 and 10^6 $TCID^{50}$/ml, were detected during the second week of infection when viremia was no longer detectable (598). There was no observable pathology within the peripheral organs; however, there was progressive neurological degeneration present in the brain of the infected hamsters (598). Most of this degeneration was attributed to WNV-induced apoptosis, as there was no observable inflammatory cell infiltrate. Some studies have also demonstrated persistence in hamsters

that survive WNV infection, with virus able to be isolated from the kidneys and urine of infected hamsters up to 247 days following the initial infection (555).

The majority of these pathogenesis experiments were performed using needle inoculations. Although this is a simple and reliable method of infection, it is not a mode that accurately depicts the natural route of infection. Even needle inoculation sc through the footpad, the closest we can get to mimicking a mosquito bite, does not accurately represent the delivery of WNV in nature. As mentioned previously, while taking a bloodmeal, a mosquito probes, all the while depositing both WNV and saliva. In fact, mice infected with WNV in the presence of salivary gland extract (SGE) succumbed to infection approximately 2 days earlier than mice infected with WNV by needle inoculation (502). Interestingly, mice exposed to mosquito feeding immediately prior to needle inoculation of WNV succumbed to infection approximately 3 days before WNV infection alone. This difference in survival time was attributed to an increase in serum and brain titers in the mice fed on by mosquitoes. Viral titers in the brain were detected earlier and at higher levels in mice exposed to mosquitoes compared to those infected with WNV alone (502). Despite these differences, in the absence of mosquito feeding or mosquito saliva, WNV-infected mice still succumbed, albeit delayed by a few days.

Non-human primates

Non-human primates do not usually develop disease following exposure to WNV. Instead, similar to the majority of human cases, they present with subclinical infections. An outbreak of WNV in Louisiana in 2002 exposed more than 1,600 nonhuman primates living in an outdoor breeding facility to WNV. Blood samples taken following the outbreak from rhesus monkeys, pigtail macaques and baboons demonstrated that 36% had developed WNV neutralizing antibodies (457). Despite the number apparently

infected with WNV, no signs of clinical illness or neurological disease were observed during the outbreak in any of the animals (457). In a subsequent study, five macaques were experimentally infected with WNV and then monitored for signs of infection and level and duration of viremia. All of the macaques developed very low, transient viremias (detectable by reverse transcriptase polymerase chain reaction) for the first 5-6 days following infection (although WNV RNA was detected in 1 of the macaques up to day 9) and neutralizing antibodies were first detectable by 9 days post infection (458). Interestingly, although no macaques showed any signs of illness, WNV RNA was present in the spinal fluid of one of the macaques, suggesting WNV had entered into the brain (458). Other studies have shown that if WNV is injected directly into the brain, some monkeys can develop neurological disease (446, 526).

Humans

WNV pathology and disease is relatively difficult to study and there is still much that is not known or understood. Reports of pathology can be derived only from autopsies completed on patients that have succumbed to the more severe neurological complications of WNV infection. The infrequent nature of symptomatic human WNV infections (at least 80% of infections are asymptomatic) and the relatively non-specific nature of the less severe disease, WN fever (approx 20% of infections), make it difficult to understand the full range of symptoms and pathology. Since experimental infections of humans are highly unethical, the time course of WNV infection in humans is still relatively unclear. It is thought that symptomatic illness manifests between 2-14 days following exposure (200, 203, 352). These symptoms, which include fever, headache, muscle and joint pain and anorexia, can persist anywhere from 3 days to several weeks (352, 435). Only about 1 in 150 patients (< 1%) will develop the more severe

neurological diseases associated with WNV infections (435). These severe WNV diseases include encephalitis, meningitis and acute flaccid paralysis and have an approximate 10% fatality rate (of those who present with neurological symptoms). Those patients who survive often have long term cognitive and neurologic impairment (352). Age appears to be the most important risk factor for developing disease. Several reports indicate that the onset of neurological symptoms is greatly increased in individuals 65 years and older (398, 407). Immunocompromised patients, particularly transplant patients, are also highly susceptible to WNV infection (240, 279). Host genetic factors may also play a role in the development of WNV disease, as flavivirus resistance in mice has been mapped to a specific gene, Oas1b (2', 5' oligoadenylate synthetase; see Chapter 2) (364, 431). Single nucleotide polymorphisms in the human OAS gene have been identified in a subset of patients infected with WNV; however, the importance of this is not yet clear (601).

The Innate Immune Response: Type I Interferon

The host's immune response consists of two distinct, but connected, arms: a swift, nonspecific early response (innate immunity) and a highly specific, late(r) response (adaptive immunity). The latter involves the production of antibodies by B cells and an effective T cell response, both of which result in long-term immunologic memory. This adaptive immune response is important for controlling viral infections, but will not be discussed in this dissertation. The innate immune response, although nonspecific, is the first line of defense against an invading pathogen. This response is also thought to help shape an effective adaptive immune response.

Cells have evolved multiple mechanisms to detect invading pathogens. Expression of specific molecules, referred to as pattern recognition receptors (PRRs), is critical for identifying specific pathogen-associated molecular patterns (PAMPs) expressed on the microbe or generated during the course of infection. These PAMPs include lipopolysaccharides (LPS) or other carbohydrates expressed on the surface of bacterial pathogens as well as DNA and single- or double-stranded RNA (or RNA motifs) that are either generated during viral replication or present in unnatural locations. In mammalian cells, multiple and often redundant pathways are engaged during viral infection that are responsible for inducing chemokines and cytokines important for eliminating or reducing the harmful effects of the virus. One critical cytokine induced following viral infection is interferon (IFN). Intense research over the past few decades has begun to dissect the complex nature of the innate immune response and we are finally beginning to understand the pathways involved in this critical response.

INDUCTION OF TYPE I INTERFERON

Key players

Since the first published report on the discovery of a molecule which interfered with influenza virus infection (235, 236), the induction and antiviral effects of IFN have been areas of intense scrutiny. Subsequent study resulted in the identification of two distinct types of IFN: type I IFN and type II IFN. Type II IFN, whose sole member is IFN-γ, is an important cytokine for the efficient development of a T cell response, but will not be discussed here. In the mid-1970s, the type I IFN group was further divided into two antigenically different species, IFN-α, produced primarily in leukocytes, and IFN-β, produced primarily in fibroblasts (196). The cloning of IFN genes resulted in an abundance of new and exciting research. It is now known that although there is only a

single human IFN-β gene, there are at least 12 distinct human IFN-α proteins, expressed from 14 different genes and pseudogenes (434). Additionally, several other type I IFNs, IFN-ε, IFN-κ, IFN-τ and IFN-ω, as well as a recently identified type III interferon, IFN-λ, are expressed in human cells.

The IFN-β promoter, which has been studied in detail, contains binding elements for several transcription factors. It is thought that the proper transcriptional activation of the IFN genes requires concerted and controlled activities of several different transcription factors, specifically the IFN regulatory factors (IRFs), NFκB and ATF-2/c-Jun (IRFs and NFκB will be discussed below). The IFN enhancer is divided into four different positive regulatory domains (PRDs) depending on the factor that it binds. IRF proteins bind to PRDI and PRDIII, NFκB binds to PRD II and ATF-2/c-Jun binds to PRDIV. The co-activator, cyclic-AMP response element binding protein (CREB) binding protein, or the closely related p300, can interact with each of these factors aiding in transcriptional activation of IFN-β (424).

The identification of viral pathogens and the subsequent induction of IFN-α/β are achieved by PRRs expressed by the host cell. There are two recognized classes of PRRs that recognize components of viral infection: toll like receptors (TLR) and RNA helicases containing the caspase recruitment domain (CARD), RIG-I and mda-5 (442). Some recent studies indicate that the dsRNA-dependent protein kinase (PKR) may also be involved in IFN induction, although the nature of its involvement is less clear.

Toll-like receptors

TLRs expressed in mammals are homologous to Toll, a molecule with antifungal activities that is expressed in *Drosophila*. To date, 13 different TLRs have been identified. TLR1-9 are common to both human and mouse, TLR10 is unique to humans

and TLR11-13 are unique to mice (511). All TLRs have an extracellular domain containing leucine-rich repeats which is important for PAMP recognition. The intracellular domain contains a Toll/IL-1 resistance (TIR) domain, which is essential for recruiting specific adaptor proteins to activate signal transduction pathways (511). Several TLRs are important for recognizing bacterial or viral nucleic acids, TLR3, TLR7, TLR8 and TLR9. TLR3 recognizes double-stranded RNA (dsRNA), which is often generated during RNA virus replication (7). This TLR also recognizes polyinosine-polycytidylic acid (pIC), a synthetic dsRNA mimetic. The substrate for TLR7 and TLR8, which are closely related, is single-stranded RNA (ssRNA) (113). This molecule is particularly important for the signaling cascade resulting in IFN-α expression. TLR9 recognizes unmethylated CpG motifs present only in bacterial and viral DNA (210, 342). These four TLRs are all expressed within endosomal compartments in cells. It is thought that this localization is important for discriminating between viral and cellular RNA/DNA. Although all four TLRs are important for various aspects of the innate immune response, only the signaling pathways of TLR3 and TLR7 (TLR8) will be discussed in detail below.

The TLR adaptor proteins

Following interaction with their specific ligands, TLRs form homo- or heterodimers which bring together the two TIR domains. TLR3 forms homodimers following its interaction with dsRNA while TLR7 can form homodimers or TLR7-TLR8 heterodimers after interacting with ssRNA. The TIR domains function to recruit specific adaptor proteins which are responsible, ultimately, for the activation of transcription factors. There are five TIR domain-containing adaptor proteins used for the various TLRs: MyD88, MAL (TIRAP), TIR domain-containing adaptor protein inducing IFN-β

(TRIF; also called TICAM-1), TRIF-related adaptor protein (TRAM) and sterile α- and armadillo-motif-containing protein (SARM). SARM appears to serve as a negative regulator of TLR signaling and TRAM and MAL/TIRAP will not be discussed, as they are thought to be only involved in TLR2 and TLR4 signaling (415). MyD88, on the other hand, appears to be involved in all TLR signaling pathways, with the exception of TLR3, which utilizes TRIF exclusively as its adaptor protein.

RIG-I-like helicase family

The second recognized class of PRRs is often referred to as RIG-I-like helicases (RLH). In contrast to the TLRs, RLH family members are localized to the cytoplasm (546). The premier member (and the one the class is named for) is a DExD/H box-containing RNA helicase, retinoic acid inducible gene (RIG-I). RIG-I contains a C-terminal helicase with intrinsic ATPase activity that was shown to interact with dsRNA (616). The N-terminal region of the molecule contains two copies of a caspase activation and recruitment domain (CARD) which was shown to be responsible for transducing signals to downstream transcription factors. The binding of dsRNA to the helicase domain results in a conformational change, revealing the CARD domains and allowing them to interact with downstream adaptor molecules (546, 616). Shortly following the identification of RIG-I, a helicase related to RIG-I, melanoma differentiation-associated gene-5 (mda-5), was also shown to recognize dsRNA (13). Interestingly, although both RIG-I and mda-5 were initially both shown to interact with pIC, it is now known that mda-5 is predominantly responsible for recognizing pIC (167, 261). RIG-I, on the other hand, was shown to be critical for recognizing 5'-triphosphates present on viral RNA (226, 438). This is an ingenious mechanism the cell employs to distinguish host RNAs from viral RNAs, as most host mRNAs contain a 5' methylated cap instead of a

triphosphate group. Interestingly, a recent report suggests that RNase L, a host endonuclease thought to cleave viral ssRNA, may be involved in IFN-β production. It is thought that when activated by 2'-5' OAS, RNase L cleaves cellular RNAs into small segments which may be recognized by RIG-I and/or mda-5 (353). Another molecule, LGP2, was recently discovered. LGP2 is closely related to RIG-I; however, although it binds dsRNA like RIG-I, it lacks the CARD domain. Since it has no CARD domain and cannot signal to IPS-1 (see below), it is thought that LGP2 serves as a negative regulator of RIG-I activities by chelating RNA molecules that might otherwise interact with RLRs (286, 476, 615).

Both RIG-I and mda-5 interact with the same downstream adaptor protein. This adapter, identified simultaneously by four independent groups, is a protein expressed on the outer membrane of the mitochondria referred to as IFN-β promoter stimulator-1 (IPS-1) (also called MAVS, Cardif and VISA) (263, 386, 510, 599). For the sake of simplicity, it will be referred to as IPS-1 for the remainder of this dissertation. IPS-1 also contains a CARD domain, presumably the site of interaction between either RIG-I or mda-5. Interaction with RIG-I or mda-5 recruits and activates secondary messenger molecules leading, ultimately, to the induction of IFN (see Signaling pathway section below).

Interferon regulatory factors

One common factor in many of the IFN induction pathways is an IFN regulatory factor (IRF), a family of transcription activator proteins. The IRF family has nine members: IRF1-7, IRF8 (ICSPB) and IRF9 (ISGF3). Four members of the IRF family have been shown to have direct roles in IFN induction, IRF1, IRF3, IRF5 and IRF7. All of the IRFs contain a conserved DNA-binding domain at their N-terminus. This region is involved in recognizing a consensus sequence on DNA referred to as the IFN-stimulated

response element (ISRE). These types of sequences are present on the promoters of the type I IFNs as well as other genes that are involved in immunity.

IRF1, as the name implies, was the first IRF identified. Shortly thereafter, IRF2 was identified. These two IRFs were shown to bind to the same DNA element. A series of experiments indicated that IRF1 is involved in the activation of IFN-α/β promoters; however, IRF2 appeared to serve as a repressor of IFN transcription and negatively regulates IFN-stimulated genes (220). IRF4 and IRF6 have not been studied intensely. IRF4 expression is limited to T and B cells and is not induced by IFN treatment (220, 550). This IRF appears to be important for lymphocyte development. To date, little is known about IRF6.

IRF8, also called IFN consensus sequence binding protein (ICSBP), was first identified as a protein that binds to an ISRE present in the major histocompatability (MHC) class I gene. As with IRF4, IRF8 also has a limited expression profile, present only in myeloid and lymphoid cell lineages. Expression in these cells can be induced by IFN-γ (220, 550). IRF9 is a subunit of the transcription factor IFN stimulated gene factor 3 (ISGF3) (see 'Type I Interferon Signaling' below). This IRF is critical for IFN-α/β-mediated signaling, as it binds to phosphorylated STAT1 (signal transducers and activators of transcription-1) and STAT2, forming the ISGF3 complex (227). This complex translocates into the nucleus where it binds to ISRE elements on DNA, inducing the expression of multiple ISGs (discussed below).

IRF5 has been shown to be involved in TLR signaling. Specifically, IRF5 is activated by MyD88 and TRAF6, downstream from TLR7 or TLR8 (503, 545). The role of IRF5 in IFN induction, however, is somewhat conflicted. One report shows that silencing IRF5 severely impairs TLR7-mediated IFN-α production (503); however, a

separate report indicated that, although the expression of some proinflammatory cytokines was impaired, IRF5 knockout mice did not show any impairment in IFN-α levels in response to various TLR ligands (545).

IRF3 and IRF7 are, perhaps, the most studied of the IRFs. They are highly homologous, but there are distinct differences. IRF3 is a transcription factor associated with IFN induction via TLR3 and RIG-I/mda-5 signaling. It is expressed constitutively in all cell types and resides in the cytoplasm. IRF3 is activated by the phosphorylation of specific serine residues in the C-terminus of the protein (396). Once phosphorylated, IRF3 forms a homodimer (or a heterodimer with IRF7), translocates into the nucleus and forms a complex with co-activator CBP /p300 (220). The complex then binds to PRDI and PRDIII elements on the IFN promoter, inducing the expression of both IFN and other ISGs (17). IRF3 can also bind to specific IFN stimulated response elements (ISRE) to induce the expression of multiple genes, including ISG54 and ISG56 (178)

IRF7 appears to work in concert with IRF3 in some cases; however, it appears to serve a different subset of innate immune functions. Unlike IRF3, IRF7 is expressed constitutively only in specific cell types (including plasmacytoid dendritic cells). In other cells, IRF7 is expressed at very low levels but can be induced by IFN stimulation (221). Because of this fact, it was initially thought that IRF7 is involved in a second 'wave' of IFN production (489). However, the finding that the contribution of IRF3 to IFN induction by some viruses is minor in the absence of IRF7 suggested that the low basal levels of IRF7 may also play an important role in the initial phase of IFN induction and the production of IFN by the subset of cells that constitutively express IRF7 is key for effective innate immune responses (223). Similar to IRF3, IRF7 is activated by the phosphorylation of specific serine residues on its C-terminus (60). The activation of IRF7

results in the formation of homodimers (or heterodimers with IRF3), translocation into the nucleus and the transcriptional activation of IFN genes (by binding to PRDI and PRDIII elements on the IFN promoter), particularly IFN-α subtypes.

The NFκBtranscription factor

NFκB was first identified as a nuclear factor in B cells which bound to the κ light chain enhancer region more than 20 years ago (509). Since then, NFκB has been shown to be involved in a multitude of different cellular processes and to regulate a variety of genes, including chemokines, cytokines, adhesion molecules, stress-response genes and regulators of apoptosis (159) following activation by more than 200 different physiological stimuli (553). NFκB consists of a family of five (in mammals) structurally related, conserved proteins: RelA (p65), c-Rel, RelB, p50 (and its precursor p105) and p52 (and its precursor p100). These subunits, which are present only as homo- or hetero-dimers, each contain a Rel homology domain (RHD) which gives them DNA binding capabilities. In resting cells, the homo- or hetero-dimers are linked to a family of proteins called inhibitors of NFκB (IκB; IκBα, IκBβ, IκBε, IκBγ, Iκbξ, BCL3, p100 and p105) (496). The activation of NFκB involves the phosphorylation of the IκB protein by a core Iκ kinase (IKK) complex (consisting of IKK1, IKK2 and NEMO). This phosphorylation targets IκB for degradation which releases NFκB allowing it to translocate into the nucleus (553).

There are three primary NFκB signaling pathways: the canonical pathway, the noncanonical pathway and the atypical pathway (553). The canonical pathway is the most common activation pathway and components of multiple signaling pathways have been shown to be involved. Several members of the TRAF family (TRAF2, TRAF5, TRAF6) have been shown to interact with the IKK complex, resulting in the phosphorylation of

IκB (553). NFκB has been shown to be activated by TLRs as well as RIG-I/mda-5 signaling (discussed below). It is also suggested that PKR signaling may also activate NFκB (discussed in Chapter 2) (161, 298). NFκB modifications, such as phosphorylation and acetylation have been documented (73), but the physiological role of these modifications is unclear.

The other two pathways have been described more recently and are, thus, not as well defined. To date, only a few stimuli have been shown to activate the noncanonical signaling pathway. Activation of this pathway appears to require only IKK1 (not IKK2 and NEMO) and an additional protein called NFκB inducing kinase (NIK) (198). It is thought that, together with NIK, IKK1 homodimers selectively phosphorylate p100 associated with RelB. This results in the partial proteolysis of p100 to form p52-RelB dimers which can translocate into the nucleus (553). Even less is known about the atypical activation pathway. This pathway appears to be activated in response to DNA damage. Stimuli that cause DNA damage, such as ultraviolet light, appear to mediate the phosphorylation of IκB in an IKK-independent fashion (433, 553).

Signaling pathways

The major players involved in IFN production were described above. The pathways involved in linking these major players together and initiating IFN synthesis is a subject of intense research. A simplified representation of the various signaling pathways discussed below (TLR3, TLR7 and RIG-I/mda-5) is shown in Figure 1.6. There is some evidence that suggests PKR may also serve as either a PRR or a downstream signaling kinase during IFN-β production, as well, although these pathways are only discussed in Chapter 2.

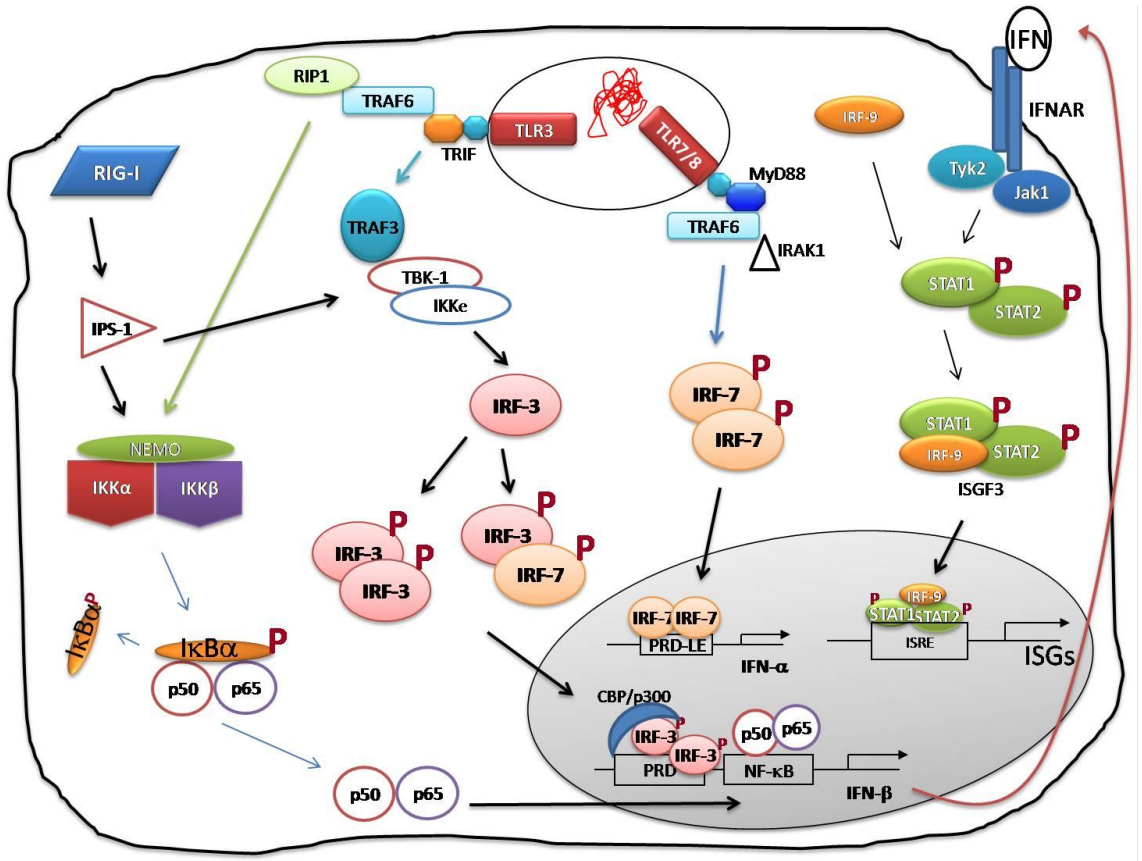


FIGURE 1.6: IFN INDUCTION AND STIMULATION PATHWAYS. Abbreviated signaling pathways for TLR3, TLR7/8 and RIG-I are shown as well as the type I IFN JAK/STAT signaling pathway.

TLR3

TLR3 is activated by dsRNA that enters the lumen of the endosomal compartment where this TLR resides. As mentioned above, dsRNA ligates TLR3 to form homodimers which brings the C-terminal TIR domains into close proximity and recruits the adaptor protein TRIF (421, 602, 603). TRIF activates two noncanonical IκB kinase homologs, IκB kinase-ε (IKKε) and TANK-binding kinase-1 (TBK1) (139, 516), presumably via a direct interaction with TRIF and the involvement of another adapter protein, tumor-necrosis factor receptor-associated factor 3 (TRAF3) (416). It has been suggested that

TBK-1/IKKε kinases are controlled upstream by NFκB-activating kinase-associated protein 1 (NAP1) (487, 488), although the exact nature of this regulation is not clear. The two kinases are responsible for phosphorylating IRF3, which forms a homodimer (or an IRF3-IRF7 heterodimer) and translocates into the nucleus. The IRF3 dimers form a complex with CBP/p300 and bind to the PRD I and PRD III domains on the IFN promoter to induce IFN-β transcription.

A second aspect of TLR3 stimulation is the activation of NFκB. Although this signaling pathway is less well defined, it is thought that TRIF recruits receptor interacting protein-1 (RIP1) which, in turn, signals downstream to activate NFκB (385). The interaction between TRIF and RIP1 appears to be mediated by TRAF6, which directly interacts with the N-terminus of TRIF. Interestingly, the association between TRIF and TRAF6 physically interferes with the association between TRIF and TBK-1, suggesting that the TLR3-mediated activation of IRF3 and NFκB are independent of each other (490).

TLR7/8

TLR7 and TLR8 have both been shown to recognize nucleic acids (specifically ssRNA) (113, 205), although they were first recognized as mediators of IFN production following treatment with imidazoquinolines (209). TLR7 and TLR8, which are closely related, are expressed in both humans and mice; however, TLR8 does not appear to be functional in mice (511). Unlike TLR3 signaling, TLR7 signaling is dependent upon MyD88 (262); there is no evidence to suggest that TRIF or any other TIR-domain containing adaptor proteins are involved in the TLR7/IRF7 pathway (208). TLR7-mediated IFN production occurs via the transcription factor, IRF7. As mentioned above, IRF7 is expressed constitutively only in specific cell populations (such as plasmacytoid

dendritic cells) while it is expressed only following IFN stimulation in other cell types. Thus, in many cell types, IRF7 serves as a factor involved in the secondary amplification of IFN induction, not necessarily in the initial induction, which is believed to occur through the RLR/TLR3 pathways.

In dendritic cells, the interaction between TLR7 and its ligand results in dimerization and the recruitment of MyD88 to its cytoplasmic tail. MyD88, as well as TRAF6, have been shown to interact with IRF7, forming a MyD88-TRAF6-IRF7 complex (262). Interestingly, it appears as though ubiquitination of IRF7 by TRAF6, which has been shown to have E3-ligase activity, is required for IRF7 activation (262). Further investigation has also indicated that two other kinases, interleukin-1 receptor-associated kinase-1 (IRAK1) and IKKα, can interact with IRF7 (222, 564), resulting in its phosphorylation. Interestingly, it has also been found that IKKε and TBK-1, which are involved in IRF3 activation, can also phosphorylate IRF7 (516); however, it is not known whether this mode of activation is involved directly in the dendritic cell pathway and/or the secondary amplification pathway. Interestingly, MyD88 also interacts with IRAK4 kinase, an interaction that is critical for the production of cytokines such as IL-6 and TNFα, two inflammatory cytokines that are strongly induced by NFκB. Interestingly, when IRAK4 is co-expressed with IRF7, NFκB promoter activity was impaired (222). This observation lead researchers to suggest that, as seen with TLR3-mediated activation, the signaling pathway for activation of NFκB by TLR7 may run parallel to the activation of IRF7 and that the two signaling pathways may interfere with each other (222).

RIG-I/mda-5

The previous two IFN induction pathways described involve the recognition of viral nucleic acids within endosomes. Two different PRRs, RIG-I and mda-5, are

important for recognizing viral nucleic acids present in the cytosol (556). RIG-I and mda-5 are related and signal via similar pathways; however, they appear to recognize distinct viral pathogens (167, 261). The interaction between RIG-I and its ligand causes a conformational change in RIG-I (revealing its CARD domain) leading to the formation of a CARD-CARD interaction between RIG-I and the adaptor protein, IPS-1. IPS-1, in turn, interacts with TRAF3 via its non-CARD regions (479). TRAF3 then recruits and activates TBK-1 and IKKε which phosphorylates IRF3 and/or IRF7 (546, 556). IPS-1 has also been shown to activate NFκB. Although the exact mechanism of this interaction is unknown, some evidence suggests it may involve an interaction through TRAF6 (599).

TYPE I INTERFERON SIGNAL TRANSDUCTION

After induction, the IFN protein is secreted from the cell where it can interact with a specific receptor on the cell that produced it or neighboring cells (independent of other activation). The type I IFN receptor (IFNAR), which binds both IFN-α and IFN-β, is composed of two components, IFNAR1 and IFNAR2 (443). Janus kinase 2 (JAK2) and tyrosine kinase 2 (Tyk2) are bound to the intracellular chain of IFNAR and, following the interaction between IFNAR and IFN, these kinases are activated and phosphorylate tyrosine residues on the receptor and on the kinases themselves. This recruits STAT1 and STAT2, which are subsequently phosphorylated by JAK2/Tyk2 and form a heterodimer (1). IRF9 interacts with the STAT1-STAT2 dimer, resulting in the formation of the transcription factor, ISGF3. ISGF3 translocates into the nucleus where it binds to ISRE promoters on DNA, inducing the expression of a multitude of ISGs (See Figure 1.6).

IFN stimulation results in the expression of hundreds of ISGs, many of which are known to be involved in establishing an antiviral environment within the stimulated cell. Although most ISGs are still undefined, there are a few that are well studied, including

ISG15, the GTPase Mx1, 2', 5'-OAS and PKR. ISG15 is an ubiquitin homologue whose many targets include proteins that are involved in the type I IFN response. Several viruses have been shown to be susceptible to ISG15 activity, including influenza virus, Sindbis virus, vesicular stomatitis virus and lymphocytic choriomeningitis virus (LCMV) (308, 422). The Mx-1 GTPase has been shown to be an effective antiviral against a variety of viruses, as well, including orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses and bunyaviruses (478). Interestingly, although Mx-1 is expressed in both humans and mice, only the human Mx-1 has been shown to have antiviral activities (478). OAS and PKR will both be described in more detail in Chapter 2. Several other ISGs have been shown to have antiviral activities, including ADAR1 (adenosine deaminase, RNA-specific 1), APOBEC (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide), the tripartite-motif-containing (TRIM) proteins such as TRIM19 (also known as PML), viperin, and IFIT1 (IFN-induced protein with tetratricopeptide repeats 1) and IFIT2 (478).

Impetus for This Research

The infection of a host cell by a pathogen is the start of a delicate balance between host defenses and viral replication. The slightest of advantages for either the host or the virus can determine the outcome of infection: survival or death. Viruses are often required to parasitize host proteins or processes for their replication; therefore, the presence or absence of these specific factors in different cell types or hosts may affect host range and pathogenesis. Hijacking specific proteins may increase the ability of the WNV to replicate, effectively shifting the balance to the virus' advantage. In fact, prior to

the initiation of this dissertation, WNV had been shown to interact with several host proteins to promote its infection.

However, the host cell employs mechanisms to identify and eliminate WNV. The type I IFN response is critical for controlling WNV infection, as previous work has shown that flaviviruses are sensitive to IFN treatment (12, 56, 397, 441, 475, 483) and flavivirus-infected IFNAR null mice demonstrate increased mortality compared to WT mice (252, 332, 519). Additionally, flaviviruses have developed mechanisms to counteract the cell's ability to induce ISGs in infected cells (35, 186, 255, 316, 505). In the past decade, the mechanism(s) behind virus-induced IFN production has been an area of intense research. Although several different molecules have been suggested as important for developing an anti-WNV environment, the pathway(s) directly involved in WNV-induced IFN production is still relatively unclear.

HYPOTHESIS AND GOALS

The overall hypothesis of this dissertation was that *although WNV recruits cellular factors to successfully replicate and spread, the host cell counteracts the virus by recognizing the replicating genome and producing separate factors which function to inhibit WNV replication.* The goal of this research was to define critical interactions occurring between WNV and the host cell and, more specifically, identify cellular factors important for impeding or promoting WNV replication. This was achieved through two specific aims. Evidence from the literature as well as our early experiments led us to the hypothesis that PKR is important for WNV-induced IFN production. Thus, the first specific aim of this study was to determine the role of PKR during WNV infection. The second specific aim was to identify host proteins that were essential for efficient WNV infection. This aim was achieved by systematically knocking down a subset of human

genes (using a small interfering RNA library) and using this as a screen to discover genes required for efficient WNV replication.

The research summarized in this dissertation expands upon our knowledge of WNV-host cell interactions. These virus-host interactions are key in determining the outcome of infection, and hence, disease manifestation. Having a better understanding of the mechanisms behind viral recognition and clearance from an infected cell/host as well as a detailed understanding of viral replication will aid in the development of potential targets for anti-WNV therapeutics and, perhaps, aid in the rational design of vaccines.

CHAPTER 2: THE DOUBLE-STRANDED RNA-DEPENDENT PROTEIN KINASE, PKR, IS A CRITICAL FACTOR FOR WEST NILE VIRUS INCUDED IFN SYNTHESIS *IN VITRO*[1]

Abstract

Cells are able to sense invading pathogens via a variety of molecules, referred to as pattern recognition receptors (PRR). Interaction of these PRRs with pathogen-associated molecular patterns (PAMPs), which include components of a viral genome or viral particle, instigates a signaling pathway(s) ultimately leading to the induction of a multitude of genes, including those for type I interferon (IFN), which are critical for developing an effective and rapid antiviral environment. Here we demonstrate that the double-stranded RNA (dsRNA)-dependent protein kinase (PKR), which has been shown to function as a PRR in cells treated with the dsRNA mimetic poly(IC), serves as a PRR in WNV-infected cells. Evidence for PKR's role as a PRR was obtained in both human and murine cells. In mouse embryo fibroblasts (MEF), we demonstrated that PKR gene knockout, post-transcriptional gene silencing of PKR mRNA using siRNA, or chemical inhibition of PKR function all interfered with IFN synthesis following WNV infection. In three different human cell lines, siRNA knockdown and chemical inhibition of PKR blocked WNV-induced IFN synthesis. Using these same approaches we demonstrated

[1] Significant portions of the work and ideas presented in this chapter have been published in Journal of Virology. Copyright permission has been granted by American Society for Microbiology providing proper citation. The citation is:
Gilfoy, F. D., P. W. Mason. 2007. West Nile virus-induced interferon production is mediated by the double-stranded RNA-dependent protein kinase PKR. J Virol 81:11148-11158

that PKR was not necessary for Sendai virus-induced IFN synthesis, suggesting that PKR is particularly important for recognition of WNV infection.

Introduction

FLAVIVIRUSES AND THE INNATE IMMUNE RESPONSE

The induction and subsequent action of IFN is one of the best early defenses a host cell has against viral infection. As detailed in Chapter 1, host cells encode a variety of PRRs which can recognize viral infection as well as antiviral molecules which can interfere with viral infection. The role of the innate immune response in controlling viruses within the Flaviviridae family has been the subject of intense scrutiny recently. Progress has been made; however, there is still much that is not known.

Studies with IFNAR knockout animals demonstrated the importance of IFN during flavivirus infections. Mice deficient in type I and/or type II IFN receptors were highly susceptible to DENV (252, 519), MVEV (332) or WNV (483), demonstrating increased viral replication and increased morbidity and mortality compared to wild type mice. Additionally, pre-treatment of cells (12, 475, 483) or mice (56, 397, 441) with IFN can protect against flavivirus infection and increase survival and in some cases, IFN treatment in humans is effective in controlling WNV disease (256).

Several PRRs have been shown to be important for controlling flavivirus infections, including TLR3, RIG-I and mda-5. Although distantly related to flaviviruses, HCV is interesting due to its ability to block signaling from both TLR3 and RIG-I/mda-5. The NS3/4A protein of HCV has been shown to cleave both TRIF (312) and IPS-1 (77, 142), molecules that are involved in IRF3 and NFκB activation. RIG-I has been shown to be involved in JEV (70, 261) and WNV infection (143, 144). Interestingly, both RIG-I

and mda-5 appear to be important for the recognition of DENV infection (334); although a recent study suggests mda-5 may also be involved in WNV infection (144). IRF3 was shown to be quite important for maintaining an anti-WNV environment. In cells devoid of IRF3, WNV spreads more effectively and maintains a high titer for a longer duration compared to wild type cells (145). It is thought that IRF3 is activated in a RIG-I-dependent fashion, as RIG-I deficient cells show delayed activation of IRF3-dependent genes, ISG54 and ISG56 (143), although, as mentioned above, a recent study suggests that mda-5 may also be involved (144).

It is interesting to note that WNV appears to block or evade TLR3-mediated activation of IRF3 (505); however, this effect appears to wane later in infection (145). A recent study suggests that the E protein of WNV can block TLR3-mediated signaling by interfering with RIP1, a protein involved in TRIF-mediated activation of NFκB (15). A WNV nonstructural protein, NS1, has also recently been shown to play a role in blocking WNV-mediated TLR3 signaling (587). Despite the blockage of its signaling, TLR3 does appear to play a role in the pathogenesis of WNV, as mice deficient in TLR3 show decreased neurovirulence (575), suggesting that TLR3 may be involved in the entry of WNV into the brain. More recently, it was found that macrophages derived from older patients expressed higher levels of TLR3 and increased levels of cytokines compared to macrophages derived from younger patients (287). However, despite the importance of RIG-I-mediated IRF3 signaling to the production of ISG56 and ISG54, levels of WNV-induced IFN in IRF3 deficient mice are similar to that seen in wild type mice (48, 101), suggesting that another pathway may also be involved in IFN induction. Following its induction, IFN is secreted from the infected cell (or neighboring activated cells) where it can interact with its receptor on the surface of that cell or neighboring cells and initiate

signaling cascades. Flaviviruses have evolved mechanisms to evade the initiation of this signaling cascade. TBEV (35), DENV (400, 401), JEV (316, 317) and WNV (186, 329, 505) have all been shown to inhibit STAT1 phosphorylation. The ability of WNV to block the IFN response appears to be a contributing factor to the differences between virulent lineage I viruses and avirulent lineage II viruses (264).

Although flavivirus-infected cells are impaired in their ability to respond to IFN stimulation, IFN is still critical for controlling infection (see above). This is likely due to the initiation of an antiviral environment in neighboring, uninfected cells. This would effectively limit the spread of WNV from cell to cell. The ISGs responsible for the anti-flaviviral response, however, are not known. The roles of two very well characterized ISGs, OAS and PKR, have been studied for flavivirus infection. OAS is important for activating RNase L, a latent cellular endonuclease. Activated RNase L, in turn, cleaves both viral and cellular RNAs, preventing viral replication and likely inducing apoptosis. PKR, on the other hand, is activated by dsRNA. Activation results in the phosphorylation of translation elongation factor 2-α (eIF2α) and shutoff of cellular and viral translation. Cells deficient in both RNase L and PKR showed no impairment in the ability of IFN-β to protect cells against DENV infection (109), suggesting other ISGs are important for controlling DENV infection. Recent studies suggest that there is an anti-WNV effect of RNase L, although it is not know whether it is associated with OAS expression. WNV levels were increased in RNase L deficient cells (497) and RNase L null mice were more susceptible to WNV infection (486) compared to wild type mice. Additionally, in certain cell types, both PKR and RNase L appear to be involved in controlling WNV infection (486). Decades ago, several species of mice were shown to be resistant to flavivirus infection. This resistance was subsequently mapped to a single gene on chromosome 5

(*flv*), which encodes and isoform of OAS, OAS1b (364, 431). Mice that are susceptible to flavivirus infection encode a truncated form of OAS1b, suggesting that OAS is important for determining the outcome of WNV infection. Interestingly, studies from some patients hospitalized with WNV infections showed polymorphisms in the RNaseL and OAS genes (601), suggesting that OAS may have some role in human WNV infections.

PKR AS A FACTOR OF THE INNATE IMMUNE RESPONSE

PKR is a double-stranded RNA sensor that is involved in a variety of cellular processes including signal transduction, apoptosis and cell differentiation and development. The protein contains two domains: an N-terminal regulatory dsRNA-binding domain (RBD) and a C-terminal catalytic kinase domain. The N-terminal RBD contains two separate dsRNA-binding motifs (RBM) of approximately 65 amino acids, separated by a 20 amino acid linker (134). The RBMs bind A-form RNA structures with no apparent sequence specificity. Mutational studies have indicated that RBM1 is more important for dsRNA binding; however, both RBM1 and RBM2 are required for optimal activity. Analysis has also shown that RBM2 interacts directly with the C-terminal catalytic domain in the absence of an activating substrate. This RNA-binding motif also appears to be important in maintaining the association between the two PKR monomers in the fully active dimeric enzyme (Figure 2.1).

In an inactive state, PKR exists in the cell as a monomer, but following activation, PKR is converted into an active homodimer (Figure 2.1). Structural studies have demonstrated the events involved in PKR activation. DsRNA or other stimuli interact with PKR through the two N-terminal RBMs resulting in conformational changes in PKR, relieving the autoinhibitory interactions and allowing homodimerization (Figure 2.1) (155). Rapid autophosphorylation in a stretch of amino acids termed the activation

segment follows dimerization. Two specific residues, Threonine (Thr) 446 and Thr451 are consistently phosphorylated during PKR activation and are thought to increase the stability of the dimer and increase the catalytic activity (108, 552, 620). In addition to dsRNA, several other ligands can activate PKR, including other proteins like PKR-associated protein (PACT) and molecules like heparin, dextran sulfate and chondroitan sulfate (151).

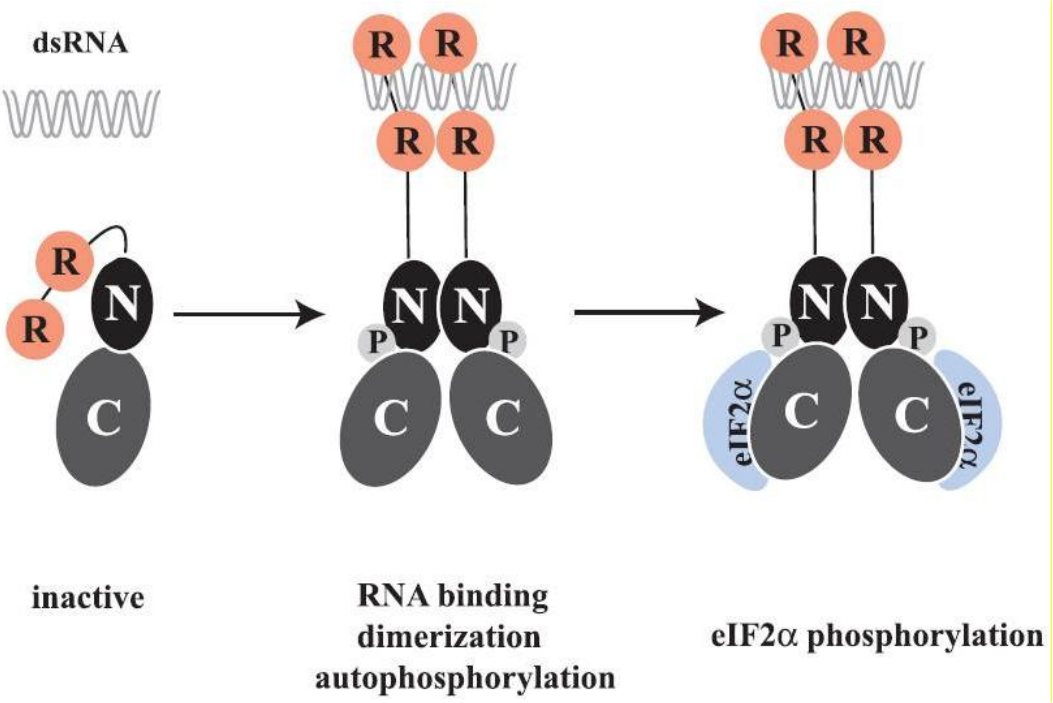


FIGURE 2.1: SCHEMATIC DRAWING OF PKR ACTIVATION. Following stimulation with a specific ligand (dsRNA), a conformational change allows PKR to dimerize and undergo autophosphorylation. This active from of PKR can then interact with, and phosphorylate, its downstream target, shown here as eIF2α. Figure was reprinted with permission from Elsevier from Garcia, MA et al. 2006. Impact of Protein Kinase PKR in Cell Biology: from Antiviral to Antiproliferative Action. Microbiol Mol Biol Rev **70:**1032-60.

An IFN-induced protein capable of blocking viral protein synthesis was first discovered in the early 1970s (146, 382). Subsequent work led to the identification of the protein responsible for this activity, a dsRNA-dependent kinase (465, 508) which was

later named PKR (91). The most well characterized activity of PKR is its phosphorylation of eIF2α, leading to the inhibition of mRNA translation (81). This activity was shown to be important for controlling viral replication (19, 384). Further studies, however, revealed even more complex roles for PKR in innate immunity.

The late 1980s brought the first suggestion that PKR is required for the expression of IFN (360). Several groups demonstrated that treatment with 2-aminopurine (2-AP), a PKR inhibitor, blocked IFN expression following treatment with pIC (625) or infection with VSV (360, 558). Additionally, cells derived from mice lacking PKR showed a similar defect in IFN production following stimulation with pIC (114). The specificity of 2-AP for PKR has not been well established, however, these studies demonstrated that treatment with 2-AP did not have an effect on the induction of several other genes, including heat shock proteins and metallothionein (360, 558, 625). Treatment also had no effect on gene induction following treatment with exogenous IFN (360, 558), suggesting that 2-AP does not affect the cell's ability to induce an antiviral environment.

These early studies began a flurry of investigations to determine how PKR affects IFN signaling. The discovery that dsRNA induces the binding of NFκB to the IFN-β promoter introduced a possible link between PKR and NFκB (569). The cloning of PKR in 1990 enabled more direct study of interactions between PKR and NFκB (383). PKR was shown to induce the phosphorylation of IκB in cell-free extracts, suggesting a possible mechanism of action (298). Experiments in a mouse cell line showed that a catalytically inactive form of PKR (K296R PKR) inhibited NF-κB reporter gene expression, suggesting that PKR activates NFκB by inducing the phosphorylation of IκB (298). The generation of mice deficient in PKR (PKR-null) confirmed the role of PKR in NFκB activation. In mouse embryo fibroblast (MEF) cells, pIC treatment resulted in the

activation of NFκB; however, in MEF cells derived from PKR-null mice, this activation was significantly impaired (607). Interestingly, priming of PKR-null MEF cells with IFN resulted in a partial recovery of NFκB activation following pIC stimulation.

The identification of the molecules involved in NFκB activation helped to further define PKR-mediated NFκB activation. PKR was shown to directly interact with the IKK complex (160, 619), which is required for NFκB activation (see Chapter 1). Cells that were deficient in PKR showed a severe impairment in pIC-induced NFκB activation; specifically, pIC failed to activate IKK in the absence of PKR (619). Expression of PKR via a vaccinia virus recombinant induced the activation of NFκB; however, co-transfection of this construct with dominant negative mutants of IKK1 and IKK2 reduced NFκB DNA binding activity and NFκB-driven reporter activity (160). Analysis of the sequence for PKR indentified two putative TRAF-interacting motifs. TRAFs are a family of proteins shown to be mediators in several different IFN induction pathways (see Chapter 1). Interestingly, three TRAF proteins, TRAF2, TRAF5 and TRAF6, were shown to co-localize and physically interact with the dimerized form of PKR. The functional role of this interaction was demonstrated when TRAF2 or TRAF5 deficient cells, transfected with PKR, failed to show NFκB activation (161). Additionally, the expression of dominant negative forms of TRAF5 and TRAF6 led to a reduction in NFκB activity (161). Although there is ample evidence suggesting PKR interacts with the IKK complex, how PKR mediates this response is still up for debate. There are conflicting reports on the requirement of catalytically active PKR for NFκB activation as well as what IKK molecule is the target of PKR. PKR has been shown to interact with both IKK1 (160) and IKK2 (46, 84). Additionally, reports have shown that a catalytically inactive form of PKR can still activate NFκB, albeit only when expressed at high concentrations (46). A

separate report showed that only PKR constructs containing the full kinase domain were able to activate NFκB (162). This group did show that PKR mutants lacking the kinase domain were still able to interact with the IKK complex. However, contrary to the two previous results, another group suggested that neither the kinase activity nor the dsRNA binding activity are essential for PKR-dependent NFκB activity (238), suggesting an indirect role for PKR in NFκB activation.

One interesting link between PKR, NFκB and IFN induction is IRF1. As mentioned in Chapter 1, IRF1 has been shown to be involved in the activation of IFNα/β promoters. Mice deficient in PKR demonstrated impaired activation of IRF1 promoter in a reporter assay (299). This impairment was rescued following transfection with wild type PKR. Interestingly, IRF1-dependent IFN-β production involves the activation of NFκB (277). This IRF1-dependent activation of NFκB was impaired in cells expressing a dominant negative form of PKR, suggesting that PKR is involved in IRF1-induced NFκB activation (277). PKR deficient cells also appear to have impaired responses to different TLR ligands, suggesting that PKR may be involved in TLR signaling, as well. Activated PKR was present following stimulation with either LPS (TLR4 ligand) or CpG (TLR9 ligand). Additional study indicated that PKR can interact directly with TIRAP, which is involved in TLR4-mediated signaling (225). In addition to TRAF2, TRAF5 and TRAF6, PKR was recently shown to associate with TRAF3 (416), a signaling molecule involved in a variety of signaling pathways, including TLR3 and RIG-I.

Therefore, in addition to being its own dsRNA sensor, PKR also appears to be involved in a variety of other signaling pathways associated with the innate immune response and IFN induction. Figure 2.2 depicts the possible involvement of PKR in various signaling pathways.

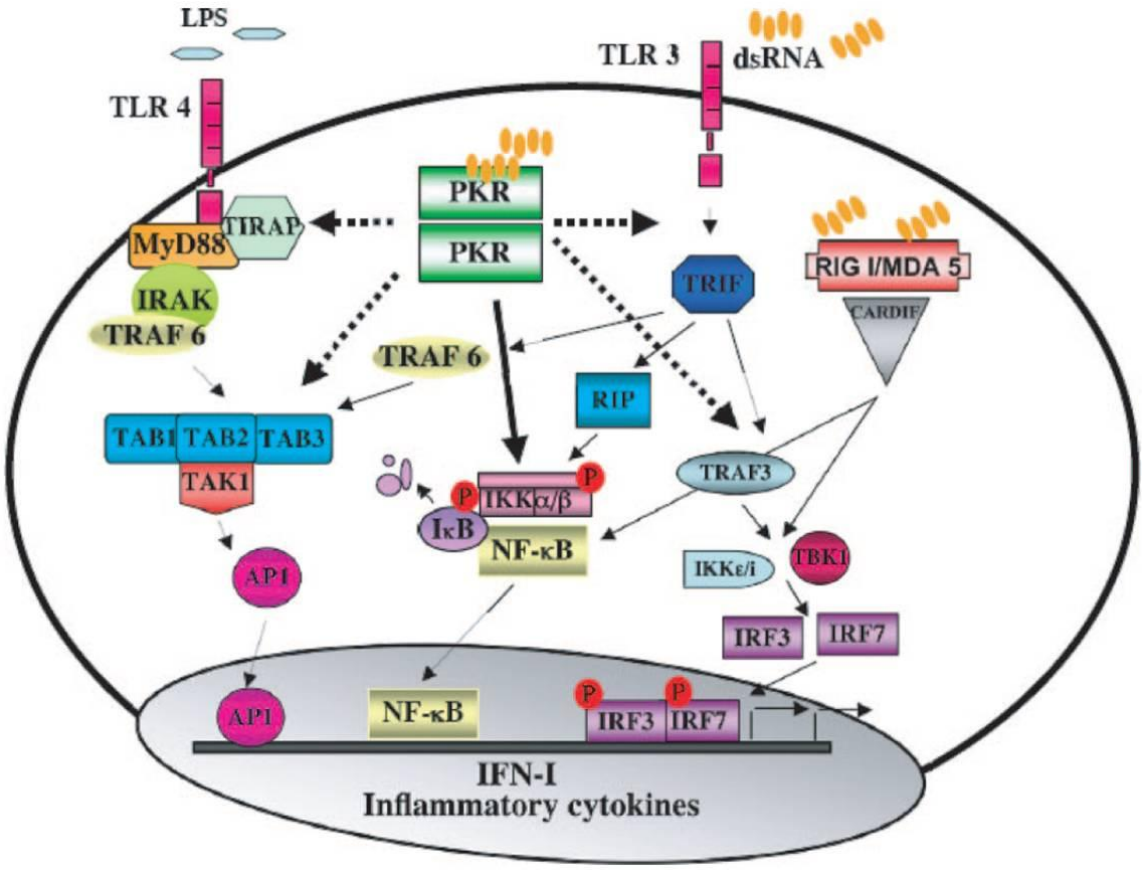


FIGURE 2.2: PKR CAN BE ACTIVATED DIRECTLY BY DSRNA OR SERVE AS AN INTERMEDIARY COMPONENT IN OTHER SIGNALING PATHWAYS. PKR has been shown to be activated by dsRNA; however, it appears to be involved in other signaling pathways, as well. PKR activity has been associated with molecules involved in TLR4, TLR3 and RIG-I/mda-5 signaling pathways. Figure was reprinted with permission from Elsevier from **Garcia, MA et al.** 2006. Impact of Protein Kinase PKR in Cell Biology: from Antiviral to Antiproliferative Action. Microbiol Mol Biol Rev **70:**1032-60.

REPLICONS AND VIRUS-LIKE PARTICLES

Replicons

Deletion studies with genetically engineered flavivirus genomes revealed that the majority of the sequences encoding the structural proteins are dispensable for flaviviral RNA genome replication. Specifically, these studies showed that subgenomic RNA

(replicons) which contain large deletions in the genes encoding the structural proteins are capable of self-replication. In fact, only sequences encoding the non-structural proteins, the 5' untranslated region (UTR), the 3' UTR (containing the 3' cyclization sequence; CS) and the 5' CS (a short region of RNA located at the 5' end of the C gene) are required for efficient replication (267, 273). However, these replicon genomes are unable to be packaged into viral progeny and are thus effectively marooned within the initial cell they infect. Replicon technology has allowed for the analysis of aspects of viral replication without the interference of any virion release and re-infection. To date, replicons have been engineered for a variety of flaviviruses, including KUNV (273), WNV (132, 475, 517), YFV (94, 254, 393), DENV (423) and TBEV (157, 197).

The flavivirus replicon has many different manifestations. Often, replicons are engineered to harbor foreign genes, expressed either as an in-frame insertion in the 5' ORF in lieu of the structural proteins, or as a polyprotein sequence added to the 3' end of the genome. Expression of the polyprotein present on the 3' UTR is usually driven by an internal ribosome entry site (IRES). The foreign genes of choice usually include a reporter gene of some kind (such as green fluorescent protein (GFP) or firefly or renilla luciferase) as well as a selection marker (such as neomycin phosphotransferase gene (NPT) or puromycin N-acetyl transferase (pac). These genes allow for easy identification and selection of replicating genomes. The addition of a reporter gene is particularly useful, as levels of reporter gene expression correlate with the level of RNA replication and viral protein expression.

Virus-like particles

Although the requirement of structural proteins for producing flavivirus particles is absolute, it is not necessary for the structural proteins to be provided in the context of

the viral genome itself. Flavivirus genomes deficient in the structural genes can be packaged into infectious particles using cells engineered to express these missing genes in trans. Packaging cell lines can be developed by using non-cytopathic replicons or eukaryotic expression plasmids to provide long-term or inducible expression of the C, prM and E proteins. Introduction of flavivirus replicons into these cells produces virus like particles (VLPs) that are functionally indistinguishable from a wild type virus particle, except that the encapsidated genomes do not encode the structural proteins (132, 157, 272, 504). VLPs are able to infect a cell as would a virus particle, however, because the structural genes are not encoded on the encapsidated viral genome, the newly replicating genomes cannot be packaged and the infection cannot spread beyond the first infected cell.

Introducing WNV genomes into cells via VLPs has several distinct advantages. First of all, delivering viral genomes via VLP infection is more efficient and less toxic than other methods, such as transfection or electroporation. The confounding problem of obtaining consistent transfection efficiency between experiments and among cell types is essentially eliminated. Additionally, this allows for quantification of viral particles and, thus, delivery to cells in carefully controlled doses (multiplicities of infection). Second, although many flaviviruses are categorized as biosafety level three (BSL3) pathogens, VLP infections can be carried out under standard BSL2 conditions, making manipulations much easier and safer. Third, VLPs can be used to deliver replicon genomes directly *in vivo*. This allows for the examination of primary cellular targets of infection within the host (48, 504), monitoring the trafficking of infected cells through the host, as well as measuring the host's response to infection (368). Recently, forms of

VLPs have been investigated as potential flavivirus vaccine candidates (131, 239, 368, 585, 586).

VLPs used in the studies presented here are primarily generated by introducing the WNV replicon into a BHK packaging cell line, which provide all three structural proteins *in trans* via a non-cytopathic Venezuelan equine encephalitis virus (VEEV) replicon (436) expressing the structural proteins of WNV and a puromycin selection gene under the control of one or more 26S subgenomic promoter(s) (132). These BHK cell lines, placed under puromycin selection, persistently harbor the VEEV replicons, are incredibly stable (586) and are capable of expressing high levels of the WNV structural proteins. The replication of the VEEV replicon does not interfere with an incoming WNV replicon (132); thus, when WNV replicons are introduced, these packaging cell lines are able to generate high titer preparations of WNV VLPs (132, 239, 586).

RATIONALE

IFN plays a key role in the early control of viral infections; therefore, the recognition of viral infection and the subsequent induction of IFN is a critical mechanism the cell utilizes to modulate viral pathogenesis. This was the foundation of the first specific aim of this dissertation, which was to determine the mechanism by which the host cell recognizes WNV infection. Early experiments from multiple groups have indicated that PKR may be involved in the induction of IFN. The *hypothesis* of this aim, that PKR was indispensible for WNV-induced IFN production, was based on a number of early studies implicating PKR in dsRNA-induced IFN expression. The hypothesis was tested by utilizing a variety of experimental methods, including the use of knockout cell lines, chemical inhibitors and siRNA technology.

Materials and Methods

CELL LINES USED AND THEIR MAINTENANCE

To accurately determine a role for PKR during WNV infection, a variety of human and mouse cell lines were utilized. Two different wild-type mouse embryo fibroblast cell lines (MEF) were kindly provided by I. Frolov (UTMB, MEF-G) and M. Gale (UT Southwestern; MEF-D). PKR-null (607) and RNase-PKR-null MEF (624) cells were provided by I. Frolov and STAT1 null MEF cells (117) were a kind gift from J. Durbin (Ohio State University). Multiple human cell lines were also used during the course of these studies. Huh7 cells, derived from liver tissue, were generously provided by S Lemon (UTMB). A549 cells, derived from lung epithelia tissue, and Hec1B, derived from uterine epithelia tissue, were kindly provided by K. Narayanan (UTMB). MRC-5 cells, a fibroblast cell line derived from fetal lung tissue, were purchased from American Type Cell Culture (ATCC).

All of the MEF-derived cell lines (WT, PKR-null, PKR-RNaseL null and STAT1 null MEF) were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1mM sodium pyruvate (NaPy; Invitrogen), 1% antibiotic/antimycotic (atb; Invitrogen), 1% non-essential amino acids (NEAA; Invitrogen) and 20ug/ml gentamycin. Hec1B cells were propagated in DMEM supplemented with 10% FBS, 1mM NaPy, 1% atb, 1% NEAA and 20ug/ml gentamycin. MRC-5 cells were propagated in Modified Eagle's medium (MEM) supplemented with 10% FBS, 1mM NaPy, 1% atb, 1% NEAA and 20ug/ml gentamycin. A549 cells were propagated in DMEM/F-12 50/50 medium supplemented with 10% FBS, 1% atb and

20ug/ml gentamycin. Huh7 cells were propagated in DMEM supplemented with 10% FBS, 1% atb and 20ug/ml gentamycin.

WNV VIRUS LIKE PARTICLE (VLP) PREPARATION

To generate the WNV VLP preparations, WNV replicons were introduced into BHK packaging cell lines (132) by two methods: electroporation of *in vitro* transcribed WNV replicons or infection with previous WNV VLP preparations. Following electroporation, a portion of the cells were plated onto a tissue culture plate and assayed by immunohistochemistry (IHC; described below) for electroporation efficiency. The remaining cells were seeded into a tissue culture flask. After the cells were allowed to adhere, the growth media was replaced with DMEM or MEM supplemented with 1% FBS, 1% antibiotic solution (atb) and 10mM HEPES (DMEM+++ or MEM+++) to help limit cell growth and buffer pH changes. Cell supernatants were harvested and the cell monolayers were re-fed with DMEM+++ or MEM+++ approximately every 24 hours until the cells were destroyed by either cytopathic effect or overgrowth. The supernatants were centrifuged to remove cellular debris (5 minutes at 1000 rpm), and then aliquoted and frozen at -80C. Titers of the VLP preparations were determined by titration and IHC as described below.

VIRUS AND VLPS UTILIZED

The WNV utilized for these studies was a low-passage virus recovered from BHK cells electroporated with an infectious cDNA clone of a human 2002 Texas isolate (475). To improve infectivity on MEF cell lines, the cDNA clone was modified to contain a Glutamic acid (Glu) to Lysine (Lys) mutation at position 138 in the E protein (305), which confers heparan-sulfate binding capability (WNV^{HS}). Sendai virus (SeV)-Cantell was obtained from the Charles River Laboratories. The vesicular stomatitis virus (VSV)

used was the Hazelhurst strain of the New Jersey serotype (obtained from R. B. Tesh, UTMB).

Several different VLP preparations were utilized for these studies. WNV replicons (described below) were packaged using a BHK packaging cell line as described above. This BHK packaging cell line stably expresses a VEEV non-cytopathic replicon encoding the structural proteins of WNV (expressed via a subgenomic promoter) and a *pac* gene (expressed via a second subgenomic promoter), which conferred resistance to puromycin (VEErep/C*-E/Pac) (132). Unfortunately, MEF cells are at least 100 times less susceptible to WNN infection compared to Vero cells. To circumvent this problem, we used a BHK cell line expressing the VEEV replicon containing the Glu-Lys mutation in the WNV E protein described above (VEErep/C*-E^{HS}/Pac) (132).

The WNV replicon (WNR NS1-5, referred to as WNV VLP in the text) contains the coding regions for the entire nonstructural proteins as well as a portion of the C protein (containing the cyclization sequences) along with the 5' and 3' UTR. WNV VLP packaged using the BHK packaging cells expressing the heparan sulfate-binding E protein are referred to as WNV VLP^{HS}. This replicon was further modified by inserting a firefly luciferase gene followed by the auto-catalytic 2A protein from foot-and-mouth disease virus (FMDV) in lieu of the structural proteins. The function of the FMDV protein was to properly cleave the luciferase gene from the remaining structural proteins during translation. This replicon, WNR C-Luc2A NS1-5, will be referred to as WNV FLuc VLP. Schematic diagrams of each of the VEEV and WNV constructs are shown in Appendix A.

VIRUS AND VLP TITRATIONS

Cell lines generally exhibit different sensitivities to virus infection. Since many different cell lines were utilized for these studies, it was necessary to get an accurate understanding of the sensitivity to WNV infection of each of the individual cell lines. If any major differences in sensitivity occurred between two cell lines, infecting with similar focus forming units (ffu) of VLP or WNV determined on a single cell type could result in drastically different levels of infection, leading to inaccurate interpretations. To overcome this problem, VLP and WNV preparations were titrated on each of the individual cell lines. These titers were used in all multiplicities of infection (MOI) calculations.

WNV was titrated in the biosafety level 3 (BSL3) in either 96-well or 24-well plates. WNV preparations were serially diluted (10-fold) in DMEM+++. Dilutions typically ranged from 10^{-1} to 10^{-8}. Monolayers of cells, in duplicate wells, were infected with 150µl (24-well plate) or 50µl (96-well plate) of WNV serially diluted (10-fold) in DMEM+++ and rocked for 1hr to allow for virus attachment and entry. The infected monolayers were overlaid with a tragacanth solution (0.6% tragacanth in MEM without phenol red, supplemented with 1% FBS and 1% antibiotic solution) and incubated undisturbed for 24hrs. The monolayers were then fixed by removing the overlay and fixing with Acetone:Methanol (1:1 v/v) for at least 30 minutes at -20C. Following fixation, the plates were transported to the (BSL2) for processing.

The titration of VLP preparations were performed similarly. Monolayers of cells plated in 96-well plates were infected, in duplicate, with 50µl of serial dilutions (10-fold in DMEM+++) of WNV VLP. Since VLPs are unable to spread from cell-to-cell, it was unnecessary to add the tragacanth overlay, so cells were incubated, with the VLPs, for

24hrs. The infected monolayers were fixed in Acetone:Methanol for at least 30min at -20C.

IMMUNOHISTOCHEMISTRY

To detect antigen-positive cells by IHC, Acetone:Methanol was removed from fixed monolayers and the plates were air-dried. The dried monolayers were rehydrated in blocking buffer [phosphate buffered saline (PBS) supplemented with 1% normal horse serum (NHS)]. After blocking for approximately 10 minutes, the monolayers were incubated with the primary antibody diluted in blocking buffer (usually at least 30 minutes at room temperature (RT)). Following three washes with PBS, the monolayers were incubated with a horseradish peroxidase-conjugated (HRP) secondary antibody of appropriate specificity diluted in blocking buffer (usually at least 30 minutes at RT). Following removal of the secondary antibody and washing with PBS, antigen-containing cells were stained by using a peroxidase detection assay (Vector VIP, Vector Laboratories), which generates a red precipitate when exposed to antigen-bound HRP. WNV antigen was detected using a WNV-specific murine hyperimmune ascites fluid (MHIAF) diluted 1:500 or 1:1000 and the corresponding goat anti-mouse IgG, HRP secondary antibody (KPL Laboratories). VSV antigen was detected using a VSV-specific polyclonal MHIAF (provided by R. Tesh, UTMB) diluted 1:1000 and the corresponding goat anti-mouse IgG, HRP secondary antibody (KPL Laboratories).

VIRUS AND VLP INFECTION

Cell monolayers plated into 48-well plates were infected with WNV VLP or WNV VLP^{HS} at a MOI of approximately 3. Seventy-five microliters of WNV VLP diluted in DMEM+++ (or DMEM+++ for mock infections) were added to each monolayer and gently rocked at 37C for 1 hour. The VLPs were then removed and 250µl

of fresh DMEM+++ was added and incubated for approximately 24 hrs at 37C prior to harvest. For studies involving inactivated WNV, the WNV VLPs were subjected to a 2 minute exposure under a ultra-violet fluorescent lamp (254 nm, 4W, 10cm), diluted in DMEM+++ and cell monolayers were infected with an approximate MOI of 3 as described above. For all WNV or WNV VLP infections, supernatants were collected at 24 hours post infection (hpi) unless otherwise indicated.

Sendai virus (SeV) and vesticular stomatitis virus (VSV) infections were carried out similarly. In short, cell monolayers were infected with either 40 hemagglutination units of SeV or VSV at an MOI of approximately 3 (based on Vero or MEF WT titrations) and incubated for approximately 1hr at 37C, rocking gently. The virus was removed following this incubation and 250ul of fresh DMEM+++ was added to each monolayer followed by an 8 hour incubation period at 37C.

POLY (I:C) TREATMENTS

For some experiments, poly(IC), a well-known dsRNA mimetic was used to stimulate IFN production in cell monolayers. In some cases, poly(IC) was added directly to the media, mimicking extracellular (or endosomal) stimulation. Other times, poly(IC) was transfected directly into the cell, mimicking intracellular (or cytoplasmic) stimulation.

For extracellular stimulation by poly(I:C), a stock of poly(IC) (EMD/Calbiochem) diluted to 2.5mg/ml in PBS was further diluted in DMEM+++ to 40ug/ml final concentration. Two hundred and fifty microliters of the diluted poly(IC) was added to cell monolayers plated in 48-well plates and incubated for approximately 24 hours at 37C prior to harvest.

For intracellular stimulation, poly(IC) was transfected into cell monolayers using DharmaFECT-1 transfection reagent. Essentially, 40ug of poly(IC) was mixed with Dharmacon Cell Culture Reagent (DCCR; Dharmacon) and 0.5ul DharmaFECT-1 (DF-1; Dharmacon) in a final volume of 50ul and incubated at RT for at least 10 minutes. The poly(IC)-DF-1 mixture (50ul total volume) was added to the cells and the volume was raised to 250ul with DMEM+++. The transfected cells were incubated at 37°C for approximately 24 hours prior to harvest.

SIRNA TRANSFECTIONS

Transfection of the siRNA was similar to transfection of poly(IC). The primary difference was siRNAs were introduced into cells by 'reverse' transfection, in which the cells in suspension were added to the siRNA-transfection reagent within the well. To this end, cells were transfected with either 30nM Dharmacon plus Smartpool PKR-specific siRNA (murine or human specific), 30nM (final concentrations, after cell addition) Dharmacon plus Smartpool non-targeting siRNA negative control (Dharmacon), RIG-I-specific siRNA (targeted to GGAAGAGGTGCAGTATATT; Ambion) or media alone using DF-1 transfection reagent (Dharmacon). The indicated siRNAs were first incubated with 0.5µl DF-1 diluted in 50µl DCCR for approximately 10 minutes at RT. The siRNA-DF-1 complexes were added to the well (50µl/1 cm^2 well) and 200µl of cells at a concentration of 10,000cells/ml was added to the complexes and incubated for approximately 72hrs at 37°C prior to stimulation with either poly(IC), WNV VLP, WNV or SeV as described above.

PKR INHIBITION

As an alternative method to silencing PKR with siRNAs, PKR kinase activity was inhibited using a PKR inhibitor. Monolayers of cells seeded into 48-well plates were

treated with 100uM PKR Inhibitor (PKR-I; CalBiochem) or 100uM PKR Inhibitor, Negative control (PKR-N; CalBiochem) diluted in DMEM+++ for 1hr at 37°C. Following incubation, the inhibitor (or negative control inhibitor) was removed and cells were stimulated with WNV VLP, WNV, SeV or poly(IC) as described above.

SDS-PAGE ELECTROPHORESIS AND WESTERN BLOT ANALYSIS

Monolayers of treated cells plated in 48-well plates were harvested in 75ul of lysis buffer (300mM NaCl, 50mM Tris-HCl pH 7.68) and frozen at -20°C. In some cases, particularly when examining phosphorylation status, 100nM Calyculin A (Upstate Cell Signaling Solutions), a phosphatase inhibitor, was added to the lysis buffer to prevent protein dephosphorylation. The monolayers were then thawed and transferred to microcentrifuge tubes by gently scraping the well with a pipette tip. The harvested cell lysates were centrifuged for 10 minutes at 13,200g at 4°C to remove cellular debris. The supernatants were transferred to a fresh tube and stored at -20°C.

Protein concentrations for the cell lysates were determined by D_C protein assay (BioRad) as per the manufacturer's protocol. A known concentration of bovine serum albumin (BSA) was used to generate a standard curve. Equal amounts of protein (2ug) were diluted in 1X NuPAGE LDS buffer (Invitrogen) supplemented with 50mM DL-Dithiothreitol (Gold Biotechnology, Inc), heated at 60°C for 10 minutes and loaded onto a NuPAGE 4-12% Bis-Tris gel (Invitrogen). Following resolution, the proteins were transferred onto an Immobilon PVDF membrane (0.45μm pore size; Millipore) and blocked with 5% instant nonfat milk (Nestle) hydrated in PBS containing 0.1% Tween-20 (Sigma) for at least one hour. The blocked membranes were probed with at least one of the following monoclonal or polyclonal antibodies diluted in the blocking buffer: monoclonal anti-β-actin (Sigma), rabbit anti-PKR (D-20; Santa Cruz), rabbit anti-

phospho-PKR threonine 451 (Cell Signaling Technologies), rabbit anti-phospho-IRF3 Serine 396 (Cell Signaling Technologies), rabbit anti-phospho-eIF2-α Serine 51 (Cell Signaling) or rabbit anti-eIF2-α (Cell Signaling Technologies). The membranes were washed 3 times with PBS containing 0.05% Tween-20 and incubated with either HRP-conjugated goat anti-mouse IgG (KPL) or HRP-conjugated goat anti-rabbit IgG (KPL) secondary antibodies. HRP-decorated protein bands were visualized using ECL plus Western Blotting Detection System (Amersham) and the signal was collected on Kodak X-ray film**.** β-actin staining was used as a loading control for all Western blot analyses performed.

IMMUNOFLUORESCENCE ASSAY (IFA)

Monolayers of WT and PKR-null MEF cells, plated onto chamber slides, were infected with WNV VLP and incubated for 24 hours at 37°C as described above. Thirty minutes prior to harvest, the cell monolayers were treated with 50ng/ml phorbol 12-myristate 12-acetone (PMA) for 30 minutes at 37°C. Following this incubation, the cell monolayers were washed 2 times with PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature and then washed 2 times with PBS. In some cases, the fixed cells were stored at 4°C in 10mM glycine. For permeabilization, the cells were incubated with PBS containing 0.1% Triton-X-100 for 10 minutes and then blocked in blocking buffer (2% bovine serum albumin, 5% normal horse serum, 10mM glycine in PBS) for 30 minutes. The fixed monolayers were probed using the following monoclonal and polyclonal antibodies: anti-WNV MHIAF polyclonal, anti-NF-κB p65 (Santa Cruz), Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) and Alexa Fluor 568 goat anti-mouse IgG (Invitrogen). Following secondary antibody incubation, the cells were incubated with DAPI (500ng/ml) for nuclear counterstaining and mounted using VectaShield (Vector

Laboratories). Stained cells were analyzed with a 1.0 Zeiss LSM 510 UV META Laser Scanning Confocal Microscope at the UTMB Infectious Disease and Toxicology Optical Imaging Core Facility.

IFN BIOASSAY

Supernatants harvested from treated cells were clarified and serially diluted in DMEM supplemented with 1% FBS, 1% antibiotics and 1mM HEPES side-by-side with either a human IFN-α standard (NIAID) or a murine IFN-β standard (NIAID). The murine IFN-β standard was produced from mouse L cells and the human IFN-α standard was produced from FS-4 human foreskin fibroblast cells. Both standards were acquired through the NIAID Reference Reagent Repository operated by either Braton Biotech, Inc. (human IFN-α) or KamTek, Inc. (murine IFN-β). Either WT MEF-G cells or Huh7 cells, depending on the IFN source, were treated with the IFN standard or supernatant dilutions for approximately 24hrs then infected with a firefly luciferase (FLuc)-expressing WNV VLP [WNV FLuc VLP; (132)]. Twenty-four hours post infection, FLuc expression was assayed by lysing the cell monolayers with a FLuc substrate as previously described (132). IFN concentration (U/ml) of each sample was determined by plotting the dilution giving 50% inhibition of WNV VLP FLuc activity and multiplying this value by the number of units of the IFN standard (human or mouse) that produced 50% inhibition in side-by-side assays. The limits of detection for these assays were approximately 2 and 5 U/ml of human and mouse IFN, respectively. Units of activity were expressed in terms of U/mg of protein in the treated monolayers as determined by protein assay (described above).

STATISTICAL ANALYSIS

A Student's t test was used to determine significant differences for these studies.

Results

IFN-STIMULATED PKR IS NOT CRITICAL FOR PROTECTION AGAINST WNV INFECTION

To determine whether PKR serves as an important IFN-stimulated gene (ISG) for IFN-mediated induction of an antiviral state, cells with WT or PKR-null genotypes were pretreated with IFN and tested for their ability to support WNV infection. Briefly, WT MEF-G cells, PKR-null MEF cells, STAT1 null MEF cells and PKR-RNaseL double null MEF cells were treated with varying concentrations of mouse IFN-β for approximately 24 hours and then infected with approximately 50 foci formation units (ffu) of WNV and assayed for IFN-induced inhibition of WNV infection. As expected, STAT1 null MEF cells, which are deficient in the IFN signaling pathway (117), did not demonstrate any IFN-stimulated inhibition of WNV infection; however, WT MEF-G cells showed 50% inhibition of WNV infection with as little as 10 units (U)/ml of IFN-β. Although PKR-null MEF cells showed a modest impairment in IFN-stimulated inhibition of WNV infection (50% inhibition of WNV infection at approximately 40 U/ml), PKR-RNaseL double null MEF cells were indistinguishable from WT MEF-G cells (Figure 2.3A). Since it seemed unlikely that RNaseL deletion would make PKR-null cells respond to IFN better, this suggested that the difference observed between the two PKR-null cell lines was due primarily to differences in how these two immortalized, clonally-derived cell lines responded to IFN treatment and that this difference was independent of the PKR genotype.

To further probe the function of PKR in establishing an anti-WNV environment, we examined levels of phosphorylated eIF2α, a functional target of PKR antiviral activity, in WNV- or VSV-infected WT MEF-G cells. Since PKR did not appear to be

critical for the induction of an IFN-stimulated antiviral response, it was expected that one of the hallmarks of the antiviral activities of PKR, the phosphorylation of eIF2α, would not occur following WNV infection. As expected, unlike VSV, WNV VLP infection did not induce eIF2α phosphorylation, (Figure 2.3B), consistent with reports that flavivirus infections do not result in a shutoff of host protein synthesis.

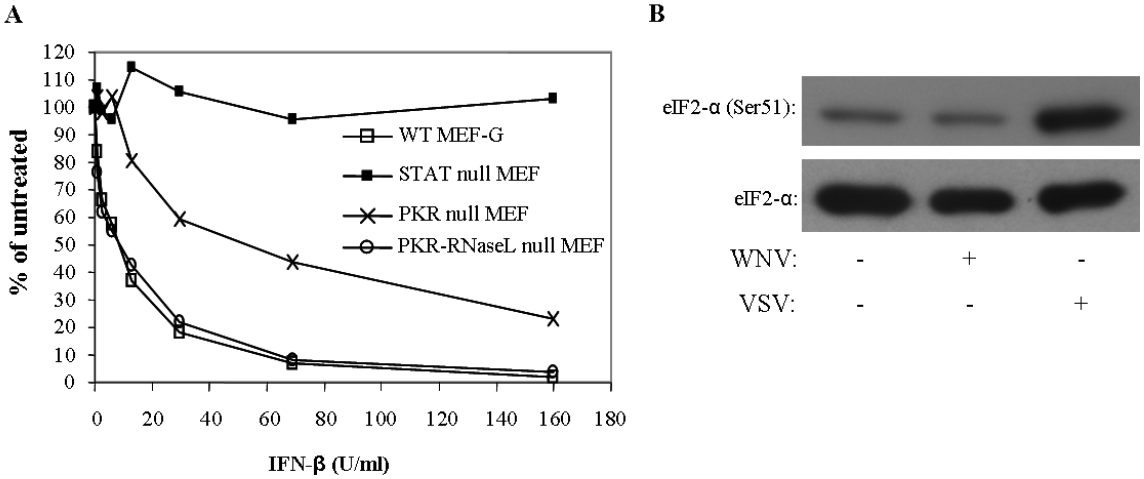


FIGURE 2.3: MEF CELLS LACKING PKR HAVE NO DEFECT IN ESTABLISHING AN IFN-MEDIATED ANTI-WNV ENVIRONMENT. (A) Monolayers of cells were treated with the indicated concentrations of IFN-β for approximately 16-24 hours, infected with approximately 50 ffu of WNV and assayed for foci production. Values are shown as the percent of foci present compared to infected, untreated cells. (B) WT MEF cell monolayers were infected with WNV VLP^{HS} (MOI = 3) or VSV (MOI = 3) for 24 or 8 hours, respectively. Cell lysates were probed for levels of phosphorylated eIF2α and total eIF2α.

WNV GROWS MORE EFFICIENTLY IN PKR-NULL MEF CELLS COMPARED TO WT MEF CELLS AT EARLY TIMES POST INFECTION

To evaluate the effects of PKR on WNV infection, WNV growth/release curves were performed on both PKR-null MEF cells and WT MEF-G cells. Because PKR-null MEF cell line and the WT MEF-G cell line demonstrate different susceptibilities to WNV infection, the WNV stock was titered on each individual cell line and, to insure equal infection rates between the two cell lines, cell monolayers were infected with similar

multiplicities of infection (MOI) based on the ffus calculated for each individual cell line. With low MOIs, (MOI = 0.1), at 24 and 48 hpi (hours post infection) WNV grew to higher titers in PKR-null MEF cells compared to WT MEF-G cells (Figure 2.4A). However, by 72 hpi PKR-null and WT MEF-G cells had similar WNV titers. There were no observable differences in either total number of cells or number of WNV-infected cells and by 48 hpi, both cell lines demonstrated 100% infection (Figure 2.4B). Interestingly, at a higher MOI (MOI = 1), the WNV titers from PKR-null and WT MEF-G cells were indistinguishable (data not shown), suggesting that the WT MEF cells are more capable of preventing viral spread, not necessarily viral infection or replication.

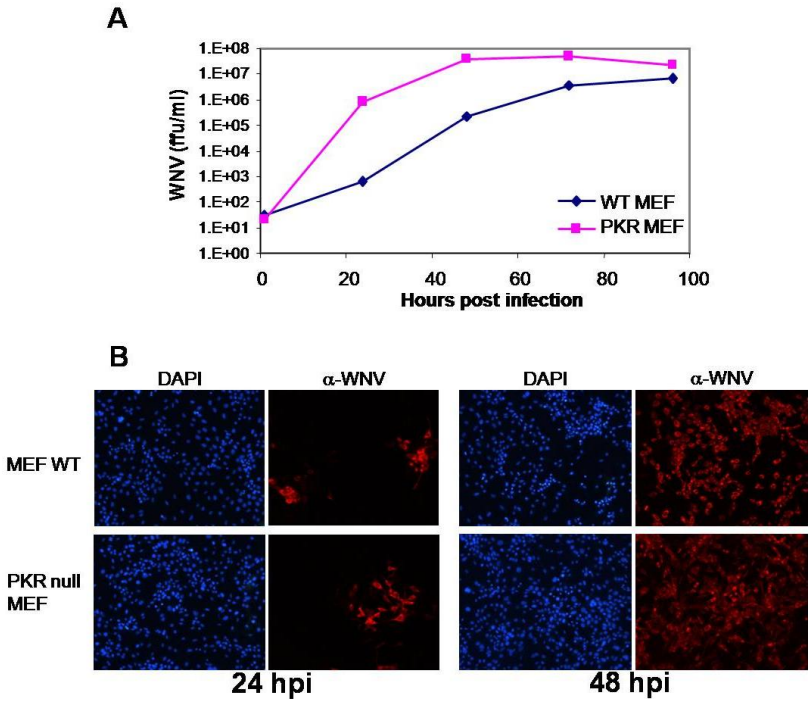


FIGURE 2.4: WNV GROWS TO LOWER TITERS IN PKR KNOCKOUT MEF CELLS. Monolayers of WT and PKR-null MEF cells were infected with WNV (MOI = 0.1) and supernatants were harvested at the indicated times. The cell supernatants were then titrated on Vero cells as described in the Materials and Methods. Panel A indicates the viral titers present at each timepoint and Panel B shows monolayers of infected cells probed with a polyclonal anti-WNV antibody.

MEF CELLS LACKING PKR DEMONSTRATE REDUCED IFN SYNTHESIS FOLLOWING INTRACELLULAR POLY(IC) STIMULATION AND WNV INFECTION

Since PKR did not appear to be serving as an IFN-induced effector molecule, we wanted to investigate the reason WNV grew to higher titers in cells lacking PKR compared to WT cells early in infection. Early work in the PKR field had demonstrated a possible role for this protein in IFN induction in response to dsRNA treatment (19, 114, 360, 542, 625). It is possible that the PKR-deficient MEF cells are unable to control WNV infection and/or spread due to a decrease in their ability to produce IFN and induce the expression of critical antiviral effector molecules. Thus, to examine whether our PKR-null cells showed any impairment in dsRNA-induced IFN production, WT, PKR-null or PKR-RNaseL double null MEF cells were treated with poly(IC) (pIC), a dsRNA mimetic, either extracellularly (pIC was added directly to the media; Ex-pIC) or intracellularly (pIC was transfected into the cells; Tx-pIC). The two cell lines that lacked PKR showed a marked reduction in Tx-pIC-induced IFN production compared to WT MEF-G cells, indicating a possible role for PKR in the recognition of dsRNA intracellularly. There was little, or no, induction of IFN following Ex-pIC treatment in any of the cell lines tested, suggesting that only the intracellular (cytoplasmic) recognition of dsRNA is important in these cells (Figure 2.5A).

To determine whether PKR is involved in the synthesis of IFN following WNV infection, WT MEF, PKR-null MEF and PKR-RNaseL double null MEF cells were infected with WNV and assayed for IFN production. IFN bioassays revealed that PKR-null and PKR-RNaseL double null MEF cells had dramatically lower levels of WNV-induced IFN compared to WT MEF-G cells (Figure 2.5B), suggesting that, in MEF cells, PKR is important for IFN production.

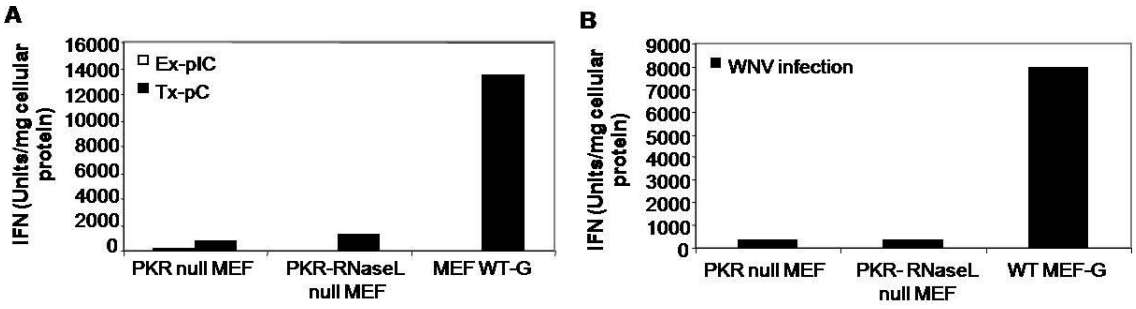


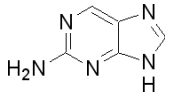
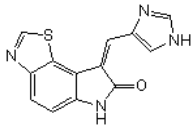
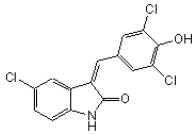
FIGURE 2.5: CELLS LACKING PKR PRODUCE LESS IFN COMPARED TO WT CELLS. (A) pIC was either transfected into the cytoplasm (Tx-pIC) or added directly to the media (Ex-pIC) of WT MEF, PKR-null MEF and PKR-RNaseL null MEF cells and, 24hr post-stimulation, assayed for IFN production by bioassay (see Materials and Methods). (B). WT MEF, PKR-null MEF and PKR-RNaseL double null MEF cells were infected with WNV at an MOI of approximately 3. Twenty-four hours post infection, the cell supernatants were assayed for IFN production by IFN bioassay. IFN units were determined by side-by-side analysis of an IFN standard and normalized to milligrams of total cellular protein. Graphs shown are representative graphs from two independent experiments.

As mentioned previously, there seemed to be some variation in how the PKR-null and PKR-RNaseL double null MEFs responded to IFN; therefore, it was necessary to ensure that that lower levels of IFN observed were due, in fact, to a specific role of PKR and not defects in these two clonally-derived cell lines. Two different methodologies were performed to corroborate the data from the knockout cell lines: chemical inhibition of PKR activity or post-translational gene silencing of PKR mRNAs (using small interfering RNA (siRNA) technology).

Although 2-aminopurine is widely accepted as a PKR inhibitor, in initial studies we were unable to demonstrate an inhibition of PKR autophosphorylation at the threonine 451 phosphorylation site (data not shown), a site critical for enzymatic activity of PKR (467). Therefore, we utilized a different inhibitor, an imidizolo-oxindole compound (PKR-I), that has been shown to be a potent inhibitor of PKR, presumably by interfering

with the ATP-binding site on the protein (245). As an added control, cells were also treated with a PKR inhibitor, negative control (PKR-N), a closely related oxindole compound demonstrated to have no anti-PKR activity (Table 1) (245).

TABLE 2.1: PKR INHIBITORS.

Compound Name	Structure	IC_{50}	Additional information
2-aminopurine		10mM (230)	Known inhibitor of multiple protein kinases, including PKR
PKR-Inhibitor (PKR-I): imidizolo-oxindole compound		100nM	Potent, ATP-binding site directed inhibitor of PKR; inhibits PKR autophosphorylation at IC_{50}=210nM (245)
PKR-Inhibitor, Negative Control (PKR-N): oxindole compound		> 100μM	Ineffective at blocking PKR autophosphorylation (245)

PKR MEDIATES POLY(IC)-INDUCED IFN PRODUCTION IN HUMAN AND MURINE CELL LINES

To test the effect of the PKR inhibitor on dsRNA-induced IFN production, WT MEF-G and a second WT MEF cell line derived from a different source, WT MEF-D cells, were treated with 100μM PKR-I or PKR-N and stimulated with Ex-pIC or Tx-pIC. In both cell types, treatment with PKR-I resulted in a significant decrease in Tx-pIC-induced IFN production compared to PKR-N treatment (Figure 2.6A). Consistent with the observations in Figure 2.3A, Ex-pIC stimulation in both WT MEF-G and WT MEF-D resulted in little or no IFN production, regardless of the treatment. These data indicate an important role for PKR in dsRNA stimulation of IFN synthesis in these cell lines.

PKR is a known ISG and, thus, expression can be induced by IFN. It was therefore necessary to eliminate the possibility that the abrogation of IFN production observed in the PKR-deficient MEF cells was due to differences in IFN signaling

downstream via the IFN receptor. To examine the effect of PKR on IFN induction in the absence of any endogenous IFN stimulation, we utilized STAT1 null MEF. Although IFN is still capable of interacting with its receptor in these cells, the absence of STAT1 prevents further downstream signaling; thus, these cells are unable to respond to IFN stimulation (117). Following treatment with PKR-I, the STAT1 null MEF cells demonstrated impairment in Tx-pIC-induced IFN production similar to that observed in the WT MEF cell lines (Figure 2.6A), indicating that the PKR-dependent abrogation of IFN induction is not a factor of any endogenous IFN signaling feedback loop.

To further support the observation that PKR is involved in p(IC)-induced IFN production and to demonstrate a more wide-spread requirement for PKR, similar studies were performed in three different human cell lines: MRC-5, a human fetal lung fibroblast cell line; A549 cells, a lung epithelial cell line; and Hec1B, a uterine epithelial cell line. All three of these cells lines were shown to be permissive for WNV, and produced IFN in response to WNV infection (data not shown). The Hec1B cells served as a particularly useful cell line for these studies, as these cells are relatively unresponsive to IFN stimulation. Although their signaling pathway is intact, the IFN receptors present on the surface of Hec1B cells bind IFN inefficiently, resulting in impaired IFN signaling (150, 568). These cells were utilized as a human equivalent to the STAT1 null MEF cells, allowing us to examine PKR-dependent IFN production in the absence of endogenous IFN signaling.

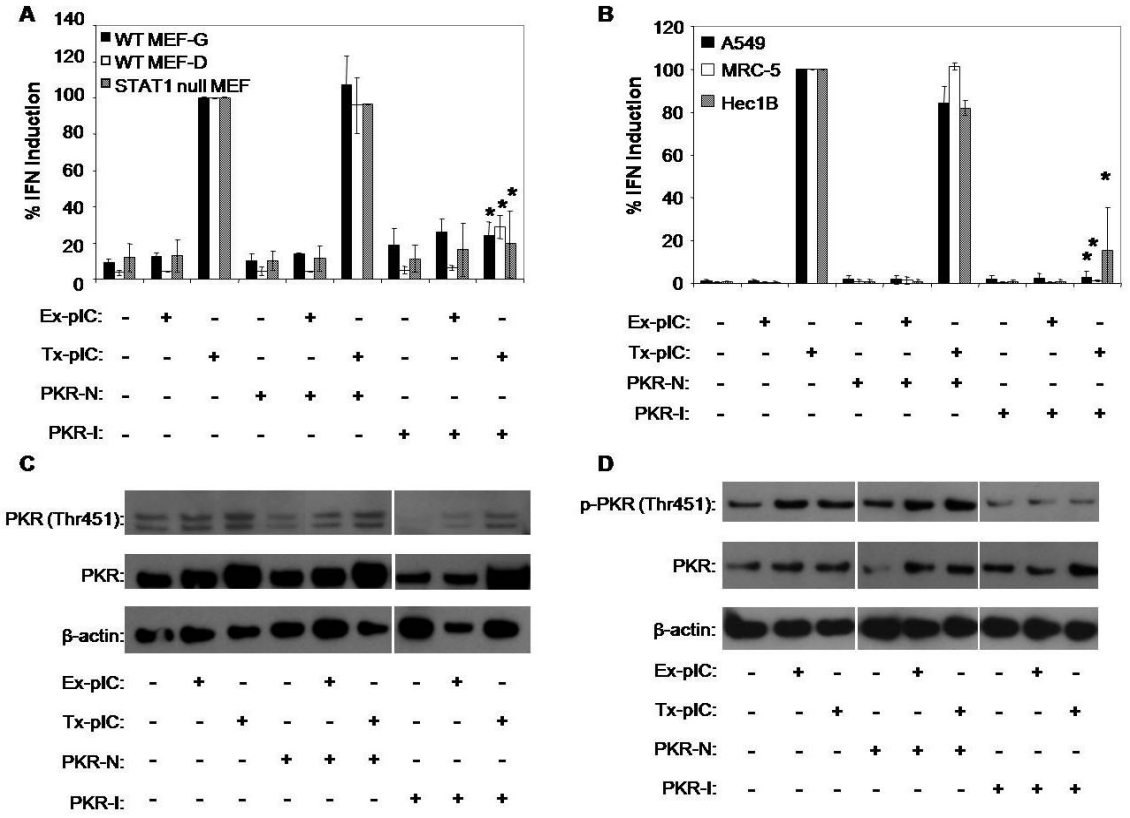


FIGURE 2.6: INHIBITION OF PKR ACTIVITY ABROGATES DSRNA-INDUCED IFN PRODUCTION. Monolayers of (A) MEFs or (B) human cell lines were treated with PKR-N or PKR-I and stimulated with Ex-pIC or Tx-pIC for approximately 24hrs. The cell supernatants were harvested and assayed for IFN production via IFN bioassay. IFN concentrations were determined by side-by-side analysis of IFN standard and values were normalized to total cellular protein. Values are shown as % IFN induction compared to mock-treated, Tx-pIC -stimulated samples. Error bars represent standard deviations between experiments and * denotes statistical significance ($p < 0.05$). (C) Western blot showing the levels of the phospho-Thr451 form of PKR, total PKR and β-actin in lysates of MEF WT-G cells and (D) A549 cells treated with the indicated compounds and exposed to pIC by the indicated methods.

In support of the data obtained from the MEF cell lines (Figure 2.4A), none of the human cell lines responded well to Ex-pIC, with all three showing little or no induction of IFN (Figure 2.6B). However, cells stimulated with Tx-pIC showed a robust

stimulation in either mock- or PKR-N-treated cells; however, there was a significant abrogation in IFN production in cells treated with PKR-I (Figure 2.6B). The data from the human cell lines is in line with the data from the MEF knockout cell lines and the PKR-I-treated WT MEF cell lines, supporting our conclusion that PKR is a key component of cytoplasmic dsRNA recognition.

Confirmation of PKR inhibition was determined by probing whole cell lysates for levels of PKR phosphorylated at the threonine 451 position, a site critical for kinase activity (467). Western blot analysis was performed on both mouse and human cell lysates treated with PKR-I. In all cell lines, mouse (Figure 2.6C) and human (Figure 2.6D) the expression of phospho-451 PKR was reduced to levels below either mock- or PKR-N-treated cells, regardless of the type of pIC stimulation. There was, however, a slight increase in phospho-451 PKR levels in cells transfected with pIC, suggesting that cytoplasmic dsRNA does induce PKR activation in the presence of PKR-I. This could be due to either the incomplete effectiveness of the PKR inhibitor or the presence of alternate PRRs. Interestingly, when probed for total PKR levels, we saw a strong induction following Tx-pIC, indicative of IFN induction (Figure 2.6C, 6D). Additionally, there appeared to be a slight increase in PKR levels following stimulation with Ex-pIC, indicating a limited induction of this gene through dsRNA-stimulation of a cell surface PRR. This suggests that that there is some IFN produced in these cells, undetectable in our bioassay, which induced the expression of ISGs such as PKR. Induction of PKR protein expression was not observed in either STAT1 null MEF cells or Hec1B cells (data not shown), confirming their inability to respond to IFN stimulation.

PKR IS A CRITICAL COMPONENT OF WNV-INDUCED IFN PRODUCTION

All of the data presented thus far implicate PKR as a key modulator of dsRNA-induced IFN synthesis. Additionally, we demonstrated that MEFs lacking PKR produce much less IFN following WNV VLP infection compared to WT MEFs (Figure 2.5B). Therefore, to further investigate the hypothesis that PKR is directly involved in WNV-induced IFN production, the cell lines utilized thus far were subjected to either PKR-I treatment or PKR siRNA transfection and then infected with WNV VLPs.

Chemical Inhibition of PKR blocks WNV-induced INF synthesis

To follow up on our observation that PKR was involved in IFN induction, WT MEF-G, WT-MEF-D and STAT1 null MEF cells were treated with 100μM PKR-N or PKR-I for 1 hour. However, instead of pIC treatment, the cells were infected with either WNV VLPs (MOI = 3). WNV VLPs, which have infectious properties nearly indistinguishable from WNV (272, 504) infect cells and initiate genome replication but are unable to produce any viral progeny. Utilizing these VLPs allows us to avoid any potentially confounding effects from secondary replication cycles obtained from WNV infection. In these studies, a special WNV VLP, VLP^{HS} was utilized. WNV VLP^{HS} carries the same genome as WNV VLP but it is packaged in a cell lines encoding an E protein with an HS-binding mutation (See Materials and Methods). In addition to WNV VLP^{HS} infection, the cells were also infected with SeV which induces IFN synthesis via the RIG-I/MAVS signaling pathway (260, 311, 377).

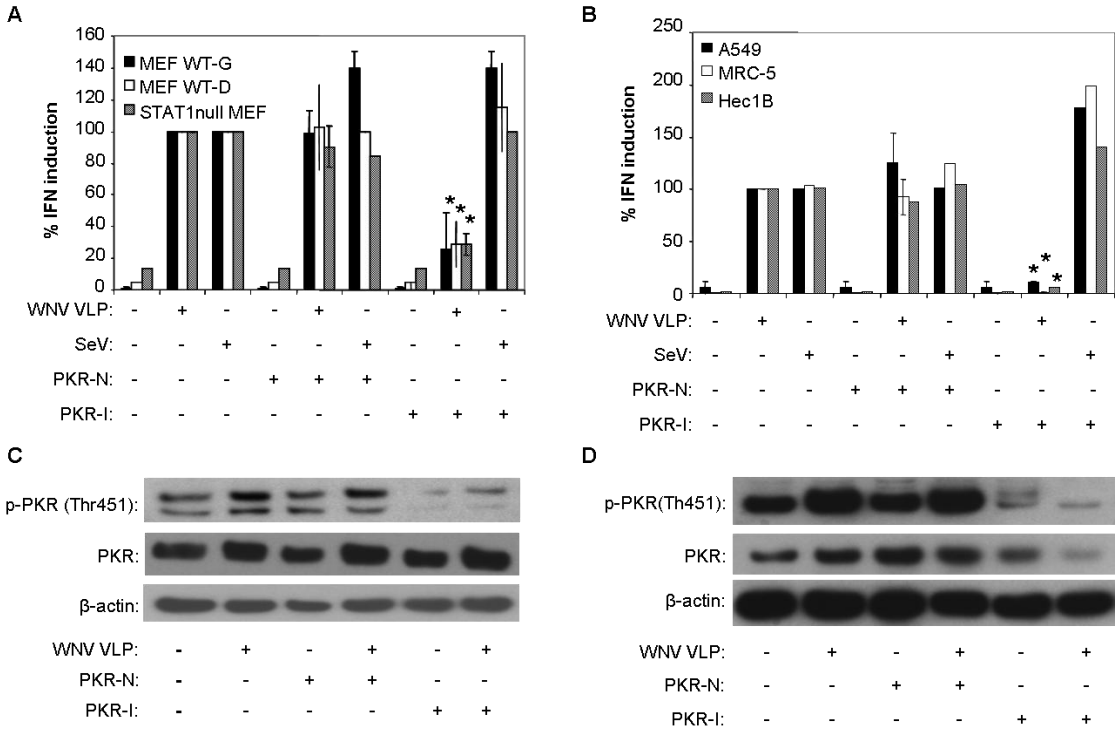


FIGURE 2.7: WNV-INDUCED IFN PRODUCTION IS BLOCKED FOLLOWING INHIBITION OF PKR ACTIVITY. Monolayers of (A) mouse cell lines or (B) human cell lines were treated with PKR-N or PKR-I and infected with WNV VLP^{HS} (MOI = 3) or SeV. Supernatants were harvested 24hr or 8hr post infection, respectively and IFN levels were measured as described. Values are shown as % IFN induction compared to mock-treated and WNV VLP^{HS}- or SeV-infected samples. Error bars represent standard deviation between experiments and * represent statistical significance ($p < 0.05$) compared to WNV VLP^{HS}-infected, PKR-N-treated cells. Western blot analysis of (C) WT MEF-G and (D) A549 cell lysates showing levels of p-PKR (Thr541) and β-actin following the indicated treatments.

Monolayers of WT MEF-G, WT MEF-D and STAT1 null MEF cells treated with either PKR-N or PKR-I were infected with WNV VLP^{HS} or SeV and incubated for approximately 24 or 8 hours, respectively. Analysis of the supernatants indicated that, although mock- and PKR-N-treated cells showed a robust IFN response to WNV infection, all three cell lines treated with PKR-I produced significantly less IFN (Figure

2.7A). As expected, SeV-induced IFN production was not affected by PKR-I treatment, indicating that the inhibitor treatment was not having any general, nonspecific effects on IFN induction. To determine whether this effect would be seen in the human cell lines, as well, A549, MRC-5 and Hec1B cells were treated with PKR-N and PKR-I and infected with WNV VLP-838^{HS} or SeV as described above. Supernatants harvested from these treated cells indicated that PKR-I treatment significantly inhibited WNV-induced, but not SeV-induced, IFN production in all three cell lines (Figure 2.7B). Incubation with UV-inactivated WNV VLP^{HS} did not induce IFN production (data not shown), consistent with previous reports indicating that WNV replication is necessary for viral recognition in cell culture (145).

Cell lysates harvested from treated cells indicated that the PKR inhibitor was effective in blocking autophosphorylation of PKR in both WT MEF-G and A549 cell lines (Figure 2.7C, 7D). Additionally, there was an increase in activated PKR following WNV infection in these cells, indicating that WNV VLP infection activates PKR. Analysis of cell lysates from the other cell lines was consistent with these observations (data not shown). In WT MEF-G cells, there was also a WNV-induced stimulation of total PKR levels (Figure 2.7C), indicative of IFN stimulation. However, this stimulation was very slight in the mock- and PKR-N-treated A549 cells; however, there was no WNV VLP^{HS}-induced stimulation of PKR expression in A549 cells treated with PKR-I. In fact, the PKR-I-treated cells demonstrated lower levels of PKR compared to mock- and PKR-N-treated cells (Figure 2.7D). This is different from the MEF cell lines and may be due to a lack of WNV-induced IFN produced in PKR-I-treated cells (which showed a near complete inhibition of IFN synthesis).

The apparent decrease in PKR expression following PKR-I treatment is a puzzling point. One possible explanation is that phosphorylated PKR can regulate the expression of PKR, so the reduction in levels of phosphorylated PKR could result in a reduction of PKR. However, this is not seen in any of the other cell types and it is not thought that this is a result of the PKR-I treatment.

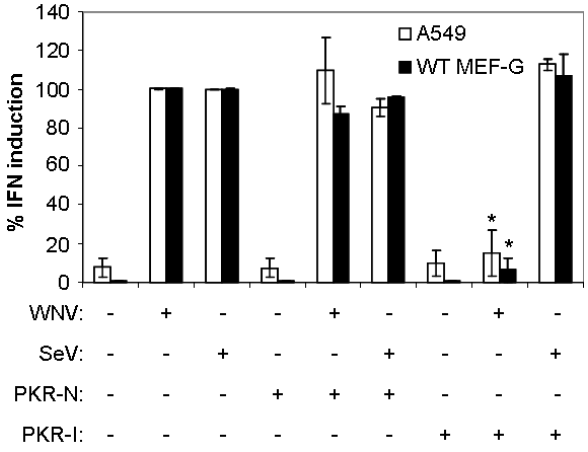


FIGURE 2.8: INHIBITING PKR ACTIVITY INHIBITS LIVE VIRUS-INDUCED IFN. Monolayers of WT MEF-G and A549 cells were treated with PKR-N or PKR-I and infected with WNV (MOI = 3) or SeV. Supernatants were assayed for IFN production as described. Values are shown as % IFN induction and error bars represent standard deviation between experiments. The asterisk represents statistical significant ($p < 0.05$) compared to mock-treated, WNV- or SeV-infected samples

Although WNV VLP have similar infective characteristics to WNV, it was important to determine whether PKR is involved in 'live' WNV-induced IFN production. To this end, WT MEF-G and A549 cells were treated with PKR-N or PKR-I and infected with either SeV or WNV at an MOI of approximately 3. Consistent with our WNV VLP^{HS} studies, treatment with PKR-I resulted in a significant abrogation of IFN production compared to mock- or PKR-N-treated cells in both WT MEF-G and A549 cells (Figure 2.8). SeV-induced IFN production was not affected by the inhibition of

PKR. Together with the WNV VLP data, this indicates a clear and important role of PKR for inducing IFN expression following WNV infection and that, as expected, VLP mimic live virus in IFN induction.

Post-transcriptional gene silencing of PKR mRNA

Post translational silencing of the PKR gene was utilized as a secondary methodology to examine PKR's role in the induction of IFN. SiRNAs are small (generally < 25 base pairs), segments of double-stranded RNA complimentary to an mRNA sequence in a target gene. Transfecting cells with siRNA results in the degradation of the target mRNA and, thus, silencing of the mRNA product (this will be reviewed in more detail in Chapter 4; (62, 121)). This technology allows us to 'knockdown' (KD) PKR expression without the problems associated with clonally-derived knockout cells which may have other abnormalities.

Our first step in applying siRNA technology to our system was to determine whether silencing PKR expression in the mouse and human cell lines resulted in an impairment of WNV-induced IFN production similar to that observed in the knockout cell lines or chemical inhibition of PKR. Each of the three MEF cell lines, WT MEF-G, WT MEF-D and STAT1 null MEF, were transfected with a PKR-specific siRNA or a non-targeting negative control (NEG) siRNA. Following a 72hr incubation with the siRNA—to allow sufficient KD—the cells were infected with either WNV VLP^{HS} or SeV. As with treatment with PKR-I, silencing PKR in both the mouse and the human cell lines results in a significant reduction in WNV-induced IFN production compared to mock- or NEG siRNA transfected cells (Figure 2.9A, 9B). As expected, silencing PKR had no effect on SeV-induced IFN production.

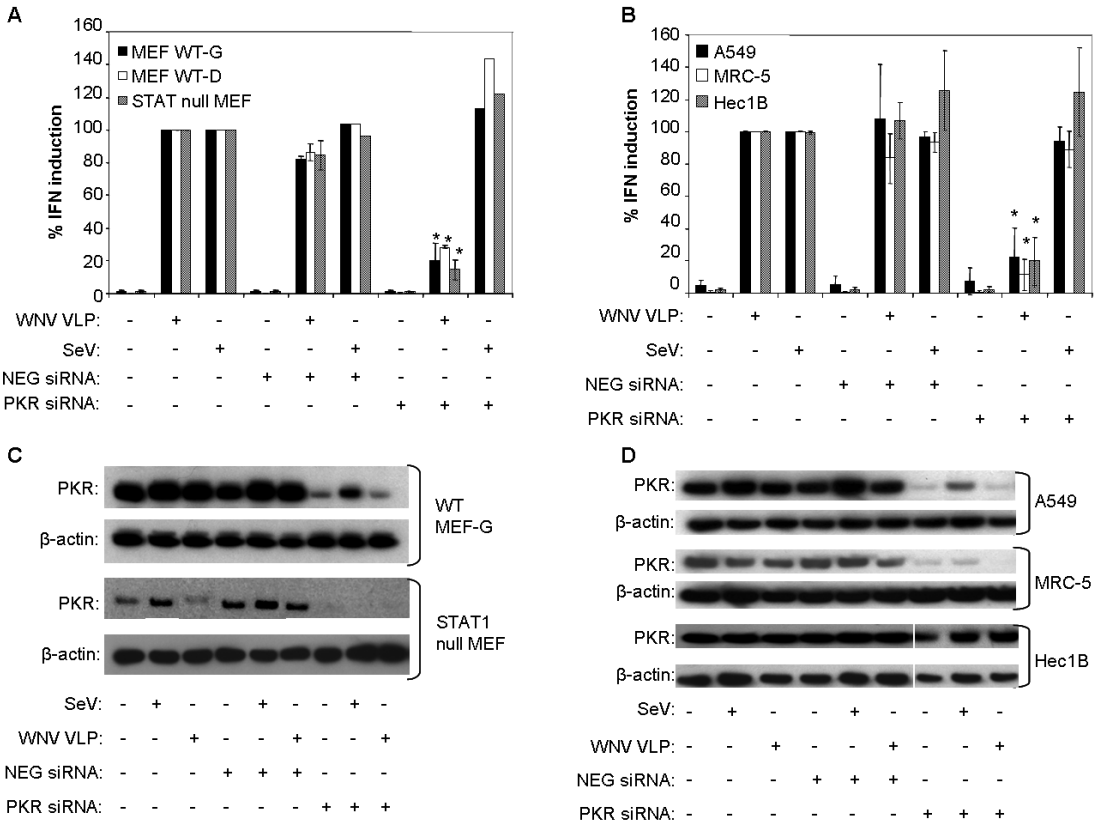


FIGURE 2.9: SILENCING PKR EXPRESSION ABROGATES IFN SYNTHESIS. Monolayers of (A) mouse cell lines or (B) human cell lines were transfected with a non-targeting (NEG) or a PKR-specific siRNA and infected with WNV VLP^{HS} (MOI = 3) or SeV. Supernatants were harvested 24hr or 8hr post infection, respectively, and IFN levels were measured as described. Values are shown as % IFN induction compared to mock-transfected and WNV VLP^{HS}- or SeV-infected samples. Error bars represent standard deviation between experiments and * represent statistical significance ($p < 0.05$) compared to WNV VLP^{HS}-infected, PKR-N-treated cells. Western blot analysis of (C) WT MEF-G and (D) A549 cell lysates showing levels of p-PKR (Thr541) and β-actin following the indicated treatments.

Analysis of PKR levels in the mouse and human cell lines demonstrated a marked reduction in PKR protein levels following transfection with PKR-specific siRNA compared to mock- or NEG siRNA-transfected cells (Figure 2.9C, D, respectively). In the WT MEF (Figure 2.9C), A549 and MRC-5 (Figure 2.9D) cell lines, infection with

WNV or SeV induced PKR expression, consistent with an autocrine ISG signaling response in these cells. Interestingly, there was also a slight increase in PKR expression in the PKR siRNA-treated WT MEF cells with SeV, suggesting stimulation of PKR in excess of the host cell's silencing machinery (Figure 2.9C). STAT1 null MEF cells (Figure 2.9C) and Hec1B cells (Figure 2.9D), on the other hand, did not demonstrate an increase in virus-induced PKR expression, consistent with the fact that these cells are unable to respond to IFN. Lysates from WT MEF-D cell demonstrated levels of PKR similar to those observed in the WT MEF-G cells (data not shown).

Although PKR appears to be quite important for WNV-induced IFN production in the cell lines tested, multiple studies have demonstrated a role for RIG-I-mediated signaling for establishing an antiviral response to WNV infection (143, 144). To determine how RIG-I-mediated signaling influences IFN production following WNV infection, A549, MRC-5 and Hec1B cells were transfected with RIG-I-specific siRNA in addition to PKR-specific and NEG siRNA and then infected with WNV VLP-838^{HS} or SeV. Consistent with the other studies, all three cell lines showed reduced WNV-induced, but not SeV-induced, IFN production following transfection with PKR-specific siRNA (Figure 2.10). Additionally, in two of the cell lines, A549 and MRC-5, transfection with RIG-I-specific siRNA, there was also a modest, but significant reduction in WNV-induced IFN production compared to mock- or NEG siRNA-treated, suggesting that RIG-I does play some role in WNV-induced IFN production. As expected, silencing of RIG-I resulted in a near complete loss in IFN synthesis in SeV-infected cells (Figure 2.10), consistent with reports that host recognition of SeV is RIG-I-dependent (260). Interestingly, silencing RIG-I in Hec1B cells had no effect on WNV-induced IFN synthesis. This suggests that either RIG-I signaling is not important for IFN production in

these cells or, perhaps, RIG-I-mediated IFN production in these cells is dependent upon IFN signaling, as RIG-I expression can be induced by IFN (244, 370). The later hypothesis, however, still needs to be tested.

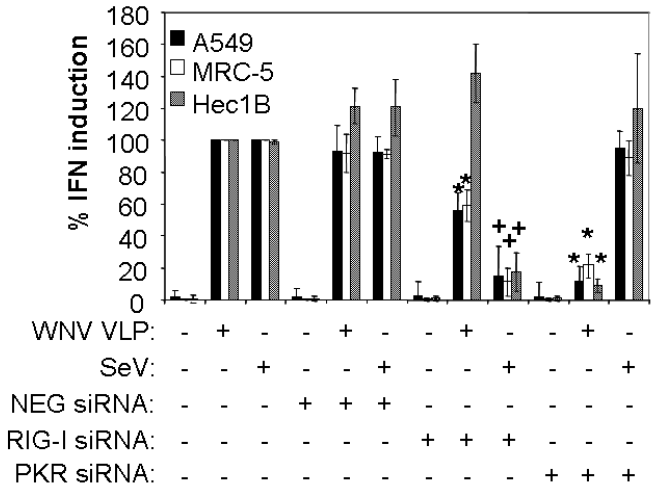


FIGURE 2.10: SILENCING RIG-I RESULTS IN A MODEST DEFECT IN WNV-INDUCED IFN PRODUCTION. Monolayers of cells were transfected with NEG, PKR-specific or RIG-I-specific siRNA and infected with WNV VLP^{HS} (MOI =3) or SeV. Cell supernatants were assayed for IFN production as described. Error bars represent standard deviation between experiments. The asterisk represent significance ($p < 0.05$) compared to mock-transfected, WNV-infected samples and a plus represents significance ($p < 0.05$) compared to mock-transfected, SeV-infected samples.

WNV-INDUCED NF-κB ACTIVATION IS IMPAIRED IN CELLS LACKING PKR

Many reports have indicated that PKR may be involved in the activation of NKκB (161, 298, 299, 619). Thus, to determine if this was the mechanism behind PKR-dependent IFN induction during WNV infection, we set out to determine whether NFκB activation was impaired in any way in cell lines lacking PKR. The status of NFκB in WT MEF-G and PKR-null MEF cells was examined by two methods: degradation of IκBα (Figure 2.11) and nuclear translocation of NFκB (Figure 2.12). Monolayers of WT MEF-G and PKR-null MEF cells plated in chamber slides were mock- or WNV VLP-838^{HS}-

infected (MOI = 5). Approximately 30 minutes prior to harvest, the cells were treated with 50ng/ml or 500ng/ml of PMA (phorbol 12-myristate 12-acetone), a known activator of NFκB (295). Monolayers were then either lysed or fixed in 4% paraformaldehyde. Cell lysates analyzed for IκBα indicate that, in WT MEF-G cells, WNV, and PMA, induced IκBα degradation at both 16 and 24 hours post infection. However, in PKR-null MEF cells, WNV-induced IκBα degradation appeared to be disrupted, without affecting PMA-induced degradation (Figure 2.11).

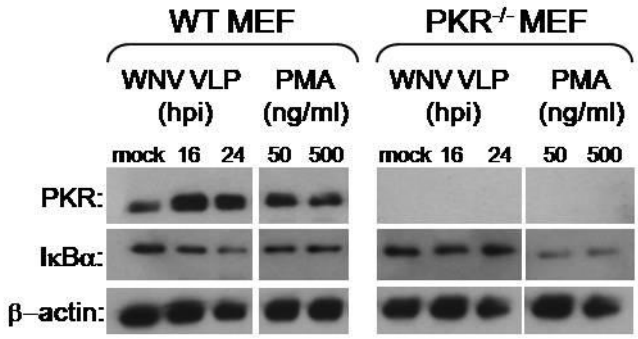


FIGURE 2.11: PKR IS IMPORTANT FOR WNV-INDUCED IκBα DEGRADATION. WT MEF-G and PKR-null MEF cells were mock- or WNV VLP-infected for either 16 or 24 hours. Approximately 30 minutes prior to harvest, selected wells of mock infected cells were treated with 50ng/ml or 500ng/ml PMA. Treated cell monolayers were harvested and assayed by Western blot analysis (Materials and Methods) for total levels of IκBα. Parallel blots prepared from the same samples were used to show levels of PKR and β-actin (loading control).

To confirm the activation status of NFκB, we looked directly at the nuclear translocation of NFκB in both WT MEF-G and PKR-null MEF cells. In WT MEF-G cells, WNV VLP infection resulted in the nuclear translocation in most infected cells; however, in PKR-null MEF cells, WNV VLP infection induced NFκB translocation at much lower frequencies than WT MEF-G cells, suggesting a defect in NFκB activation (Figure 2.12). PMA treatment, on the other hand, induced translocation in both cell types, indicating that NFκB activation via PKR-independent mechanisms is not impaired in

PKR-null MEF cells. Taken together, the impairment in both IκBα degradation and NFκB nuclear translocation following WNV VLP infection in the PKR-null MEF cells suggests that PKR activation may, in fact, be involved in WNV-induced NFκB activation. Whether this PKR-mediated activation of NFκB is directly involved in IFN induction is something that needs to be investigated in more detail.

WNV INDUCES NF-κB ACTIVATION AND PKR PHOSPHORYLATION, BUT NOT IRF3 ACTIVATION, EARLY IN INFECTION

The inhibition of NFκB activation in the PKR-null MEF cells was an interesting observation. Our initial hypothesis was that PKR is important for early recognition of WNV and other PRRs, such as RIG-I, recognizes WNV later. This hypothesis is consistent with data indicating that IRF-3 phosphorylation and dimerization is not observed in WNV-infected cells until at least 20 hours post infection (145, 505). However, due to the large levels of IFN detected in cells at 24 hours post infection, it is likely that another pathway must be involved during the initial phases of infection. Our published data suggests that this alternative pathway involves the activity of PKR.

To determine the timecourse of PKR, IRF3 and NFκB activation, WT and PKR-null MEF cells were infected with WNV VLPs and then harvested, at the indicated times, for Western blot analysis (Figure 2.13). By 16 hours post infection, WNV induced IκBα degradation in WT MEF cells, followed by a subsequent decrease in IκBα levels at 20 and 24 hours post infection. In PKR-null MEF cells, however, although there was a slight decrease in IκBα levels at 16 hours post infection, it was not consistent throughout the course of infection. Interestingly, analysis of total PKR levels showed an induction at 16 hours post infection, indicative of endogenous IFN stimulation. Analysis of phosphorylated PKR and IRF3 on parallel blots shows that WNV VLP infection induces

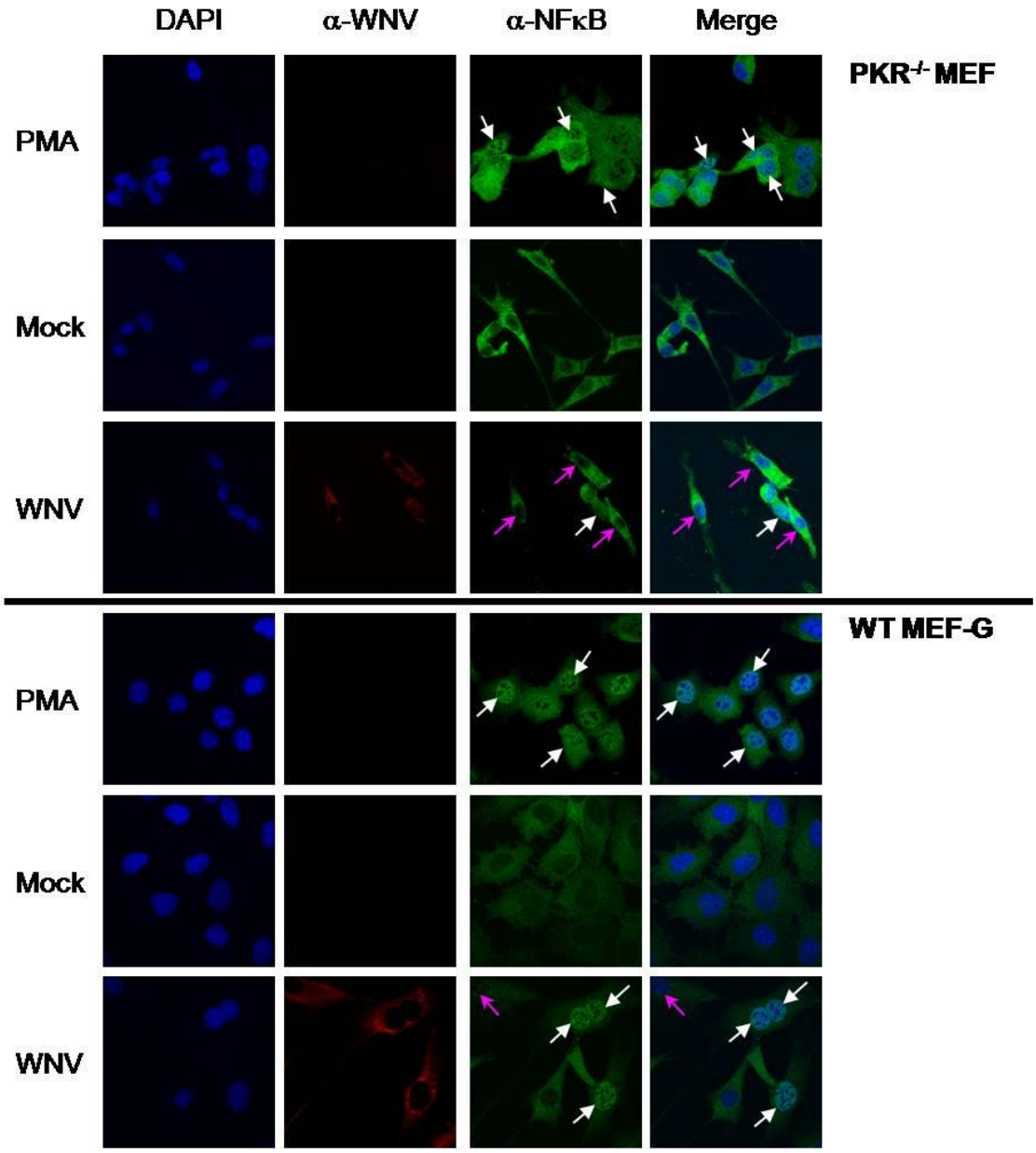


FIGURE 2.12: NFκB NUCLEAR TRANSLOCATION IS IMPAIRED IN PKR DEFICIENT MEF CELLS. WNV-induced NF-kB activation in WT MEF cells and PKR-null MEF cells. Twenty-four hours post infection with WNV VLPs or 30min post treatment with 50ng/ml PMA, cell monolayers were fixed and probed for WNV antigen and NFκB (p65) (Materials and Methods). The cells were also incubated with DAPI (500ng/ml) for nuclear counterstaining. White arrows indicate cells with NFκB located in the nucleus; red arrows represent WNV antigen-positive cells with NFκB localized to the cytoplasm.

PKR phosphorylation by 16 hours post infection, although there appears to be a slight increase at 12 hours post infection (Figure 2.13).Although at very low levels, IRF3 phosphorylation, is not observed until at least 20 hours post infection (Figure 2.11), after the first indication of IFN stimulation.

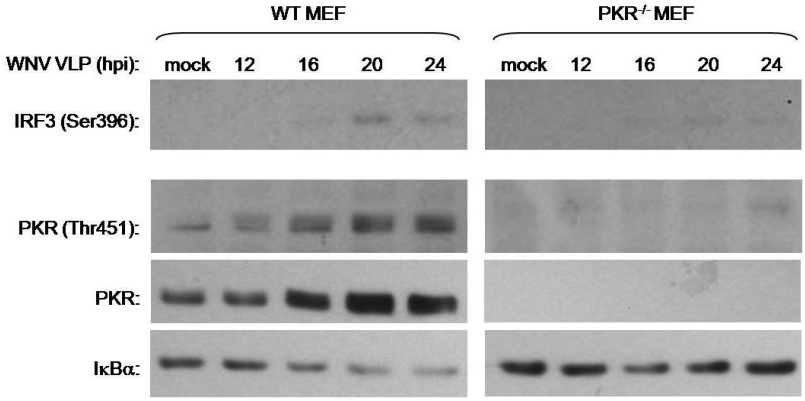


FIGURE 2.13: ACTIVATION OF PKR CORRELATES WITH IκBA DEGRADATION. Western blot analysis of lysates harvested from WNV VLP-infected (at the indicated times post infection) WT and PKR-null MEF cells. Monolayers of cells were harvested and assayed for PKR and IκBα. Parallel blots prepared from the same samples were prepared and assayed for phosho-PKR and phospho-IRF3 levels as described in the Materials and Methods.

Interestingly, there appears to be lower levels of phosphorylated IRF3 in the PKR-null MEF cells (Figure 2.13), although whether this is directly related to the PKR-null genotype is not known. It is possible that there is cross-talk between PKR and IRF3. The activation of PKR, however, appears to correlate with the activation of NFκB and the induction of ISG expression (PKR). Even though only low levels of phosphorylated IRF3 are present at the later timepoints, this finding does not prove that IRF3 is not active. The activity of IRF3 relative to our ability to detect phosphorylated forms is not known. Furthermore, the PKR-null MEF cells appear to have even lower levels of phosphorylated IRF3 as well as lower levels of IRF3 (detected by IRF3-specific Western blot analysis; results not shown). Despite this low level of IRF3, these PKR-null MEF

cells respond like WT cells to SeV, thus even low levels of IRF3 can promote the induction of IFN by this pathway.

Discussion

The most well characterized activity of PKR is its phosphorylation of eIF2α in response to dsRNA activation. This results in a translational shutoff in the infected cell. PKR is an ISG and its induction and inhibition of translation of viral RNA has been shown to be a critical aspect of the antiviral response to a number of viruses. Mice deficient in PKR displayed increased mortality to ECMV infection (624) and PKR antiviral activity was shown to be important for controlling VSV infection (19). Cells derived from PKR-null mice were more sensitive to VSV-induced apoptosis compared to WT cells and PKR-null mice were more susceptible to VSV infection compared to WT mice (19). The importance of PKR for establishing an antiviral response is highlighted by the fact that many viruses have evolved mechanisms to interfere with PKR activity. Influenza virus NS1 protein has been shown to inhibit PKR activation/activity, presumably by binding to dsRNA, the ligand of PKR (341). The importance of this block is demonstrated in mutant Influenza viruses which are unable to block the activation of PKR. These mutant viruses showed defects in virus protein synthesis, consistent with an increase in eIF2α phosphorylation (195). HCV is able to block PKR activity, as well. HCV NS5A protein was shown to directly bind the catalytic domain of PKR, blocking its activity (152). Sub-genomic replicons of HCV have also been shown to replicate to higher levels in PKR-null MEF cell lines (69).

In vitro studies with DENV showed that PKR was not essential for the development of an IFN-induced antiviral environment (109). This observation was

similar to what we observed, as we saw no significant impairment in IFN-β-mediated inhibition of WNV infection in cell lines lacking PKR, although an impairment has been observed in some types of primary neuronal cells (486). WNV infection does not induce eIF2α phosphorylation, consistent with the observation that flaviviruses do not usually block translation. Interestingly, a different group showed that eIF2α was transiently phosphorylated in response to WNV infection in neuronal cells. The effect of eIF2α phosphorylation on viral translation was not examined (375). The differences between our observations and their observations could be due to the different cell types, MEF cells versus neuronal cells. Additionally, it is possible that eIF2α is phosphorylated transiently and was missed in our single timepoint assay. Mice lacking both PKR and RNase L were more susceptible to WNV infection compared to wild-type mice (486). These animals exhibited increased viremia and viral loads in the peripheral tissues, earlier entry into the brain and higher viral titers in the central nervous system than wild type mice (486). Based on these studies, authors concluded that PKR acted as an ISG to establish an antiviral state; however, our studies argue that PKR is, instead, serving in a PRR role.

Over the last few years, much attention has been paid to TLR3, TLR7/8 and RIG-I/mda-5 signaling. Great strides have been made to understand the complex signaling pathways involved in IFN induction and the antiviral response. Many early studies had implicated PKR as a key modulator of IFN production (360, 558, 625) and, although most of the more recent work has focused on the IFN-inducible properties of PKR, it is still important to determine how PKR fits into the other PRR pathways (or if it serves as a PRR in and of itself).

In this chapter, we have investigated the means by which the cell recognizes WNV infection and induces the expression of IFN, a cytokine critical for controlling

infection. Our preliminary experiments indicated that PKR may be an important modulator of WNV infection, as WNV grew to higher titers in PKR-null MEFs. Because multiple reports have shown that PKR is important for inducing IFN expression in response to pIC (dsRNA) (114, 625) and VSV infection (360, 558), PKR-mediated IFN induction during WNV infection was investigated. Three different experimental methods clearly demonstrated that PKR plays a role in WNV-induced IFN production. First, MEF cells deficient in PKR generated lower levels of IFN compared to WT MEF cells following challenge with WNV VLPs. Secondly, chemical inhibition of PKR activity resulted in impaired IFN production in response to intracellular pIC or WNV VLP infection. This same inhibitor had no effect on SeV-induced IFN production, indicating that the inhibitor did not have a non-specific effect on IFN gene expression. Finally, transfection of cells with PKR-specific siRNAs significantly impaired both intracellular pIC-induced and WNV VLP-induced IFN production, but had no effect on SeV-induced IFN production. Consistent with the cytoplasmic localization of PKR, extracellular treatment of cells with pIC had little or no effect on IFN induction. Additionally, consistent with previous reports (145), UV-inactivated WNV VLPs failed to induce IFN, indicating that WNV replication is required for IFN induction in these cells.

As discussed in the introduction to this chapter, both TLR3 and RIG-I/mda-5 have been shown to be important for mediating flavivirus infections. RIG-I and/or mda-5 are involved in the recognition of both JEV (70, 260) and DENV (334). In HeLa cells, WNV genome expression blocks IRF3 dimerization and nuclear translocation in response to extracellular pIC stimulation, suggesting a block in TLR3-mediated signaling. This block appears to inhibit IFN gene expression following stimulation with extracellular pIC (505). In another cell type, however, this block was not permanent, as IRF3 activation

was observed later in infection. Recent studies have indicated that either WNV E protein (15) or NS1 (587) may be involved in blocking TLR3-mediated activation of IRF3.

RIG-I has also been shown to be important for mediating the innate immune response to WNV infection. To directly address the role of RIG-I as a PRR, three human cell lines were transfected with RIG-I specific siRNAs. Silencing RIG-I gene expression partially blocked WNV VLP-induced IFN production in two of the three cell lines tested, although not to the same extent as seen following PKR gene silencing. Interestingly, the cell line that was not affected by RIG-I knockdown was also the cell line unresponsive to IFN. RIG-I is an IFN inducible gene, although the importance of IFN induction on RIG-I activity in these cell lines was not tested. Fredericksen et al. has reported that RIG-I is a key component of the innate immune response against WNV infection, as RIG-I-null MEF cells show a delay in the activation of ISG54 and ISG56 (143), two IRF3-stimulated antiviral molecules (178).

Further study has suggested that mda-5 may also be important in the activation of IRF3-mediated genes (144). Additionally, inhibition of IPS-1 signaling appears to have a detrimental effect on IRF3 activation, as WNV-infected cells transfected with HCV NS3/4A (which cleaves IPS-1) show a severe impairment in IRF3 nuclear localization and ISG56 induction (144). The defect in IRF3 activation following inhibition of IPS-1 activation was attributed to a role for mda-5 in sensing WNV infection; however, no direct evidence supports this hypothesis. Like PKR, mda-5 senses dsRNA; thus, mda-5, like PKR, could also play a role in recognizing WNV infection. However, loss of IFN induction following inhibition of PKR activity or following knockdown of PKR expression suggests that mda-5 alone is not sufficient for WNV-induced IFN production,

although determining whether PKR and mda-5 signaling pathways are connected may be of particular interest.

The difference observed between these studies and our own could be explained by the differences in the experimental methods used to examine the antiviral response. Fredericksen et al. examined levels of ISG54 and ISG56 as an indication of an effective antiviral response. These gene products are important ISGs, however, their expression is also driven by IRF3 transcriptional activation (178). When examining the role of PKR, however, we studied expression levels of IFN, which is driven by a promoter containing binding sites for multiple transcription factors, including those for IRFs and NFκB (359, 424). Evidence suggests that, although IRF3 is important for controlling WNV infection (101, 145), it may exert its antiviral effects in an IFN-independent fashion, as mice deficient for IRF3 do not have defects in the level of circulating IFN (48, 101).

The data presented here clearly demonstrate the importance of PKR for the induction of IFN following WNV infection. However, the question remains as to how PKR is inducing IFN. As mentioned in the introduction to this chapter, PKR has been linked to a variety of signaling pathways. PKR activity has been shown to activate NFκB by inducing the phosphorylation of IκBα (160, 298, 619). It is possible that PKR, induced by WNV, phosphorylates IκBα, permitting NFκB to translocate into the nucleus and activate the transcription of IFN-β. WNV infection has been shown to activate NFκB (78); thus, we wanted to examine a possible link between PKR and NFκB in response to WNV infection. PKR-null MEF cells clearly show an abrogation of both WNV-induced IκBα degradation as well as WNV-induced NFκB nuclear translocation compared to WT MEF cells, suggesting that PKR may signal downstream to NFκB. However, we have not yet made a direct correlation between NFκB signaling and IFN induction.

One initial hypothesis to describe the different roles of RIG-I and PKR during WNV infection was that PKR is important for the early recognition of WNV infection, while RIG-I is involved at later times post infection. This hypothesis was supported by data from us and others indicating that IRF3 is not activated until at least 24 hours post infection (145, 505). However, at 24 hours post infection, we can detect high levels of IFN, suggesting another pathway may be active earlier. Timecourse infection studies indicate that, consistent with previous studies, IRF3 is activated approximately 24 hours post WNV VLP infection; however, IκBα degradation and PKR phosphorylation can be observed in the infected cells as early as 16 hours post infection. Additionally, PKR expression is induced in WT MEF cells by 16 hours post infection, indicative of endogenous IFN signaling (PKR is an ISG). It is very interesting that PKR-null MEF cells appear to have decreased levels of phosphorylated IRF3, as well. Despite the reduced levels of IRF3 detected, PKR-deficient mice do not appear to have any defect in their ability to produce IFN in response to SeV infection, indicating that, although low, the levels of IRF3 (both non-phosphorylated and Ser396-phosphorylated forms) present in these cells are sufficient to induce IFN transcription. However, our observation suggests there may be some crosstalk between the two signaling pathways. Perhaps RIG-I- or mda-5-mediated signaling is connected to PKR-mediated signaling. Our results indicating that silencing RIG-I (mda-5 has not been tested) results in a partial inhibition of WNV-induced IFN signaling may support this suggesting; however, more detailed studies will have to be done.

It is not known whether PKR serves directly as a PRR or as a downstream signaling molecule. There are several possibilities. PKR could, as described above, be directly responsible for detecting dsRNA and signal downstream via NFκB. However, if

the binding of other elements to the IFN-β promoter is required for proper induction, this cannot be the only explanation. The discovery that PKR interacts with TRAF proteins, particularly TRAF3 (416) and TRAF6 (248), is interesting. Both TRAF3 and TRAF6 are involved in signaling mediated by RIG-I and/or TLR3, which signal via IRF3. It is possible that PKR acts downstream of these PRRs. However, as mentioned, IRF3 is activated much later than PKR and NFκB following WNV infection, suggesting that PKR activity via these pathways may not be required.

Another possible role of PKR is through IRF1. IRF1 can bind to the PRD-I domain of the IFN-β promoter (440) and, in some cases, is involved in the induction of IFN-β (148, 371, 477, 549). HCV was shown to block the activation of IRF1, in addition to blocking the activity of PKR (26, 87, 257, 437). Mutations interfering with the PKR binding domain of NS5A prevented this block and resulted in the induction of IRF1 dependent antiviral genes and a reduction in HCV replication (437). Studies have shown that PKR is involved in IRF1 signaling, as mice deficient in PKR showed impaired IRF1 promoter activity (277) and the expression of the dominant negative form of PKR resulted in impaired IRF1-mediated NFκB activation (299). It is possible that the activation of PKR induces IRF1 activation, leading to the induction of IRF1 and/or NFκB-dependent IFN-β. However, this link is speculation, as the activation or activities of IRF1 has not been studied during WNV (or any other flavivirus) infection.

CHAPTER 3: PKR IS A CRITICAL COMPONENT OF IFN SYNTHESIS IN PRIMARY CELLS

Abstract

Sensing invading pathogens early is critical for establishing an effective defense. Many PRRs are responsible for recognizing (and responding) to viral infections, quickly creating an antiviral environment in the infected cell and the surrounding cells and tissues. Several PRRs have been shown to be important for establishing this antiviral response to WNV infection, including RIG-I, TLR3 and, as discussed in Chapter 2, PKR. To help confirm our observations on the important role of PKR in WNV recognition in the human and mouse cell lines, primary bone marrow derived dendritic cells (BMDCs) were harvested from mice and assayed for WNV-induced IFN production following PKR inhibition. Unsorted BMDCs treated with a chemical inhibitor of PKR produced significantly lower levels of WNV VLP-induced IFN compared to those treated with a negative control inhibitor. Additionally, CD11c+ DCs sorted from the BMDC populations showed a similar phenotype. Sorted CD11c+ DCs derived from IRF3-null mice were not deficient in the ability of WNV to induce IFN expression; however, when these cells were treated with the PKR inhibitor, that IFN induction was nearly completely ablated. Consistent with previous reports, SeV-induced IFN production was impaired in these IRF3-null DCs. Finally, the inhibitor studies were confirmed by harvesting BMDCs from PKR-null mice. Sorted CD11c+ populations of the BMDCs derived from these mice induced little or no IFN following WNV infection, although SeV infection induced levels were comparable to cells derived from WT mice.

Introduction

In Chapter 2, several pieces of data were presented indicating a key role for PKR during WNV-induced IFN production. As useful as cell lines are for delineating virus-cell interactions, there are problems associated with them. In fact, we observed distinct differences in the ability of several of the cell lines to respond to IFN stimulation (Figure 2.1). Therefore, it was important to assay the role of IFN in a more natural system. One system for studying flavivirus infections and the innate immune response are dendritic cells (DCs). These cells may be among the first cells which come into contact with flaviviruses and they are important immunologic sentinels, able to quickly recognize and respond to viral infections.

DCs are crucial for the innate and adaptive immune responses to infections. They are particularly important for recognizing pathogens and linking the innate and adaptive immune responses. DCs represent a diverse population of cells composed of many different subtypes which, although having many common features, are quite distinct. These DC subtypes can be categorized into two major groups plasmacytoid DCs (pDCs) and conventional DCs (cDCs) (380). cDC populations can also be differentiated based on their tissue localizations, such as skin DCs (Langerhans), mucosal-associated DCs, lymphoid tissue-associated DCs (spleen or thymus DCs) and interstitial tissue DCs (lung and liver DCs). Although it was initially suggested that DCs are all derived from a single myeloid precursor, more recent studies indicate that different DC populations can be generated along distinct developmental pathways originating from precursors from different hemopoietic lineages. Regardless of their origin, immature DCs are thought to reside in the periphery tissues of origin and, upon stimulation by pathogens, will produce cytokines and chemokines essential for the activation of an immune response and begin

migrating to the lymph nodes (576, 595). During migration, the DCs will up-regulate surface molecules, such as MHC class I or class II, and mature, allowing them to interact with a variety of immune cells, including T cells (574). The cDC, also referred to as myeloid-derived DCs (mDCs), are important for the capture, processing and presentation of antigens; pDCs, on the other hand, are important for the rapid induction of IFN-α (23, 65, 93, 138, 241) and are not usually associated with antigen presentation. Although the primary role of mDCs is antigen presentation to T cells, they do produce IFN in response to viral infection, albeit at lower levels than pDCs (93).

In mice, it is difficult and impractical to isolate specific subpopulations of DCs from specific tissues or blood. DCs are rare in tissues so yields from a single mouse are quite low. In fact, less than 60,000 Langerhans (420) can be isolated from the ear of one mouse, only between 1-10 x 10^5 DCs from a single spleen or thymus (571) and approximately 1 million DCs from the blood of a mouse (234). However, it was found that bone marrow precursors can be used to generate up to 1 x 10^8 DCs cells from the femurs of a single mouse (233, 346), providing a useful and relatively simple method to generate large quantities of mouse DCs. Depending on the stimulation, these bone marrow precursor cells can generate, in addition to mDC and pDC populations, B cells, polymorphonuclear neutrophiles and macrophages. (574). For mDC cells, culturing the precursor cells with granulocyte macrophage-colony stimulated factor (GM-CSF) is essential (574). However, pDC generation requires culturing the bone marrow precursors with Flt-3 ligand. The presence of GM-CSF actually appeared to block the differentiation of the bone marrow cells into pDC populations (163).

DCs have been shown to be important for flavivirus infections. Most flaviviruses are introduced into the host by the bite of an infected mosquito or tick. Initial infection

and replication is thought to occur in dendritic cells (DCs) present in the skin (Langerhans cells) (253, 597). Following infection, the Langerhans DCs migrate to the lymph nodes (253) where they can help stimulate the innate and adaptive immune responses. Although this is thought to be an important aspect of inducing an efficient immune response, no one has shown that these DCs are infected by WNV. Several flaviviruses, however, have been shown to productively infect other subsets of DCs (non-Langerhans), including DENV (301, 314, 361, 408), YFV (22, 107, 451) and WNV (103, 524). In fact, a specific lectin, DC-SIGN (DC specific ICAM3-grabbing non-integrin), expressed on DCs is thought to help mediate viral entry for both DENV (408) and WNV (103).

Interestingly, there are differences in the ability of mDCs and pDCs to respond to WNV infection. Although mDCs produce IFN in response to WNV infection, the levels are much lower than those produced by pDCs (524). Additionally, a productive WNV infection was not required for IFN induction in pDCs (no WNV antigen was ever detected in these cells); however, a productive WNV infection was essential for IFN induction in mDCs (524). The differences observed between these two cell types are likely dependent upon the use of different PRR signaling pathways. pDCs are prominent IFN-α producers (65) due to the constitutive expression of IRF7 (IRF7 is only expressed following IFN stimulation in other cell types) (93, 241). Inhibiting endosomal acidification ablated the ability of pDCs to produce IFN-α following WNV infection, suggesting an essential role for PRRs present within endosomes, such as TLR7/8 or TLR9 (524), which are involved in the activation of IRF7. The precise role of IRF7 in WNV-treated pDCs, however, has not been determined. mDCs, on the other hand, appear

to recognize WNV via a cytoplasmic PRR, presenting a possible role for PKR in these cells.

RATIONALE

In this chapter, the role of PKR *in vitro* was translated to an *ex vivo* model. Cell lines, although excellent tools for studying molecular biology, are not always indicative of an *in vivo* model. Thus, to begin to understand the role of PKR in a more biologically relevant setting, WNV-induced IFN induction was examined in bone marrow-derived dendritic cells following treatment with a PKR inhibitor or in cells derived from PKR deficient mice. It was *expected* that, consistent with *in vitro* observations, the inhibition of PKR activity or the absence of PKR expression would result in a severe impairment in WNV-induced IFN production in bone marrow-derived dendritic cells. Thus, we *hypothesized* that PKR, not IRF3, is critical for the production of IFN following WNV infection in myeloid-derived DCs.

Materials and Methods

MICE

C57BL/6J mice were provided by G. Milligan (UTMB). IRF3-null mice (48) and PKR-null mice (kindly provided by T Moran; Mount Sinai School of Medicine), both on C56BL/6J backgrounds, were maintained in a breeding colony at UTMB. All mice were housed in sterile microisolator cages under specific pathogen-free conditions in the AAALAC-approved UTMB animal facility. All procedures were conducted in accordance with the UTMB Institutional Animal Care and Use Committee.

PREPARATION OF BONE MARROW-DERIVED DENDRITIC CELLS

Mice between 4 and 12 weeks of age (depending on the experiment; all mice within an experiment were age-matched) were sacrificed and the femurs (and, in some cases, their tibias) from both legs were extracted and cleaned of as much muscular tissue as possible. Both ends of each bone were cut off and the marrow was flushed out with Hanks balanced salt solution (Sigma) using a 25 gauge needle and syringe. The media containing the bone marrow cells were passed through a 26-gauge needle to break up any large clumps of cells. The suspended cells were centrifuged at 1200 rpm for approximately 10 minutes and resuspended in 80 ml Iscove's modification of DMEM (IMDM) supplemented with 10% FBS, 1% NaPy, 1% atb, 20ug/ml gentamycin and 20ng/ml GM-CSF (eBioscience). The suspended cells were transferred to eight 100mm petri dishes (less than 2 x 10^6 leukocytes per dish) and incubated at 37°C. Three days and six days post harvest, an additional 5ml growth media supplemented with 20ng/ml GM-CSF was added to each dish. The cells in suspension were collected for use at eight days post harvest. Two populations of cells were utilized for the experiments: unsorted, BMDC populations or sorted, CD11c+ DC populations.

SEPARATION OF CD11C+ DENDRITIC CELLS

In some cases, CD11c+ DCs were separated by positive selection using MACS magnetic cell sorting (Miltenyi Biotec) as per manufacturer's protocol. Briefly, cells were centrifuged at 1200rpm for 10 minutes and then resuspended in 400µl of separation buffer (PBS pH 7.2, 0.5% bovine serum albumin, 2mM EDTA; 0.22µm filter sterilized). One hundred microliters of CD11c MicroBeads (Miltenyi Biotec) was added to the cells and then they were incubated at 4°C for 15 minutes. Following incubation, 2ml of separation buffer was added to the cells and mixed. The cells were centrifuged for 10

minutes at 1200rpm and then resuspended in 500μl separation buffer. Meanwhile, an LS MACS Column (Miltenyi Biotec) was placed into the magnetic field of a MACS Separator (Miltenyi Biotec) and rinsed one time with 3ml separation buffer. The cell suspension was added to the column, allowing the unlabeled cells to pass through. The column was then washed three times with 3ml separation buffer, collecting the unlabeled cells in a centrifuge tube. To recover the CD11c+ cells, the column was removed from the magnetic field and the labeled cells were flushed into a clean centrifuge tube using the plunger supplied with the column.

VIRUS AND VLPS UTILIZED

The initial infectivity studies were performed using both WNV VLP and WNV VLP^{HS} described in Chapter 2; however, since the DCs were much more susceptible to WNV VLP^{HS} (see Results), these VLPs were utilized for the remaining studies (See Appendix A). The SeV utilized for these studies was identical to the stock utilized in Chapter 2.

VLP AND VIRUS INFECTIONS

WNV VLP infections of BMDCs and sorted CD11c+ DCs were carried out as described in Chapter 2. It was difficult and impractical to titrate the WNV VLP stock on each of the dendritic cell preparations. Therefore, MOI calculations were based on WNV VLP titrations performed on Vero cells (titration described in Chapter 2). Because dendritic cells are much less sensitive to WNV and WNV VLPs compared to Vero cells (Results; (524)), unless otherwise stated, an MOI of approximately 150 was utilized for the *ex vivo* studies. Although not 100% of the cells were infected (20-30% were antigen

positive), this was the highest MOI we were able to achieve with the WNV VLP preparations available.

For WNV VLP infections, 100µl of undiluted VLP or DMEM+++ (mock infections) was added to the appropriate wells (96-well plate) and incubated for 24 hours at 37C. Since WNV VLPs cannot spread from cell-to-cell, they were not removed from the cell monolayer. For SeV infections, approximately 200 HA units of SeV was added to the appropriate wells, incubated for 1 hour at 37C and then the plates were centrifuged (1200 rpm, 5 min) and the virus was removed and replaced with 100µl fresh DMEM+++. Following harvest, Tritxon-X-100 was added to SeV-infected supernatants at a final concentration of 0.1% to inactivate any remaining virus.

For one experiment, the DCs were infected in suspension. In this case, 50,000 cells were added to 5ml polystyrene tubes and then centrifuged for 5 min at 1200rpm. The cells were resuspended in 200µl of the appropriate WNV VLP and incubated for 24 hours at 37°C, rocking gently throughout the entire incubation period.

IMMUNOFLUORESCENCE

To assay the susceptibility of DCs to WNV infection, 50,000 unsorted BMDCs were infected, in suspension, with either WNV VLP or WNV VLP^{HS} (DMEM+++ alone for mock-infection). Twenty-four hours post infection, the cells were centrifuged (1200rpm for 5 min), washed one time with DMEM+++ and then added to a Shandon double cytofunnel (Thermo Electron Corporation) and centrifuged onto glass slides (cytospin 500rpm for 5 min). The cells were fixed with 4% paraformaldehyde and IFA was performed as described in Chapter 2. The following antibodies were used to detect WNV antigen: anti-WNV MHIAF primary antibody and a goat anti-mouse IgG Alexa Fluor 568 secondary antibody.

FLUORESCENCE ACTIVATED CELL SORTING (FACS)

FACS analysis was performed on CD11c-sorted DCs harvested from WT mice. Following the separation of CD11c+ DCs, the cells were washed 2 times with plain IMDM, centrifuging 1200 rpm for 8 minutes. The cells were then washed 1 time with FACS media (IMDM supplemented with 1% atb, 10% FBS, 0.1% sodium azide). The cells were resusepended at 2 x 10^7 cell/ml and 50µl were added to a 96-well v-bottom plate (500,000 cells/well). The cells were incubated with anti-mouse CD16/CD32 (Fc Block; BD Pharmingen) for 30 minutes, on ice, in the dark and then centrifuged for 4 minutes at 1000 rpm (4°C). Cells were resuspended in 100µl FACS media and then incubated with the following fluorescent-tagged antibodies for 30 minutes, on ice, in the dark: anti-CD11c-PE (Phycoerythrin) (Miltinyi Biotec), anti-I-A/I-E-PE (MHC II) (BD Pharmingen), anti-CD80-FITC (Fluorescein) (BD Pharmingen) or anti-F4/80-PE (Caltag Laboratories). Control cells unstained or incubated with the proper isotype control for each of the antibodies listed above were also run along-side. Following the antibody incubation, the cells were washed 2 times with FACS media and resuspended in 200µl of 1% formaldehyde. The stained and fixed cells were transferred to Falcon 2054 tubes and an additional 200ml of 1% formaldehyde was added. The cells were sorted on a Becton-Dickinson FACSCanto instrument at the Flow Cytometry and Cell Sorting Core Facility at UTMB.

PKR INHIBITION

Dendritic cells were plated into 96 well plates at 1 x 10^5 cells/well. Plates were centrifuged for 5 minutes at 1200rpm to ensure all of the cells were at the bottom of the well. Similar to the treatments described in Chapter 2, growth media was carefully removed from the DC cell monolayer and 100µl of DMEM+++ containing 100nM PKR-I

or PKR-N (DMEM+++ alone for mock treatment) was added to the appropriate wells and incubated for 1 hour at 37°C. Following incubation, the cells were mock-, WNV- or SeV-infected as described above.

IFN-β ELISA ASSAY

IFN-β ELISA assays were done per manufacturer protocol (PBL Biomedical Laboratories). Briefly, cell supernatants were diluted 1:3 or 1:5 into the provided dilution buffer and 100µl of the diluted supernatants were added to the microtiter plate. Additionally, dilutions of the provided IFN-β standard were prepared and added to the microtiter plate to generate a standard curve. The plate was covered with a plate sealer and incubated in the dark for 1 hour at RT. Following incubation, the plate was washed 4 times with 1X wash solution (PBL Biomedical Laboratories). One hundred microliters of diluted antibody concentrate (PBL Biomedical Laboratories) was added to each well and then the plate was incubated, in the dark, for 1 hour at RT. The plate was then washed 4 times with 1X wash solution and then 100µl of the diluted HRP-conjugated secondary antibody (PBL Biomedical Laboratories) and incubated, in the dark, for 1 hour at RT. Finally, the plate was washed 4 times with 1X wash solution and incubated with 100µl tetramethyl-benzidine (TMB) substrate for 15 minutes at RT. The reaction was stopped by the addition of 100µl of stop solution and the absorbance was read at 450nm. IFN-β levels in the samples were determined from the curve generated from the dilutions of the IFN-β standard.

STATISTICAL ANALYSIS

A Student's t test was used to determine significant differences for these studies.

Results

As mentioned in the introduction, although the cell lines utilized in Chapter 2 provided a great deal of information regarding the role of PKR during WNV infection, the use of transformed, clonally derived cell lines is artificial. If the cells demonstrated differences in their ability to respond to IFN-β treatment (Figure 2.3), it is possible they would display other differences as well. Therefore, it was important for us to confirm the role of PKR in another model system. Due to the fact that DCs are important cells for both innate and adaptive immune responses and that these cells are likely among the first to come into contact with WNV during a natural infection, these cells seemed to be the best model system for studying the role of PKR during WNV-induced IFN production.

Although both mDCs and pDCs produce IFN following WNV infection, only mDCs are productively infected (524). However, since WNV infection is not required for IFN induction, it is thought that pDCs recognize WNV via PRRs expressed within endosomal compartments, such as TLR7 or TLR8 (524). These TLRs signal downstream through the IRF7 transcription factor, which are expressed constitutively only in these cells (IRF7 expression requires IFN stimulation in other cell types) (241). In mDCs, on the other hand, WNV-induced IFN production is thought to be mediated by cytoplasmic PRRs (524), suggesting a possible role for PKR in this cell type.

BONE MARROW-DERIVED DENDRITIC CELLS ARE SUSCEPTIBLE TO WNV INFECTION

To determine susceptibility of our DC preparations to WNV infection, 50,000 unsorted DC cells were placed in 5ml polystyrene tubes. We had previously observed that mouse cell lines are significantly more susceptible to infection with WNV engineered to bind heparan-sulfate (data not shown). Since DCs are naturally less susceptible to WNV infection compared to many other cell types (524), we wanted to determine whether using

heparan-binding WNV VLP (WNV VLP^{HS}) would improve infection. Therefore, unsorted DCs were infected, in suspension, with 200µl of either WNV VLP or WNV VLP^{HS} at an MOI of approximately 700 (based on Vero cell MOI) and then incubated for 24 hours. Following incubation, the cells were centrifuged onto glass slides and then fixed with 4% paraformaldehyde. WNV antigen could be detected in DCs infected with both WNV VLP and WNV VLP^{HS}; however, WNV VLP^{HS} infection resulted in significantly more WNV antigen-positive cells (~20-30%) than WNV VLP infection (< 1%) (Figure 3.1). Neither WNV VLP nor WNV VLP^{HS} resulted in 100% infection of DCs, even with a Vero-based MOI of 700. The low susceptibility of DCs to WNV infection is consistent with previous results from our (524) and other laboratories (103).

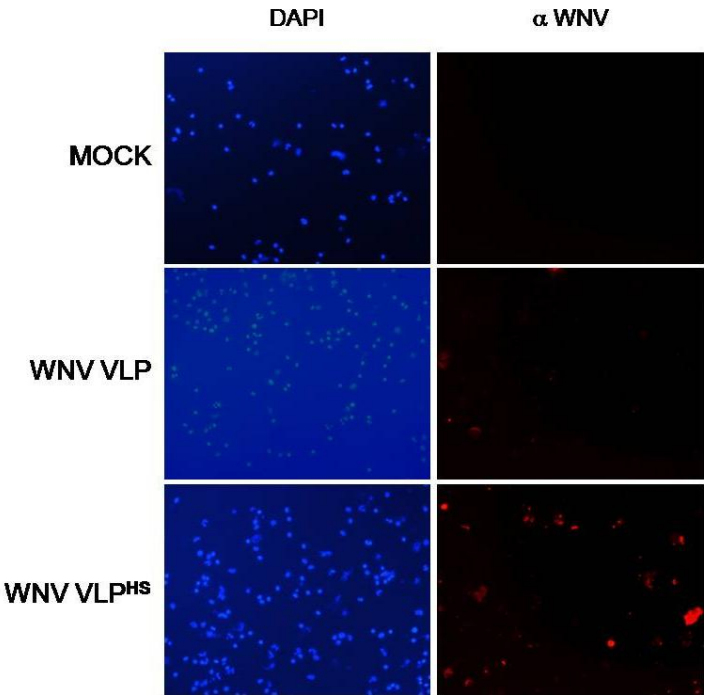


FIGURE 3.1: DENDRITIC CELLS ARE SUSCEPTIBLE TO WNV INFECTION. Unsorted GM-CSF-cultured dendritic cells were infected, in suspension, with WNV VLP or WNV VLPHS at a Vero MOI of 700. Twenty-four hours post infection the cells were centrifuged onto glass slides and assayed, by IFA (See Materials and Methods) for the presence of WNV antigen. Blue staining is cellular nuclei (DAPI) and red staining is WNV antigen.

This is a very low infection rate and could argue against the importance of these cells *in vivo*. However, these are cells infected *ex vivo* (removed from their natural environment and manipulated prior to infection), which could cause changes in their maturation level and, hence, their susceptibility to WNV infection. Furthermore, dendritic cells are thought to function as sensors of pathogen infection and, thus, it may not be required to have a large number of cells infected. Once infected, DCs could quickly become activated/mature, migrate into the lymph node and activate a large number of immune cells. Thus, a small number of infected cells could still have a large impact on the host's immune response against WNV infection.

INHIBITION OF PKR ACTIVITY RESULTS IN IMPAIRED IFN PRODUCTION IN BONE MARROW-DERIVED DENDRITIC CELLS

To assay the role of PKR in WNV-induced IFN production, bone marrow-derived lymphocytes were harvested from WT mice and cultured in GM-CSF to promote the generation of BMDCs. The unattached cells were collected eight days post harvest and the unsorted BMDC populations were plated into 96-well plates at 100,000 cells/well. The unsorted BMDCs were then treated with 100nM PKR-I, 100nM PKR-N or media alone for 1 hour at 37C and then infected with either WNV VLP^{HS} or SeV. Twenty-four hours post-infection, the supernatants were collected and assayed for IFN-β levels. Analysis indicated that PKR-I-treated BMDCs infected with WNV VLP^{HS} secreted significantly lower levels of IFN-β compared with PKR-N- or mock-treated BMDCs (Figure 3.2); however, SeV-induced IFN-β production was unaffected by PKR inhibition. These results are consistent with our previous results in both mouse and human cell lines

(See Chapter 2) and clearly suggest a role for PKR during WNV-induced IFN production in unsorted BMDC populations.

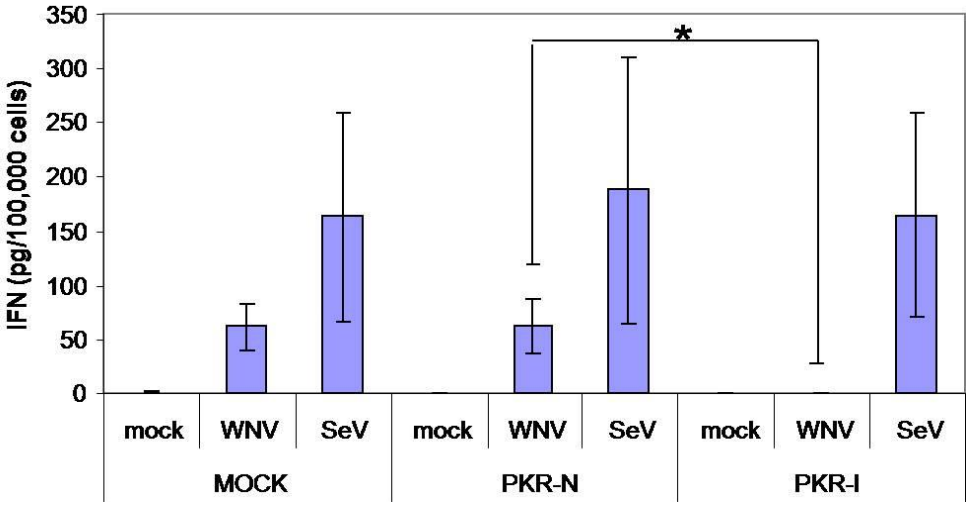


FIGURE 3.2: INHIBITION OF PKR IN DCS ABROGATES IFN-B PRODUCTION. IFN-β production in primary dendritic cells. Unsorted dendritic cells were plated at 100,000 cells/well, treated with PKR-N, PKR-I or media alone for approximately 1 hour and then infected with either WNV VLP^{HS} or SeV. Twenty-four hours post infection, cell supernatants were collected and assayed for IFN-β production by ELISA (See Materials and Methods). Error bars represent standard deviations from the mean between two independent experiments and the asterisk represents statistical significance ($p < 0.01$).

IFN PRODUCTION IN CD11C+ DENDRITIC CELLS IS INFLUENCED BY PKR, BUT NOT IRF3, ACTIVITY

As mentioned in Chapter 2, IRF3 has been implicated as an important signaling molecule for establishing an anti-WNV environment within the infected cell (143, 145). Additionally, siRNA targeted against RIG-I (which induces IFN production via an IRF-3-dependent pathway) resulted in a partial, but significant, reduction in WNV-induced IFN production in human cell lines (Figure 2.10). To investigate the role of IRF3 in WNV-induced IFN production in DCs, we harvested BMDCs from both WT and IRF3 knockout mice. In this case, to ensure we were looking at a population of DCs, the cells collected following the eight day culture in GM-CSF were positively selected for CD11c+

expression, a DC marker. Analysis of these sorted cells indicated that approximately 73% of the cells were CD11c+ (results not shown). Additionally, the sorted cells were positive for MHC class I (85%) and CD80 (50%), indicating these were mature mDCs (results not shown). The cells were negative for F4/80 (< 0.4%), a macrophage marker, indicating there was no macrophage contamination (results not shown).

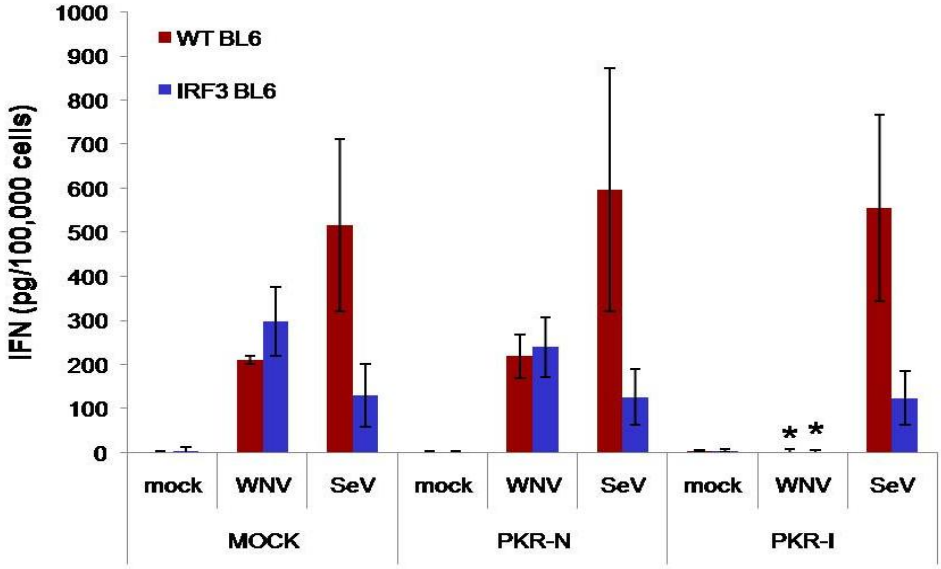


FIGURE 3.3: PKR, BUT NOT IRF3, IS INVOLVED IN WNV-INDUCED IFN-B PRODUCTION. IFN-β production in CD11c+ DCs derived from WT and IRF3 deficient mice. CD11c+ DCs derived from WT and IRF3 deficient mice were plated at 100,000 cells/well, treated with PKR-N, PKR-I or media alone for 1 hour and then infected with WNV VLP^{HS} or SeV. Cell supernatants were collected and assayed for IFN-β production by ELISA (See Materials and Methods). Error bars represent standard deviations from the mean between two independent experiments. Asterisks represent statistical significance ($p < 0.05$) compared to WNV VLP^{HS}-infected, PKR-N-treated cells.

These cells were treated with PKR-I or PKR-N and then infected with WNV VLP^{HS} or SeV and assayed, 24 hours post infection, for IFN-β production. Consistent with the previous experiment, sorted CD11c+ DCs derived from both WT and IRF3 knockout mice showed a marked reduction in WNV VLP-, but not SeV-, induced IFN-β production following PKR-I treatment compared to mock or PKR-N treatment (Figure

3.3). SeV-induced IFN-β production was impaired in the CD11c+ DCs derived from IRF3 knockout mice; this is consistent with multiple reports implicating RIG-I as a key PRR for SeV infection (311, 377). Interestingly, there was essentially no difference in WNV VLP-induced IFN-β production between WT and IRF3 CD11c+ DCs, suggesting that, in these cells, IRF3 is not critical for WNV-induced IFN production (Figure 3.3).

DENDRITIC CELLS DERIVED FROM MICE LACKING PKR ARE DEFECTIVE FOR WNV-INDUCED IFN PRODUCTION

To confirm the data obtained from treating DCs with a chemical inhibitor of PKR, DCs were harvested directly from PKR deficient mice. These PKR-null DCs, along with WT and IRF3-null DCs, were infected with WNV VLP^{HS} or SeV and incubated at 37C. Supernatants from the infected cells were collected at 12, 16, 20, 24 and 36 hours post infection and assayed for IFN-β production. Analysis showed a significant impairment in the ability of DCs derived from PKR deficient mice to generate IFN-β following WNV VLP^{HS} infection. These cells, however, had no problem producing IFN-β following SeV infection, indicating that there was no general defect in the ability of these knockout cells to produce IFN. In contrast, following WNV VLP infection, IFN-β levels in IRF3-null CD11c+ DCs were nearly identical to levels observed in WT DCs, indicating that IRF3 does not appear to contribute to WNV-induced IFN production in these cells. SeV-induced IFN production, however, was impaired in the IRF3-null CD11c+ DCs, consistent with our previous data. Interestingly, even at 36 hours post infection there was little IFN-β in supernatants collected from WNV VLP-infected PKR-null CD11c+ DCs. Similar results were observed at 48 hours post infection (data not shown) indicating that other recognition/signaling pathways are not able to compensate for the absence of PKR up to 48 hours post infection in these cells.

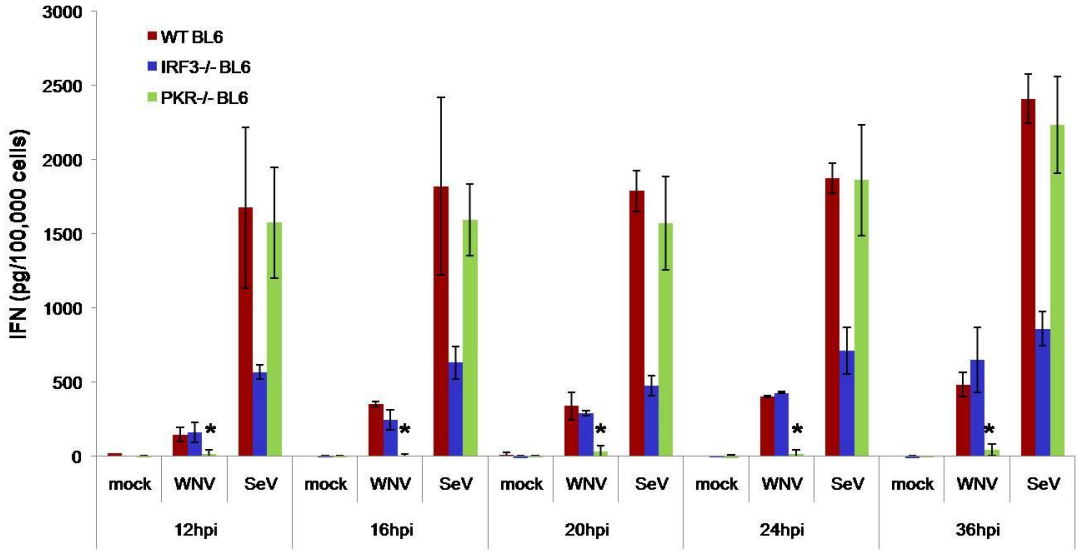


FIGURE 3.4: IFN-B PRODUCTION IS ABROGATED IN PKR-NULL CD11C+ CELLS. IFN-β production in DCs derived from WT, IRF3 deficient or PKR deficient mice. CD11c+ cells were plated at 100,000 cells/well and infected with either WNV VLP^{HS} or SeV. At the indicated times post infection, the supernatants were collected and assayed for IFN-β production by ELISA (See Materials and Methods). The graph shown is representative of two independent experiments. Error bars represent standard deviations from the mean between the replicates within the experiment. Asterisks represent statistical significance ($p < 0.05$) compared to WNV VLP^{HS}-infected WT DCs.

Discussion

Dendritic cells are very important for the induction of an effective immune response. Their ability to produce cytokines and chemokines upon viral infection as well as their ability to effectively present antigen to T cells provide an important link between innate and adaptive immunity. DCs are important for a variety of viral infections, and flaviviruses are certainly no exception. The success of the live attenuated YF-17D vaccine is thought to be due, in part, to the role of DCs (22, 451). The YF vaccine strain infects and activates DCs through the recognition of several membrane and endosomal PRRs, including TLR2, TLR7, TLR8 and TLR9, resulting in the induction of multiple

cytokines (451) and helping to drive an effective T cell response (22, 451). The association between DENV and DCs has been studied in great detail. DENV was actually shown to infect Langerhans (DCs present in the skin) (597), presumably resulting in their migration into the lymph nodes. Infection of DCs by DENV results in the induction of multiple cytokines and chemokines (75, 215).

Recently, WNV was shown to induce the expression of IFN in human DCs, although the pathways involved and the level of induction varied between mDC and pDC populations (524). The observation that IFN production in pDCs does not require WNV infection and that inhibitors of endosomal acidification blocked WNV-induced IFN production (524) suggested the use of PRRs that are expressed on the cellular membrane or within endosomal compartments. Combined with the fact that pDC constitutively express high levels of IRF7 (93, 241), it seemed likely that, in pDCs, WNV induces expression via an IRF7-dependent pathway. Although it is possible that PKR plays a role downstream of TLR7/8, we utilized mDCs, not pDCs, as an *ex vivo* model to confirm the role of PKR during WNV infection.

In these studies, we examined WNV-induced IFN production in mDCs following treatment with a PKR inhibitor or in cells derived from PKR-deficient mice. Results obtained from bone marrow derived dendritic cells are consistent with the previous studies in cell lines and strongly support a role for PKR during WNV-induced IFN production. CD11c+ DCs isolated from WT and IRF3-null mice demonstrated a severe impairment in WNV-induced IFN production following treatment with a PKR inhibitor. SeV induced lower levels of IFN in IRF3-null mice, consistent with multiple studies indicating the crucial role of RIG-I for the recognition of this virus (261, 377); however, PKR inhibitor treatment did not affect SeV-induced IFN production in either WT or

IRF3-null mDCs. Interestingly, the IRF3-null genotype had no apparent effect on WNV-induced IFN production; cells derived from WT and IRF3-null mice produced similar levels of IFN. This is not all-together unexpected; although IRF3-null mice appear to be more susceptible to WNV infection compared to WT mice (with higher viral loads in the periphery), this does not translate to a change in IFN levels (48, 101). When examined in more detail, Daffis et al. demonstrated that the role of IRF3 in WNV-induced IFN production is cell type specific, as primary cortical neurons, but not macrophages, showed significant differences in IFN levels following WNV infection (101). Thus, it is likely that IRF3 is not important in these mDCs. Consistent with the PKR inhibition studies, WNV-induced IFN production was nearly completely ablated in mDC cells derived from mice deficient in PKR. Although we hypothesized in earlier studies that PKR is important early in infection and IRF3 signaling is important late in infection, we did not see any increase in IFN production in PKR-null mDCs, even up to 48 hours post infection (results not shown). This is consistent with the lack of an effect in the IRF3-deficient cells, but suggests that, at least in these cells, the presence of IRF3 in the absence of PKR is not sufficient for IFN production, even at times when IRF3 is likely to be activated. Both IRF3 and NFκB (presumably activated by PKR) are required to bind to the IFN promoter to induce transcription; however, it is possible that other IRFs, such as IRF1, could replace IRF3, providing a redundant pathway and explaining the ability of IRF3-deficient BMDCs to induce IFN expression.

These data, along with preliminary data in PKR-null mice (results not shown), indicate that PKR is crucial for the expression of IFN following WNV infection. Although IRF3 deficiency alone was not sufficient to impair WNV-induced IFN synthesis, it would be interesting to examine where there is a connection between PKR

and IRF3. Previous studies in human mDCs indicate that WNV infection does, in fact, induce IRF3 phosphorylation and nuclear translocation (524). Based on this result and in light of the finding of reduced IRF3 phosphorylation in MEF cells deficient in PKR (Chapter 2) it is possible that there is cross-talk between PKR-mediated signaling and IRF3 activation. It is possible that, although the pathways may not be directly connected, the absence of PKR (or PKR activity) ablates the ability of IRF3 to become activated, thus preventing IRF3-mediated transcription from 'rescuing' IFN production in cells deficient in PKR (or in cells treated with PKR inhibitors). Perhaps IRF3 activity alone is not required for IFN production, but a combination of PKR and IRF3 activity is critical. This is speculation at this point and would have to be examined; however, this provides an interesting avenue of future study. Examining each of these pathways in more detail would do much to advance our understanding of how infected cells recognize WNV infection.

CHAPTER 4: IDENTIFYING HOST PROTEINS INVOLVED IN WNV INFECTION AND REPLICATION

Abstract

The lifecycle of intracellular pathogens, like viruses, is intimately tied to the cell in which they resides. RNA viruses, which usually encode a very limited number of viral proteins, must rely on the host cell's macromolecular processes to infect and replicate efficiently. WNV has been shown to interact with or require several different proteins for its own replication; however, it is likely that many other such interactions are required. In order to identify novel host proteins which WNV requires to infect cells and replicate efficiently, a siRNA library consisting of 3 independent siRNAs targeted against approximately 5500 different human genes was used to systematically knockdown (KD) host gene expression and examine the effect of this KD on WNV infection and replication. Using FLuc-expressing WNV VLPs and a Renilla luciferase (RLuc)-expressing cell line, 51 genes were identified which, when knocked down, resulted in a greater than 60% reduction in WNV VLP-dependent FLuc activity with at least 2 of the 3 individual siRNAs for that gene. By subjecting these candidate genes to several confirmatory steps, including screening of the siRNAs in several different cell lines, screening with a *lacZ*-expressing WNV VLP, screening with WNV infection, screening with a heterologous virus and screening of siRNAs purchased from a second source, we identified ten candidate genes required by WNV for efficient replication.

Introduction

VIRUS INTERACTION WITH HOST CELL MACHINERY

For all intracellular pathogens, the life of the pathogen is intimately tied to the life of the cell. RNA viruses, which encode minimal amounts of genetic material, must be able to utilize host cell processes to infect and replicate efficiently. The ability of viruses to hijack host proteins is often a determinant of host (organism or cell) susceptibility, particularly during the binding and entry into the cell. The presence or absence of specific receptors on cell surfaces is often a key factor in determining viral tropisms. In addition to requiring host translational and transcriptional machinery, viruses often utilize components of cellular trafficking, components of signaling pathways as well as other factors which enable the virus to establish an environment permissive for efficient replication. The use of cellular factors to promote viral infection has been observed for multiple RNA viruses, including WNV.

A few proteins, including La protein (an RNA binding protein involved in aspects of transcription) and polypyrimidine tract-binding protein (PTB), have been shown to interact with a number of RNA viruses. The binding of La protein to the 5' UTR of coxsackievirus B3 virus was demonstrated to be a requirement for IRES-mediated translation (459). La protein has also been shown to interact with Sindbis virus (106, 154), VSV (588, 589) and DENV (425); however, although it is thought these interactions help mediate viral genome replication, the functional requirements are not clear. In the case of DENV, La protein was also shown to interact with the NS3 and NS5 protein, although the role of this interaction is not clear (154). PTB protein has been shown to interact with two picornaviruses, poliovirus and ECMV (207). This interaction

is thought to be required for viral infection, as depletion of PTB protein inhibits viral translation. Two flaviviruses, DENV (106) and JEV (276), have also been shown to interact with PTB protein at the 3' UTR of the virus genomes; however, a functional requirement for these interactions has not been identified. Two other host proteins are known to interact with DENV, the human heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 (411) and vacuolar ATPase (116). Although the importance of the former interaction is unknown, the ATPase is thought to be involved in DENV entry and egress.

Several host protein-WNV interactions have been identified, as well. The cellular protein translation elongation factor 1 α (EF1α) interacts with the 3' UTR of the WNV genome (39). Mutational analysis indicated that interfering with the binding between the 3' UTR and EF1α did not affect viral translation efficiency, but had a detrimental effect on viral minus-strand RNA synthesis (104). Two other cellular proteins also interact with the WNV genome; however, T cell intracellular antigen-1 (TIA-1) and TIA-1-related protein (TIAR) both interact with the minus strand 3' stem loop structure (313). WNV growth was reduced in TIAR null MEF cells and, TIA-1 null MEF cells displayed slower growth kinetics compared to WT MEF cells. Later studies indicated that these interactions prevent the formation of WNV-induced stress granules and processing bodies (124), which are sites of translational control and mRNA aggregation and degradation under times of stress. This is a possible mechanism by which WNV avoids host-mediated translational shutoff. Another host protein, c-yes, appears to be required for WNV maturation and egress, although, in this case, a direct physical interaction is not apparent.

RNA INTERFERENCE

The concept of RNA interference was first identified nearly two decades ago in plants. One of the first descriptions of this phenomenon came when mRNAs expressing pigmentation genes were transfected into Petunias. Surprisingly, instead of observing an increase, nearly one-quarter of the transfected Petunias showed a reduction in pigmentation (566), suggesting that the mRNAs were able to suppress the expression of the pigmentation genes. RNA interference (RNAi), often referred to as post-translational gene silencing, was soon brought to the spotlight again when, in 1998, Fire and Mello identified RNA interference was mediated by dsRNA in *Caenorhabditis elegans* (137). This discovery earned them a Nobel Prize in Physiology and Medicine in 2006. The identification of the component responsible for RNA interference resulted in an onslaught of fresh research and propelled RNAi from a biological phenomenon to a powerful biological tool for examining specific target gene function.

In the few years since its recognition, much progress has been made on understanding the mechanism of RNAi. It is now thought that many cells, from plants to mammals, normally use RNAi to regulate gene expression. This is achieved by the generation of small, dsRNA molecules called microRNAs (miRNAs) (25). miRNAs are processed from precursor molecules, which frequently originate from RNA polymerase III transcripts (25). The maturation of mRNAs from these transcripts is achieved by processing through two members of the RNase III family, Drosha and Dicer (135). Drosha processing creates a stem loop precursor miRNA (pre-miRNA) approximately 70 nucleotides (nt) in length. The pre-miRNAs are further processed by Dicer into mature miRNAs approximately 19-25 nt long (33). The antisense strand of the miRNA is loaded into the RNA-induced silencing complex (RISC) (192), guiding it to the target mRNA,

triggering its cleavage or translational inhibition (363). Although one protein, Argonaute2, has been shown to be a part of RISC (325, 455), the identification of other proteins involved in the complex remain elusive. The requirements for the recognition of mRNAs are not clear; however, it is thought that the 5' 7-8 nt of the miRNAs are important.

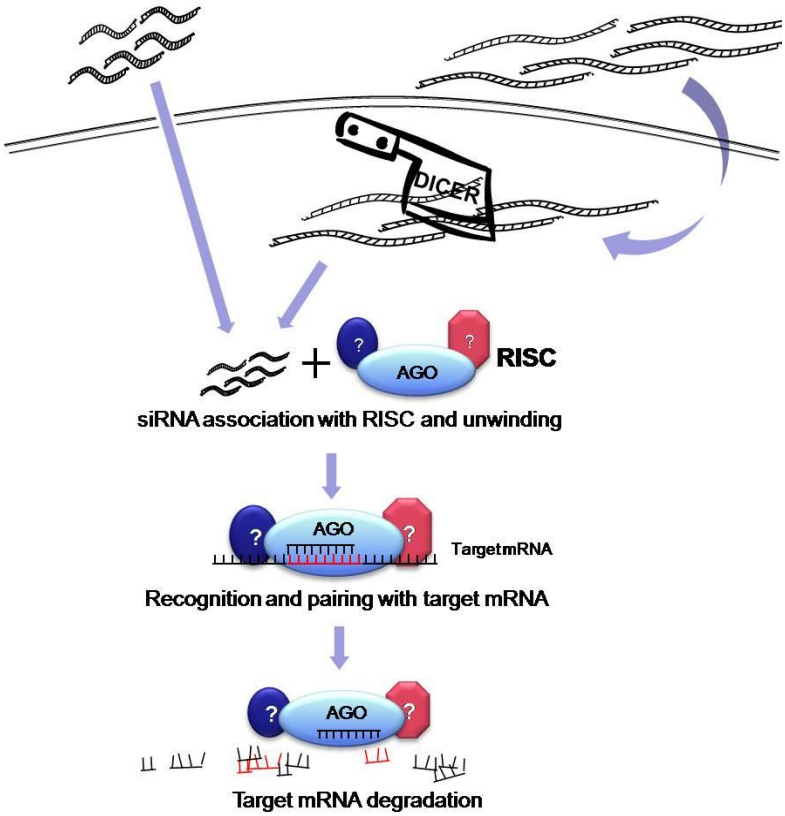


FIGURE 4.1: MECHANISM OF RNA INTERFERENCE. Double-stranded RNA or small interfering RNAs are introduced into cells. The dsRNAs are cleaved by Dicer into siRNAs and associate with RISC. The RISC-siRNA complex interacts with mRNAs containing sequence homology. RISC is targeted to mRNAs with partial sequence homology to the siRNAs, resulting in its degradation and subsequent silencing of the gene product.

It has subsequently been shown that, in plants and non-mammalian systems, the introduction of long dsRNA segments results in the formation of small interfering siRNA (siRNA), which are similar to miRNAs (Figure 4.1). The generation of these siRNAs

from long dsRNA is thought to occur through the same pathway as the generation of miRNAs. The introduction of long strands of dsRNA into mammalian cells, however, is not feasible. Long segments of dsRNA are recognized by the host cell, which, instead of silencing the expression of a single target gene, causes global changes in gene expression. In 2001, Elbashir et al. was the first to demonstrate the direct introduction of siRNAs induced RNAi-mediated gene silencing in mammalian cells. These siRNAs, which mimic miRNAs, are usually approximately 21 nt long with a 2 nt overhang at the 3' end (122) and mediate gene silencing through RISC as described for miRNAs (Figure 4.1). Transfected siRNAs, however, were small enough to remain 'hidden' from the host's dsRNA recognition system (204, 389).

Before the identification and utilization of RNA interference, gene knockdown in mammalian systems was restricted primarily to mouse knockouts, which, in addition to being expensive and technically difficult, limits the field to the study of genes not essential for proper growth and development. The use of RNAi technology has expanded the accessibility and range of gene knockdown, opening the door for studying functions of specific genes and/or gene products in cellular processes (4, 32, 63, 194, 229, 296, 523). This has also proven to be useful for studying viral infections. Several viruses have been shown to be sensitive to RNAi treatment (18, 188, 292, 379), suggesting a possible role of RNAi technology in antiviral therapies. Additionally, the development of RNAi libraries, which contain siRNAs for human genes, has been very effective at identifying host proteins that are important for the replication of different viruses (50, 79, 409, 456).

RATIONALE

The goal of this study was to identify novel host factors that are essential for efficient WNV infection and/or replication. To achieve this goal, siRNA technology was

used to systematically knockdown nearly 5,500 genes and then assay for the ability of WNV VLP to infect and replicate within specific cell lines. It was *expected* that silencing specific genes would result in a reduction in WNV VLP-driven FLuc activity, representative of a reduction in WNV infection and/or replication. It was *hypothesized* that, due to the expression of minimal genetic material, WNV would require specific host co-factors to infect cells and efficiently translate and replicate its genome.

Materials and Methods

CELL LINES AND THEIR MAINTENANCE

Five different cell lines were utilized for these studies: Huh7 rpool, HeLa rpool, Hec1B rpool, SK-N-SH rpool and a Huh7 cell line stably expressing a FLuc-expressing WNV replicon (Huh7 26.5.1a.1; described in the Results section). All these cell lines were modified to stably express Renilla luciferase [RLuc; *Renilla muelleri* (Sea Pansy)], enabling us to easily monitor cell number (described in Results section). Huh7 rpool and Hec1B rpool cells were maintained as described in Chapter 2. HeLa cells, a cervical epithelial cell line, were maintained in DMEM supplemented with 10% FBS, 1% atb and 20ug/ml gentamycin. SK-N-SH cells, a human neuronal epithelial cell line, were maintained in MEM supplemented with 10% FBS, 1% atb, 1% NEAA, 1% NaPy and 20ug/ml gentamycin. Huh7 26.5.1a.1 cells were maintained in DMEM supplemented with 10% FBS, 1% atb, 20ug/ml gentamycin and 400μg/ml G418.

REPLICONS, WNV VLPS AND WNV

The WNV utilized for these studies is the same low-passage stock of a Texas 2002 isolate (wild type WNV, not WNV^{HS}) as described in Chapter 2. In addition to WNV VLP and WNV FLuc VLP (described in Chapter 2), several other replicons were

utilized for these studies. WNR C-βgal2A NS1-5 (132) is similar to WNR C-FLuc2A NS1-5 described in Chapter 2 except, instead of the FLuc gene, this replicon encodes the *lacZ* gene followed by the FMDV 2A protein. VLPs produced by electroporating this replicon RNA into the VEErep/C*-E/Pac BHK packaging cell line are referred to as WNV βgal VLPs. VEEV VLPs expressing FLuc were generously provided by I. Frolov (UTMB). These VLPs consist of a replicon, VEErep/GFP/Luc, encoding GFP and FLuc under the control of two separate subgenomic promoters packaged into VEEV particles as described previously (570).

In addition to the WNV VLPs, we utilized a Huh7 cell line stably expressing a WNV replicon. This cell line was derived by electroporating Huh7 cells with RNA harvested from BHK cell lines stably expressing a FLuc-encoding WNV replicon (475). The replicon expressed in these cells was WNR NS1-5 containing a minipolyprotein gene cassette in the 5'UTR. This minipolyprotein encodes a FLuc gene and a NPT gene separated by the autocatalytic FMDV 2A protein. Expression of the minipolyprotien was driven by an encephalomyocarditis virus internal ribosome entry site (IRES).

Schematic diagrams of each of the WNV replicon and VEEV constructs can be found in Appendix A.

MTT ASSAY

Cell viability was determined by a standard MTT assay. In short, 100μl of MTT solution (10mg/ml methylthiazolyldiphenyl-tetrazolium bromide diluted in MEM without phenol red) were added to monolayers of treated cells and incubated for 3 hours at 37°C. Following incubation, the solution was removed and 100% isopropanol was added to solublize the metabolized MTT product. The plates were agitated for 15 minutes at room temperature and absorbance of the MTT product was measured at 560nm.

siRNA LIBRARY AND INDIVIDUAL siRNAs

The library, purchased from Ambion, consisted of siRNAs targeted against approximately 5,500 human genes known to be involved in various disease processes. Because of their involvement in disease or disorders, this set of genes was referred to as the human druggable genome. The siRNAs—three independent siRNAs for each of the 5,500 genes—arrived lyophilized onto blackwalled 96 well plates. Each plate contained a non-targeting, negative control siRNA as well as a FLuc-specific siRNA, which was used as a positive transfection control. This allowed for proper standardization within each of the plates.

The STAT1 siRNA was purchased from Ambion. WNV-specific siRNAs were designed by and purchased from Ambion. The sequences for each of the WNV siRNAs are: CUAAAGAGAUAUGAAGACAtt (WNV1); GAUAUGAAGACACAACUUUtt (WNV2); UGAAGACACAACUUUGGUUtt (WNV3). The Negative control (NEG) siRNA and FLuc siRNA (Firefly luciferase GL2 + GL3) used as controls were purchased from Ambion, although a Dharmacon plus Smartpool non-targeting control siRNA was purchased to use alongside Dharmacon siRNAs. SiRNAs targeted against the candidate genes identified in the siRNA library and subsequent secondary screens were purchased from Dharmacon for independent confirmation.

siRNA TRANSFECTIONS

Unless otherwise stated in the text, all transfections were performed with a final siRNA concentration of 30nM.

All transfections for the siRNA library screen were ‘reverse’ transfections. The siRNAs, already lyophilized onto the blackwalled 96 well plate, were rehydrated with

25μl DCCR (Dharmacon) containing 0.25μl DF1 (Dharmacon) (per well) and incubated at RT for at least 10 minutes (no longer than 30 minutes). Seventy-five microliters of Huh7 cells diluted to 1.33 x 10^5 cell/ml (10,000 cells/well) were added to each well and the cells-siRNA mixture was incubated for 72 hours at 37°C. These conditions resulted in a final siRNA concentration of 30nM.

The siRNA transfections for each of the confirmation steps were performed similarly. However, in this case, the siRNAs were not lyophilized onto the plates. Instead, solutions of the siRNAs (usually diluted in water at 2μM) were diluted into the DCCR-DF1 mixture and incubated for at least 10 minutes (no longer than 30 minutes) at RT. The siRNA-DF1 mixture was added to individual wells of a blackwalled 96 well plate (25μl/well) and Huh7 cells, at a concentration of 10,000 cells/well, were added to each well. The siRNA transfection conditions for each of the cell types were similar with the exception of HeLa cells which were trasnsfected with only 0.1μl/well of DF1 (instead of 0.25μl/well) and only 5,000 cells/well (instead of 10,000 cells/well).

In some cases, cells were transfected with pools of the Ambion siRNAs. In this case, equal volumes of each siRNA were mixed together and the cells were transfected with the pool of siRNAs as described above.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

To assay the effect of siRNAs on WNV antigen accumulation, monolayers of siRNA-transfected, WNV-infected cells were assayed for WNV antigen expression by ELISA. The media from the treated cell monolayers was removed and the cells were fixed with Acetone:Methanol (1:1 v/v mixture) for at least 30 minutes at -20C. Following fixation, the Acetone:Methanol was removed and the cells were air dried. Monolayers were rehydrated with ELISA buffer (PBS supplemented with 1% NHS and 0.1% Tween-

20) for approximately 20 minutes and incubated for 1 hour with a mouse hyperimmune ascites fluid (MHIAF) anti-WNV polyclonal antibody diluted 1:2000 in ELISA buffer. Following this incubation, the cells were washed 3 times with PBS supplemented with Tween-20 (PBS-T), 5 minutes per wash, and then incubated for 1 hour with an HRP-conjugated goat anti-mouse IgG secondary antibody diluted in ELISA buffer. The cells were then washed 3 times with PBS-T and 1 time with PBS. 50µl of TMB (Sigma) substrate was added to each of the wells and incubated at RT until a blue color developed. The reaction was stopped by the addition of 50µl 1M HCl and the plate was read at 450nm.

FIREFLY LUCIFERASE AND RENILLA LUCIFERASE ASSAYS

Since the siRNA-transfected cells were infected with a FLuc-expressing WNV VLP, infection/replication levels were monitored by assaying FLuc activity. The media from the infected cells was removed and 50µl of FLuc substrate [40mM Tricine, 8mM magnesium acetate, 33mM DTT, 0.13mM EDTA, 0.1% Triton-X-100, 0.53mM ATP, 0.47mM D-luciferin (Molecular Imaging Products Company) at pH 7.8] was added to each well. Following a 30 second incubation on a plate shaker, the plate was read on a TR717 microplate luminometer (Applied Biosystems). Although FLuc substrate is commercially available, it is extremely expensive; therefore, to cut down on costs of the library screen, we prepared our own substrate.

Following the FLuc assay, the cells were immediately assayed for Renilla luciferase (RLuc). This assay was used to monitor cell number, as, mentioned above, the cell lines used for the screen stably expressed the RLuc gene. To assay for RLuc activity, 50ml of RLuc substrate [100mM EDTA, 10mM Tris, 5ug/ml coelenterazine (CTZ; NanoLight Technologies)] was added directly to the wells (FLuc substrate was not

removed). Following a 30 sec incubation on a plate shaker, the plate was read on the a TR717 microplate luminometer (Applied Biosystems).

ONPG ASSAY

Beta-galactosidase (βgal) activity from siRNA-transfected cells infected with WNV bgal VLPs was measured by 2-Nitrophenl β-D-galactopyranoside (ONPG) assay.. Briefly, the WNV βgal VLPs were removed from the cell monolayers and approximately 20µl of lysis buffer (300mM NaCl, 50mM Tris-HCl pH 7.68) was added to each well. The plate was subjected to one freeze-thaw cycle to ensure complete cell lysis and 5µl of each sample was added, in duplicate, to a fresh 96 well plate. Serial two-fold dilutions of a βgal standard (Promega) were assayed alongside the samples to generate a standard curve (5µl/well of each dilution, in duplicate). 100µl of the ONPG substrate [4mg/ml ONPG (Sigma) dissolved in 0.1M sodium phosphate buffer, 1mM magnesium chloride, 50mM β-mercaptoethanol] was added to each well. The plate was incubated at 37°C for approximately 30 minutes. The reaction was stopped by the addition of 150ml 1M sodium carbonate and the OD was measured at 420nm.

SDS PAGE AND WESTERN BLOT ANALYSIS

SDS-PAGE and Western blot analysis to examine protein knockdown was performed as described in Chapter 2. The membranes were blocked with blocking buffer (5% nonfat dry milk (Nestle), 0.1% Triton-X-100 in PBS) for at least 30 minutes at RT (often, the blots would be blocked overnight at 4°C). The blocked membranes were probed with at least one of the following monoclonal or polyclonal antibodies diluted in the blocking buffer: monoclonal anti-β-actin (Sigma), polyclonal mouse anti-STAT1 (N-term) (Transduction Laboratories), polyclonal rabbit anti-PROS030 (PSMA1) (Santa Cruz) or polyclonal rabbit anti-PSMA2 (Cell Signaling). The membranes were washed 3

times with PBS containing 0.05% Tween-20 and incubated with either HRP-conjugated goat anti-mouse IgG (KPL) or HRP-conjugated goat anti-rabbit IgG (KPL) secondary antibodies. HRP-decorated protein bands were visualized using ECL plus Western Blotting Detection System (Amersham) and the signal was collected on Kodak X-ray film**.**

Results

GENERATION OF RENILLA-LUCIFERASE CELL LINES

Silencing certain genes, particularly those involved in cell cycle regulation, may have an impact on the number of cells present in individual wells. Additionally, the knockdown of some genes may have a toxic effect on the transfected cells. Since cell numbers affected by either of these outcomes would alter signal of virus-specific reporters, it was necessary to develop a system in which we could easily and effectively monitor the number of cells following siRNA transfection. Although MTT assays are effective at determining cellular viability, this test would require two separate plates for each of the 210 plates comprising the screen. To help streamline the screening process, we decided to generate cell lines that constitutively express Renilla luciferase (RLuc). This would allow us to easily assay cell number by measuring RLuc activity in the transfected wells while, in the same plate, monitoring WNV-dependent FLuc activity in the same well.

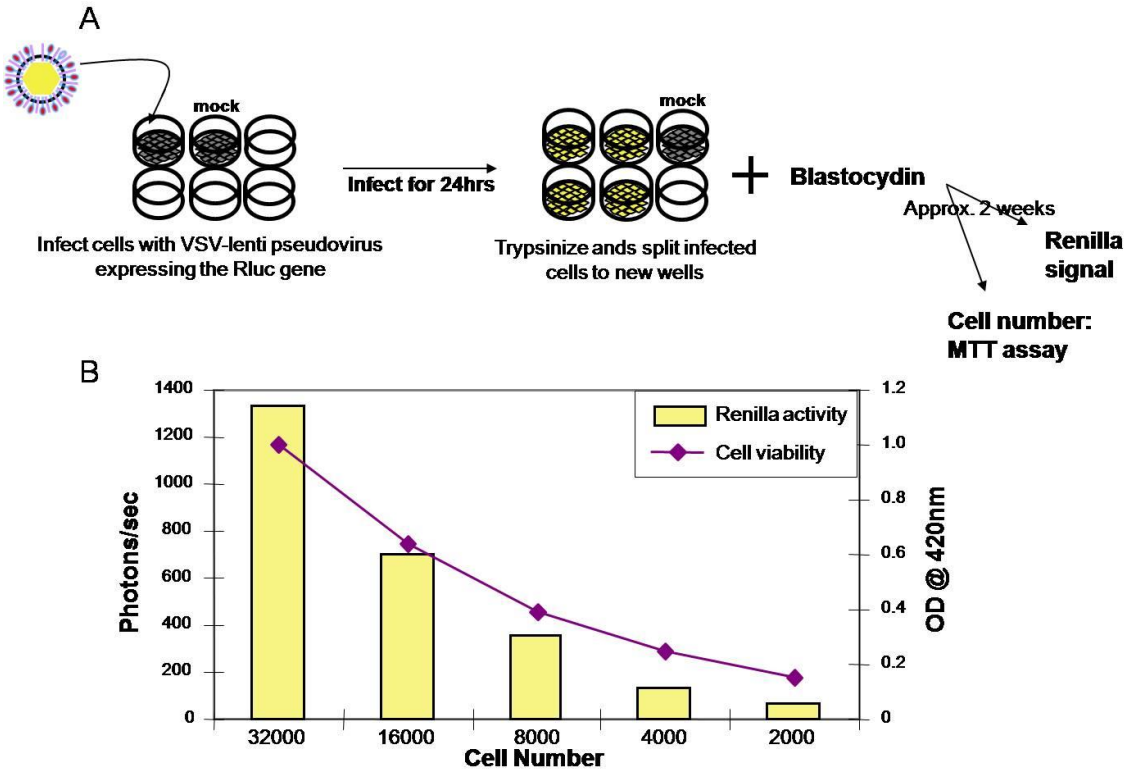


FIGURE 4.2: RLUC EXPRESSING CELL LINES CAN BE USED TO MONITOR CELL NUMBER. (A) Schematic diagram of the generation of Rpool cell lines. (B) Monolayers of Huh7 cells were plated onto duplicate 96 well plates and assayed for either RLuc activity or cell viability (MTT assay) (See Materials and Methods).

Cell lines expressing RLuc were generated using a VSV pseudotype virus. This virus contains the packaging 'shell' of VSV (VSV glycoproteins) but, instead of containing the VSV genome, the pseudotype virus contains a portion of a lentivirus that is required for gene integration, an RLuc gene and a blasticidin resistance gene (allowing for selection). The use of the lentivirus vector allowed the RLuc gene to become integrated into the cell's genome, resulting in stable RLuc expression. Monolayers of each of the cell lines, Huh7, HeLa, Hec1B and SK-N-SH, were plated into 6 well plates and infected with the VSV pseudotype virus for approximately 1 hour. Following the incubation, 3ml fresh DMEM+++ was added to the monolayers and the cells were

incubated for approximately 24 hours at 37°C. Mock infected cells were prepared side-by-side. Twenty-four hours post infection, 2μg/ml blasticidin was added to the media and the cells were monitored to follow antibiotic-mediated cell death in non-infected cell cultures and to demonstrate the blasticidin resistant gene transduction had efficiently occurred in the pseudotype-infected cultures. After approximately 2 weeks of antibiotic selection, when all the mock-infected cells were dead, the surviving infected cells were harvested and propagated in normal growth media. A schematic representation of the protocol is shown in Figure 4.2A.

Figure 4.2B shows a representative graph of the correlation between RLuc activity and cell number (as measured by MTT assay). Two-fold dilutions of the Huh7 cells were plated onto a blackwall 96-well plate (RLuc activity) and a clearwall 96-well plate (MTT assay) and incubated overnight at 37°C to allow the cells to set down and attach. An RLuc assay was performed on the blackwall plate and an MTT assay was performed on the clearwall plate. The RLuc activity measured in each of the cell densities correlated well with the absorbance obtained from the MTT assay, indicating that RLuc levels could be used as a marker for cell number. Although Figure 4.2B shows only Huh7 cells, a similar correlation was observed in the HeLa, Hec1B and SK-N-SH cells. To simplify matters, although all cell lines used from this point on express RLuc (rpool), they will simply be referred to by the cell name only (i.e. Huh7 cells, not Huh7 rpool cells).

DEVELOPMENT OF A SCREENING PROTOCOL AND SCREENING THE LIBRARY

In order to screen such a large number of plates (210 plates total), we needed to develop an effective, high-throughput screening protocol. The first step in developing this protocol was to determine the timeline for siRNA incubation that allows for optimum

host protein knockdown while preventing cell overgrowth. To determine the optimum siRNA incubation period, we used siRNAs targeted against a representative gene, STAT1. Monolayers of Huh7 cells were transfected with STAT1-specific siRNA or a non-targeting, negative-control siRNA (NEG) and incubated for 24, 48 or 72 hours at 37°C. At each timepoint, cell lysates were harvested and assayed, by Western blot, for levels of STAT1. Results showed that by 48 hours, STAT1 protein expression was knocked down by 82%; however, by 72 hours post transfection, levels were knocked down by 90% (Figure 4.3A). We tried to harvest a 96 hour post transfection sample, however, at this time, the cells were over-confluent and were beginning to show signs of cytopathology.

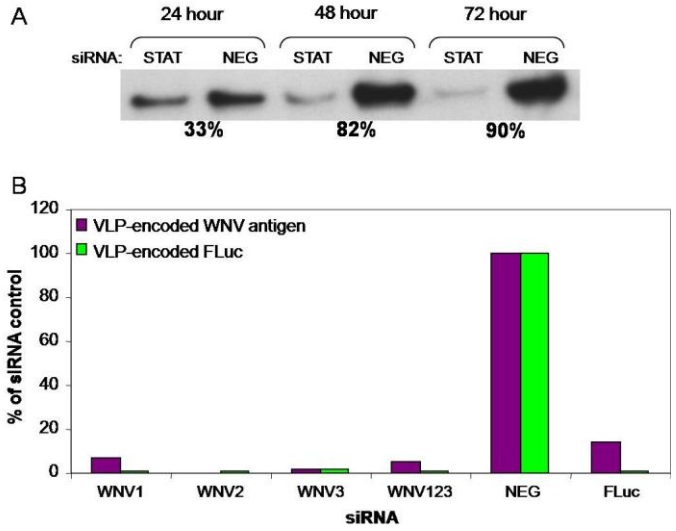


FIGURE 4.3: SIRNA TRANSFECTION IS EFFECTIVE AT SILENCING GENE EXPRESSION. (A) Western blot analysis of STAT1 protein levels following siRNA transfection. Monolayers of Huh7 cells were transfected with STAT1-specific or NEG siRNA and then harvested at the indicated times post transfection. Blots were probed for levels of STAT1. Percentages shown depict percent STAT1 siRNA-mediated knockdown compared to NEG siRNA as measured by denso-spot analysis. (B). WNV VLP-encoded FLuc activity is consistent with WNV VLP antigen expression. Monolayers of Huh7 cells were transfected with three different WNV-specific siRNAs, a FLuc-specific siRNA or NEG siRNA and incubated for 72 hours. The transfected cells were infected with WNV FLuc VLP and assayed for either FLuc expression or WNV antigen expression (ELISA; See Materials and Methods).

To reduce the complexity of the screening assay, we used WNV VLPs that express FLuc (WNV FLuc VLP) (132) as a model for WNV infection. WNV VLPs are particularly useful as they mimic WNV infection and replication in nearly all aspects but can be used safely at biosafety level 2 conditions. Additionally, because WNV VLPs contain a WNV genome that lacks structural proteins, they cannot be packaged and released. Therefore, our screen will not identify any genes involved specifically in these stages of WNV infection. Although WNV VLPs mimic WNV infection, we wanted to ensure that the FLuc activity detected from WNV FLuc VLP-infected cells accurately represented WNV infection and/or replication. To this end, monolayers of Huh7 cells on two duplicate plates were transfected with siRNAs targeted against WNV (WNV1, WNV2, WNV3 or the 3 siRNAs combined) or FLuc side-by-side with NEG siRNA and incubated for approximately 72 hours. The transfected cells were infected with WNV FLuc VLP for an additional 24 hours and then assayed for FLuc activity and WNV antigen accumulation. Results indicated that the WNV-specific and FLuc-specific siRNAs effectively knocked down WNV-driven FLuc activity and WNV antigen accumulation to similar levels ($\geq$ 90% knockdown compared to NEG siRNA) (Figure 4.3B). This indicated that FLuc activity was representative of WNV antigen accumulation and, therefore, served as a representative measure of VLP infection and genome translation/replication.

The high-throughput screening protocol is outlined in Figure 4.4. Each plate provided by Ambion contained 80 individual siRNAs, duplicate wells of two different NEG siRNAs and FLuc siRNAs (which served as a positive transfection control). The siRNAs were shipped in 70 96 well plates, in triplicate (A, B, C), to represent the 3 individual siRNAs for each of the 5500 genes. The siRNAs were transfected into Huh7 cells as outlined in

the Materials and Methods and incubated for approximately 72 hours at 37°C to allow for sufficient gene knockdown and then the transfected cells were infected with WNV FLuc VLPs for an additional twenty four hours. At harvest, each plate was first assayed for FLuc activity, which monitors WNV infection/replication, and then RLuc activity, which monitors cell number.

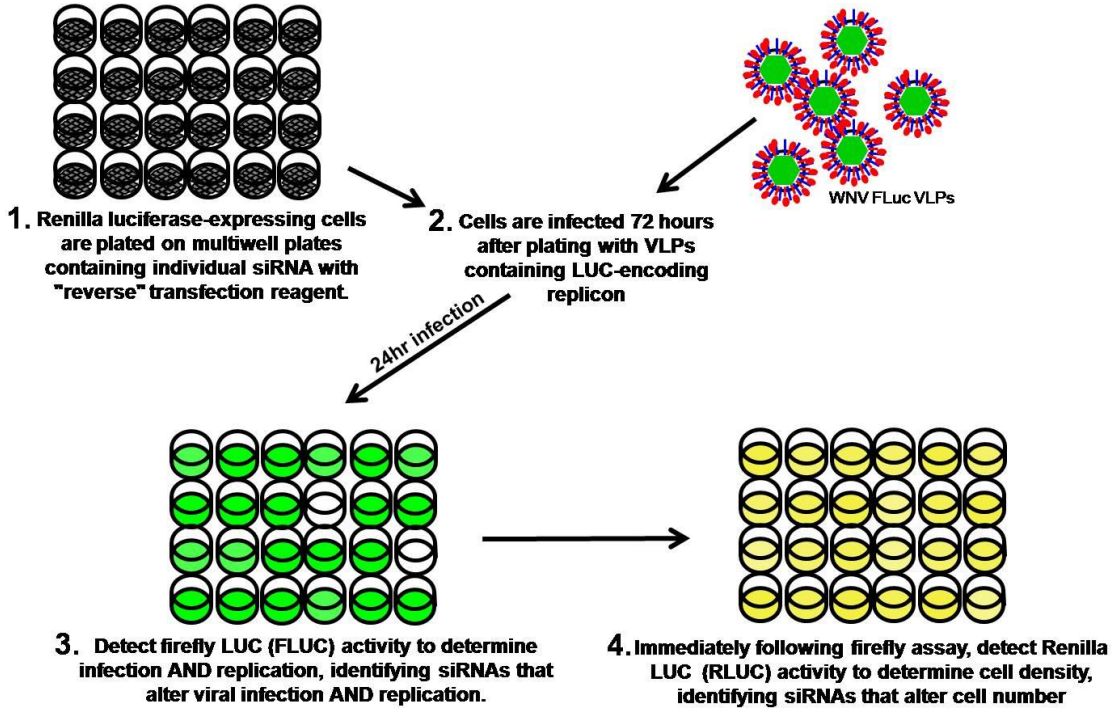


FIGURE 4.4: SCHEMATIC DIAGRAM OF HIGH-THROUGHPUT SCREENING PROTOCOL. Huh7 cells were added to plates containing siRNAs rehydrated in transfection reagent and incubated for 72 hours. The transfected cells were infected with WNV FLuc VLPs for an additional 24 hours and then assayed for FLuc and RLuc activity.

Analysis of the data obtained from the screen was multi-tiered and is shown in Figure 4.5. First, the FLuc activity from each individual siRNAs was normalized to the FLuc activity of the NEG control siRNAs on the same plate. This gave percent FLuc activity for each siRNA. Second, the RLuc activity of each individual siRNA was normalized to the RLuc activity of the NEG control siRNAs on the same plate. This gave

percent RLuc activity. Finally, to control for cell number (either due to differences in cell seeding or the effects of individual siRNAs on the cells), the percent FLuc activity was normalized to the percent RLuc activity to determine the percent specific FLuc activity for a specific siRNA.

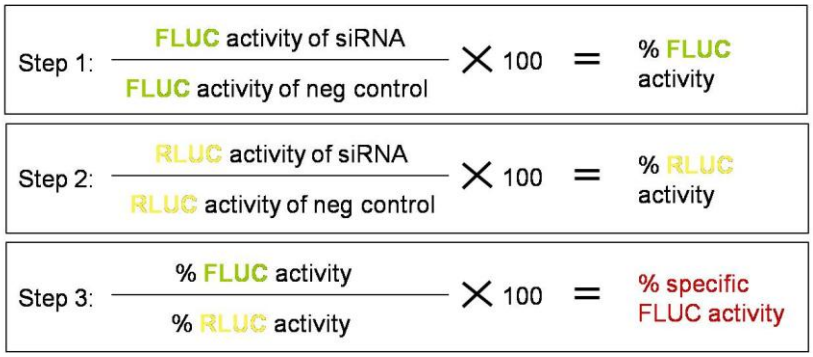


FIGURE 4.5: THREE STEP DATA ANALYSIS OF SIRNA LIBRARY SCREEN. Cartoon demonstrating the data analysis used to determine % specific FLuc activity for each individual siRNA.

Prior to the screen, we determined a set of criteria for considering a gene a potential 'hit'. Because off-target effects can be observed with siRNA knockdown (80, 242, 243, 492) and the fact that not all siRNAs will be equally effective (due to efficiency of silencing, half-lives of RNAs, levels of RNAs, half-lives of proteins, or levels of proteins), we decided that at least 2 of the 3 individual siRNAs for a specific gene must fall at or below our arbitrary cutoff. Genes were considered 'hit's when the % specific FLuc activity was ≤40%, consistent with a 60% reduction in WNV infection/replication compared to NEG siRNA transfected cells (for at least 2 of the 3 siRNAs). We excluded any siRNA that reduced % RLuc activity by 40% to eliminate wells in which significant changes in cellular viability would be likely to result in a non-sepecfic reduction in a WNV infection/replication. The siRNA library screen identified 64 genes (which we termed 'hits') which appeared to be involved in WNV infection and/or replication.

Following completion of the siRNA screen we decided to add a fourth tier in the analysis of the library—a duplicate run-through of the entire siRNA library. Of the 64 'hits' identified in the first screen, 51 repeated in the duplicate screen. Figure 4.6 depicts a flow chart demonstrating the number of hits from the two independent screens and Table 4.1 contains a list of the 51 candidate genes identified by the screen. A complete list of all of the 5500 genes in the library can be seen in Appendix B.

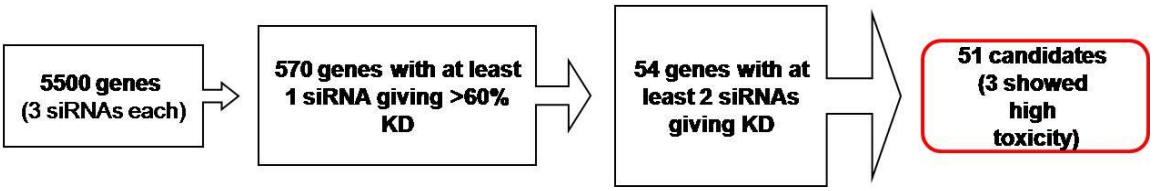


FIGURE 4.6: FLOW CHART OF SIRNA HITS. The 51 candidate genes identified showed knockdown of WNV replication/infection of at least 60% with 2 of the 3 individual siRNAs for that gene. siRNAs which resulted in a reduction of more than 40% in cell number, as measured by RLuc activity.

TABLE 4.1: LIST OF CANDIDATE GENES OBTAINED FROM SIRNA LIBRARY SCREEN.

Gene Symbol[1]	siRNA[2]	% specific FLuc[3]	RefSeq Accession Number	Full Gene Name
ACCN3	A	9	NM_020321; NM_004769; NM_020322	amiloride-sensitive cation channel 3
	B	24		
	C	49		
APP	A	98	NM_201414; NM_201413	amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease)
	B	34		
	C	38		
ASNS	A	31	NM_133436	asparagine synthetase
	B	27		
	C	5		
C13orf23	A	20	NM_025138; NM_170719	chromosome 13 open reading frame 23
	B	109		
	C	8		
CAD	A	29	NM_004341	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase
	B	58		
	C	25		
CEL	A	23	NM_001807	carboxyl ester lipase (bile salt-stimulated lipase)
	B	231		
	C	13		
DMPK	A	26	NM_004409	dystrophia myotonica-protein kinase
	B	155		
	C	35		
DRD3	A	152	NM_033663; NM_033658; NM_033659	dopamine receptor D3
	B	12		
	C	33		

ENPP1	A	31	NM_006208	ectonucleotide pyrophosphatase/phosphodiesterase 1
	B	10		
	C	245		
FLJ46361	A	200	NM_198577	FLJ46361
	B	11		
	C	28		
FOLR3	A	66	NM_000804	folate receptor 3 (gamma)
	B	32		
	C	11		
GPRC5B	A	12	NM_016235	G protein-coupled receptor, family C, group 5, member B
	B	77		
	C	4		
GPX3	A	16	NM_002084	glutathione peroxidase 3 (plasma)
	B	69		
	C	46		
HLCS	A	45	NM_000411	holocarboxylase synthetase (biotin-[proprionyl-Coenzyme A-carboxylase (ATP-hydrolysing)] ligase)
	B	12		
	C	127		
HYAL3	A	26	NM_003549	hyaluronoglucosaminidase 3
	B	18		
	C	225		
ILKAP	A	3	NM_176799; NM_030768	integrin-linked kinase-associated serine/threonine phosphatase 2C
	B	12		
	C	100		
ITGAL	A	6	NM_002209	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)
	B	21		
	C	35		
KIAA0999	A	33	NM_025164	
	B	24		
	C	170		
KIAA1434	A	14	NM_019593	KIAA1434
	B	63		
	C	17		
MAN2A2	A	28	NM_006122	mannosidase, alpha, class 2A, member 2
	B	15		
	C	145		
MAPK8	A	111	NM_139049; NM_139047	mitogen-activated protein kinase 8
	B	15		
	C	46		
MORC	A	37	NM_014429	microrchidia homolog (mouse)
	B	36		
	C	36		
MYH1	A	30	NM_005963	myosin, heavy polypeptide 1, skeletal muscle, adult
	B	209		
	C	28		
NDUFB5	A	33	NM_002492	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16kDa
	B	32		
	C	216		
NDUFS2	A	119	NM_004550	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase)
	B	18		
	C	39		
NEK2	A	10	NM_002497	NIMA (never in mitosis gene a)-related kinase 2
	B	6		
	C	141		
P2RY10	A	13	NM_014499; NM_198333	purinergic receptor P2Y, G-protein coupled, 10
	B	30		
	C	367		

PGPEP1	A	33	NM_017712	pyroglutamyl-peptidase I
	B	24		
	C	392		
PKD1L2	A	51	NM_182740; NM_052892	polycystic kidney disease 1-like 2
	B	165		
	C	29		
PKD1L3	A	9	NM_181536	polycystic kidney disease 1-like 3
	B	140		
	C	42		
PNMT	A	27	NM_002686	phenylethanolamine N-methyltransferase
	B	11		
	C	227		
PRKAA2	A	110	NM_006252	protein kinase, AMP-activated, alpha 2 catalytic subunit
	B	45		
	C	10		
PRKWNK2	A	213	NM_006648	WNK lysine deficient protein kinase 2
	B	19		
	C	26		
PRSS8	A	24	NM_002773	protease, serine, 8 (prostasin)
	B	24		
	C	96		
PSMA1	A	30	NM_002786; NM_148976	proteasome (prosome, macropain) subunit, alpha type, 1
	B	18		
	C	15		
PSMA2	A	36	NM_002787	proteasome (prosome, macropain) subunit, alpha type, 2
	B	4		
	C	103		
PSMA5	A	11	NM_002790	proteasome (prosome, macropain) subunit, alpha type, 5
	B	12		
	C	10		
PSMA6	A	21	NM_002791	proteasome (prosome, macropain) subunit, alpha type, 6
	B	18		
	C	5		
PSMB2	A	64	NM_002794	proteasome (prosome, macropain) subunit, beta type, 2
	B	6		
	C	12		
PSMB3	A	9	NM_002795	proteasome (prosome, macropain) subunit, beta type, 3
	B	2		
	C	202		
PSMB5	A	7	NM_002797	proteasome (prosome, macropain) subunit, beta type, 5
	B	10		
	C	159		
PSMB6	A	37	NM_002798	proteasome (prosome, macropain) subunit, beta type, 6
	B	13		
	C	5		
PSMC3	A	3	NM_002804	proteasome (prosome, macropain) 26S subunit, ATPase, 3
	B	5		
	C	12		
PSMD14	A	3	NM_005805	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14
	B	0		
	C	108		
PTAFR	A	13	NM_000952	platelet-activating factor receptor
	B	38		
	C	116		
RRM1	A	44	NM_001033	ribonucleotide reductase M1 polypeptide
	B	15		
	C	40		

SERPINH1	A	15	NM_001235	serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)
	B	8		
	C	182		
SORCS3	A	36	NM_014978	sortilin-related VPS10 domain containing receptor 3
	B	15		
	C	102		
STK38L	A	21	NM_015000	serine/threonine kinase 38 like
	B	220		
	C	32		
TIF1	A	80	NM_003852; NM_015905	transcriptional intermediary factor 1
	B	26		
	C	4		
TNFRSF10B	A	7	NM_003842; NM_147187	tumor necrosis factor receptor superfamily, member 10b
	B	9		
	C	131		

[1] Table represents a single replicate of the screen; however, only the genes that were 'hit's' in both of the screens are shown here
[2] The 3 individual siRNAs for each gene are shown, labeled A, B, C
[3] % specific FLuc activity represents the % FLuc normalized to % RLuc. These genes were considered 'hits' because at least 2 of the 3 individual siRNAs (A, B, C) demonstrated ≤40% specific FLuc activity.

SECONDARY CONFIRMATORY SCREENS OF POTENTIAL CANDIDATE GENES

To help ensure that the initial screen identified genes that were required for WNV infection/replication, the 3 individual siRNAs for each of the identified 51 candidate genes were assayed in multiple secondary systems. The results of these secondary confirmatory screens can be seen in Table 4.2. First, to ensure there was no cell-type specificity to the identified candidate genes, the siRNAs were transfected into three additional cell lines, Hec1B cells, HeLa cells and SK-N-SH cells. To enable us to monitor cell number, cell lines stably expressing RLuc were generated for each of the cell types as described above. Each of the 3 independent siRNAs for the 51 candidate genes were transfected into the 3 cell lines and then assayed, as described for the siRNA library above, for FLuc and RLuc activity. The genes were judged to be 'positive' (transfection with 2 of the 3 individual siRNAs resulted in a ≥ 60% reduction in WNV infection/replication) or 'negative' as shown in Table 4.2. Candidate genes which were not positive were eliminated from further consideration. Although these genes may be

interesting to study further, we were primarily interested in genes that were universally important for WNV infection/replication.

TABLE 4.2: CONFIRMATION OF CANDIDATE GENES.

	Cell line confirmation[2]				WNV βgal VLP[3]	VEEV VLP[4]	Huh7 26.5.1a.1[5]
Gene[1]	Huh7	HeLa	Hec1B	HTB-11			
ACCN3	+	+	+	+	+	-	-
APP	+	+	+	+	+	-	-
ASNS	+	+	+	+	+	-	-
C13orf23	+	+	+	-	+	-	-
CAD	+	+	+	-	+	+	+
CEL	+	+	+	+	+	-	-
DMPK	+	+	+	-	+	-	-
DRD3	+	-	+	-	+	-	-
ENPP1	+	+	+	-	+	-	-
FLJ46361	+	+	+	+	+	-	-
FOLR3	+	-	+	-	-	-	-
GPRC5B	+	+	+	-	+	-	-
GPX3	+	+	+	-	+	-	-
HLCS	+	-	+	-	+	-	+
HYAL3	+	-	+	+	+	-	+
ILKAP	+	+	+	+	+	-	-
ITGAL	+	+	+	+	+	-	-
KIAA0999	-	+	+	-	-	-	+
KIAA1434	-	+	-	-	-	-	+
MAN2A2	+	-	+	+	+	-	-
MAPK8	+	+	+	+	+	-	-
MORC	+	-	+	+	+	-	-
MYH1	+	+	+	-	+	-	+
NDUFB5	+	-	+	+	+	-	-
NDUFS2	+	-	+	-	+	-	-
NEK2	+	-	+	-	+	-	-
P2RY10	+	-	+	-	+	-	-
PGPEP1	+	+	+	+	+	-	+
PKD1L2	+	-	+	-	-	-	+
PKD1L3	+	-	+	+	+	-	-
PNMT	+	+	+	+	+	-	+
PRKAA2	-	-	+	-	-	-	-
PRKWNK2	+	-	+	+	+	-	-

PRSS8	+	+	+	+	+	-	+
PSMA1	+	+	+	+	+	-	+
PSMA2	+	+	+	+	+	-	+
PSMA5	+	-	-	+	+	-	+
PSMA6	+	+	-	+	+	-	+
PSMB2	+	+	-	+	+	-	+
PSMB3	+	+	+	-	+	-	+
PSMB5	+	+	+	-	+	-	+
PSMB6	+	+	-	+	+	-	+
PSMC3	+	-	+	+	+	+	+
PSMD14	+	-	+	-	+	+	+
PTAFR	+	+	+	-	+	-	-
RRM1	+	+	+	+	+	-	+
SORCS3	+	-	+	+	+	-	-
STK38L	+	-	+	-	-	-	-
TIF1	+	+	+	+	+	+	+
TNFRSF10B	+	+	+	+	+	-	+

[1] Genes were judged '+' or '–' based on the ability of 2 of the 3 individual siRNAs to reduce WNV VLP infection/replication by ≥ 60% with no major effects on cell number. Genes highlighted in blue were '+' in all 4 cell lines and the WNV βgal VLPs

[2] siRNAs were transfected into Huh7, HeLa, Hec1B and SK-N-SK cells and assayed for their ability to inhibit WNV infection/replication

[3] siRNAs were transfected into Huh7 cells, infected with WNV βgal VLPs and assayed for their ability to inhibit WNV infection/replication

[4] siRNAs were transfected into Huh7 cells, infected with VEEV VLPs and assayed for their ability to inhibit VEEV infection/replication. A '+' symbolizes a reduction in VEEV infection/replication

[5] siRNAs were transfected into Huh7 26.5.1a.1 cells and assayed for their ability to reduce the replication of a replicating WNV replicon. A '+' symbolizes a reduction in WNV replicon replication. Those with a '-' symbol are potentially involved in an entry stage of WNV infection

Second, in order to ensure that the reduction in FLuc activity observed in the siRNA screen was due to specific regulation or degradation of the FLuc gene, βgal-encoding WNV VLPs were used as an alternative reporter. Each of the three individual siRNAs for the 51 candidate genes was transfected into Huh7 cells and then infected with WNV βgal VLPs. Interestingly, siRNAs for several of the genes did not cause a decrease

in WNV-driven βgal activity (Table 4.2). This suggests that the initial identification of these genes was likely a result of off-target or non-specific effects.

Finally, the 3 independent siRNAs for each of the 51 candidate genes were transfected into Huh7 cells and infected with a heterologous VLP, VEEV. The use of a heterologous VLP allowed us to address two potential concerns. First, we were interested in identifying genes which were involved specifically in WNV infection/replication. There are likely to be many cellular proteins, such as specific translation or transcription factors, that are necessary for the replication of all RNA viral pathogens. Secondly, the inability of siRNAs to block the infection or replication of a different virus would suggest that silencing the gene does not simply create an environment that is not conducive to virus infection (as opposed to having a direct impact on WNV infection/replication). Analysis of siRNA-transfected, VEEV VLP-infected cells indicated that only 2 of the genes were able to affect VEEV infection/replication (Table 4.2).

In order to try to delineate gene products that may be involved in WNV entry—as opposed to WNV replication—we transfected Huh7 cells (Huh7 26.5.1a.1) that persistently harbor a WNV replicon with the siRNAs for the original 51 candidate genes. This replicon, as described in the Materials and Methods, encodes the FLuc gene via a minipolyprotein at the 3' end of the genome. This allows us to easily monitor the effects of the siRNAs on WNV replicon replication. The Huh7 26.5.1a.1 cells, which were also engineered to express RLuc, were transfected with each of the individual siRNAs for the 51 candidate genes and then assayed for FLuc and RLuc activity. Analysis indicated that twenty-eight of the 51 genes had no effect on WNV replicon replication, suggesting that these gene products may be involved at an entry step of WNV infection. These genes are designated by bold lettering in Table 4.2.

These secondary screens narrowed down the 51 initial candidate genes to only 16. These 16 genes are highlighted in Table 4.2. The remaining confirmatory steps will be restricted to these 16 genes.

CANDIDATE GENES REDUCE WNV INFECTION AND/OR REPLICATION

We often use WNV VLPs as surrogates for WNV. The VLPs have, thus far, proven to be an accurate representation of WNV infection. However, we wanted to confirm from WNV VLP infections with live WNV infection. Thus, we compared the siRNA-mediated inhibition of WNV antigen accumulation following WNV infection with the inhibition of FLuc activity following WNV FLuc infection (Figure 4.7). In this case, the 3 individual siRNAs for each of the 16 candidate genes were pooled together. This pool was used to transfect Huh7 cells. Using a pool of siRNAs enables one to transfect with the same concentration of total siRNA, while reducing the concentration of an individual siRNA, potentially minimizing off target effects.

Equal volumes of each of the individual siRNAs for the 16 candidate genes were mixed together and transfected into Huh7 cells and then infected with WNV (MOI = 0.2) or WNV FLuc VLP and incubated for approximately 24 hours. Following incubation, WNV infection/replication was monitored by assaying for WNV antigen accumulation (ELISA) or WNV-driven FLuc activity (FLuc assay). WNV-specific siRNAs, which served as a positive transfection control, resulted in a greater than 80% reduction in both WNV antigen accumulation and WNV VLP-driven FLuc activity.

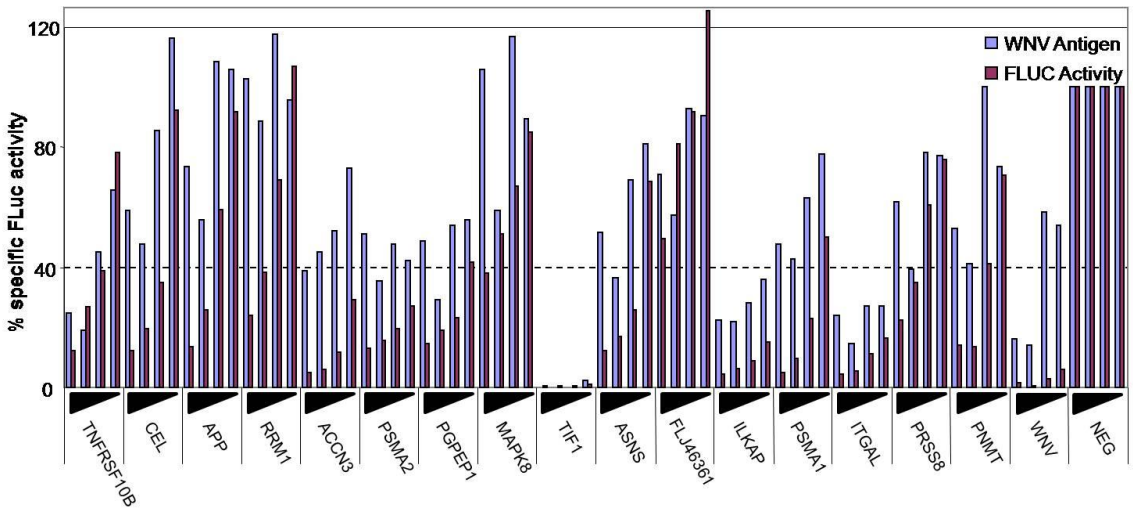


FIGURE 4.7: SILENCING THE CANDIDATE GENES IMPAIRS WNV INFECTION. Comparison of the effects of silencing the 16 candidate genes on WNV infection versus WNV VLP infection. Huh7 cells were transfected with 15nM, 7.5nM, 3.75nM or 1.88nM (final concentrations of all three siRNAs together) of pools of the 3 siRNAs for each of the 16 candidate genes and then infected with either WNV (MOI = 0.2) or WNV FLuc VLP. WNV antigen accumulation was measured by ELISA (Materials and Methods) and WNV VLP-driven FLuc activity was measured by FLuc assay. The dashed line represents the arbitrary cutoff established in the previous experiments.

All of the siRNAs resulted in a concentration dependent decrease in WNV VLP-driven FLuc activity. In fact, FLuc activity was reduced by greater than 60% for all of the siRNAs. Interestingly, the siRNAs for several of the genes, particularly ACCN3, PSMA1, PGPEP1, TIF1, ASNS, ILKAP, PSMA1 and ITGAL, showed significant reduction in WNV-driven FLuc activity at concentrations as low as 3.5nM. All of the siRNAs resulted in some reduction in WNV infection; however, the degree was less dramatic compared to the WNV VLP infection. In fact, siRNAs targeted against a few of the genes failed to satisfy our original criteria. However, the reduced effect of the siRNAs following WNV infection is likely due to secondary amplification of the virus, which is not seen with WNV VLP infections. Since siRNA transfection only results in a partial

removal of the target and 100% of the cells are not transfected, the ability to spread may make the virus less sensitive to the removal of specific co-factors.

FINAL SCREENING RESULTED IN 9 CONFIRMED CANDIDATE GENES

As a final confirmation of the candidate genes required for efficient WNV infection/replication, siRNAs from an independent source (Dharmacon plus Smartpools) were tested for their ability to block VLP-transduced FLuc activity in Huh7 cells. These Dharmacon plus Smartpools consisted of pools containing 4 independent siRNAs targeted against the gene of interest, which have been modified to reduce off-target effects. Huh7 cells were transfected with 25nM or 50nM of each of the Dharmacon smartpools. Unfortunately, a Dharmacon smartpool siRNA could not be purchased for one of the candidate genes, FLJ46361, due to a problem with the GenBank sequence. However, transfection of Huh7 cells with siRNAs targeted against the remaining 15 genes gave some surprising results. SiRNAs from only 10 of the original candidate genes caused a reduction in WNV VLP-driven RLuc activity at or below our original criteria (Figure 4.8). Silencing the genes ACCN3, ASNS, CEL, PNMT, PRSS8, PSMA1, PSMA2, RRMI and TNFRSF10B resulted in impaired WNV replication, suggesting that these genes are required for efficient WNV infection/replication. The siRNA to an additional gene, MAPK8, also impaired WNV VLP-driven FLuc activity, but only at the highest concentration tested (Figure 4.8).

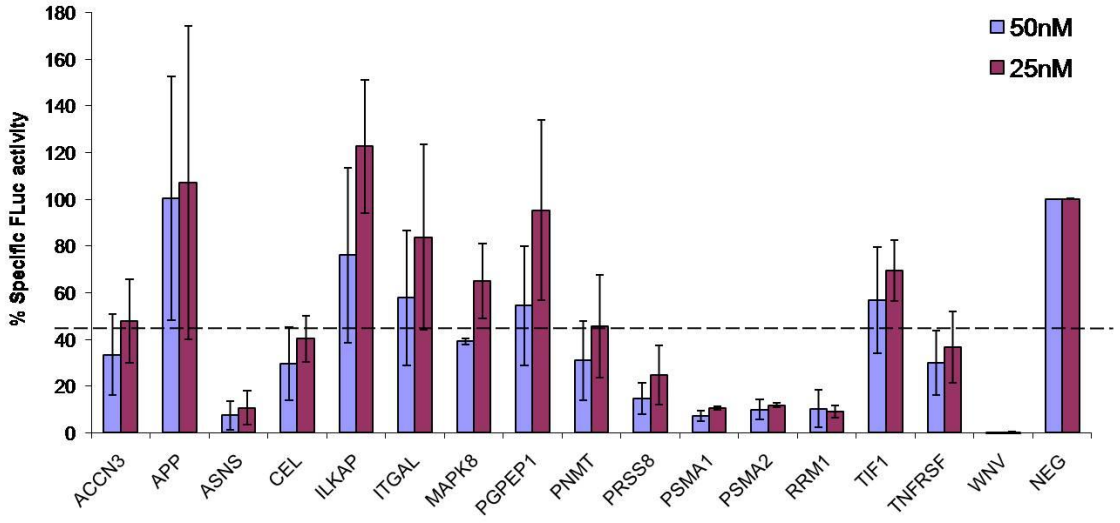


FIGURE 4.8: THE IDENTIFICATION OF 10 FINAL CANDIDATE GENES. Huh7 cells were transfected with Dharmacon plus Smartpools and infected with WNV FLuc VLPs. Twenty-four hours post infection, the cells were assayed for FLuc and RLuc activity. The dashed line represents the arbitrary cutoff established in the previous experiments.

THE PROTEASOME APPEARS TO BE CRITICAL FOR WNV INFECTION

Interestingly, 2 of these 10 confirmed candidate genes were subunits of the proteasome. In fact, 2 of 3 individual siRNAs (Ambion) and the pools of 4 siRNAs (Dharmacon) targeted against two alpha subunits, PSMA1 and PSMA2, were shown to consistently reduce WNV infection or replication (> 60% decrease in RLuc-normalized FLuc activity) when transfected into cells, with little or no effect on cell number. Additionally, silencing PSMA1 or PSMA2 had no discernable effect on VEEV VLP infection/replication (Table 4.2). To demonstrate that the siRNAs were effective in silencing the target genes, Western blot analysis was performed on PSMA1 and PSMA2 siRNA-transfected Huh7 cells. Analysis indicated that transfection of PSMA2 siRNA resulted in reduced PSMA2 expression compared to mock or NEG control siRNA transfection (Figure 1B). Interestingly, silencing PSMA1 expression also resulted in a

decrease in PSMA2 expression. Since the proteasome is a complex of multiple gene products, it seems likely that posttranslational silencing of one of the subunits would result in an inability to assemble the complex and the destruction of the "excess" levels of other subunits of the complex.

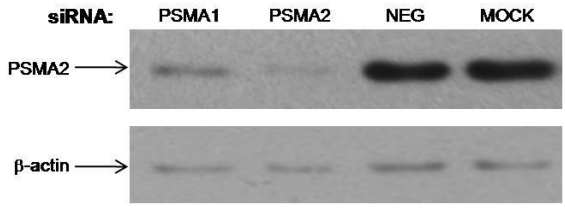


FIGURE 4.9: PSMA1 AND PSMA2 SPECIFIC SIRNAS EFFECTIVELY SILENCE PSMA2 EXPRESSION. Monolayers of Huh7 cells transfected with PSMA1, PSMA2 or NEG siRNAs were harvested and assayed, by Western blot, for levels of PSMA2 and β-actin (loading control).

Discussion

In this chapter, we employed a library consisting of siRNAs targeted against nearly 5,500 human genes to identify host co-factors required for efficient WNV infection and/or replication. Prior to the completion of this dissertation, several co-factors involved in WNV infection and/or replication have been identified, including those involved in WNV entry and genome translation/replication. The first virus-cell interaction that occurs during the infection process is the interaction between the virus particle and specific receptors on the target cell surface that facilitates viral entry. Although the precise cellular receptor for WNV has not been definitively identified, several have been suggested as potential co-receptors. An integrin, αvβ3, has been suggested as a potential receptor for WNV infection (83) and the expression of DC-SIGN on cell surfaces has been shown to promote WNV infection (103). Additionally, ICAM-1 is thought to facilitate entry of WNV into the central nervous system (102). Four other

cellular proteins, EF1α (39), TIA-1, TIAR (124, 313) and c-Yes (214), are also thought to be involved in various aspects of the WNV replication cycle.

Three of these genes, EF2α, TIA-1 and TIAR, were identified via a direct interaction with the WNV genomes. The identification of direct interactions is particularly useful for identifying host co-factors that are directly responsible for the formation of viral RNA-cellular protein complexes. However, although a direct interaction was detected, this does not necessarily indicate a functional requirement. Additionally, there are likely a multitude of proteins that are required for various aspects of WNV infection that do not directly interact with the viral genome or the viral proteins. An example of this is the c-Yes protein, which is required for proper maturation and egress of WNV particles, but its activity does not appear to be dependent upon a direct physical interaction (214). RNAi technology introduced a powerful, simple and rapid technique for identifying host factors that are essential for viral replication.

Screening of the siRNA library and subsequent confirmation assays identified ten candidate genes which appear to be required for efficient WNV infection and/or replication. The small number of candidate genes identified (less than 0.2% of the original 5,500 genes) was somewhat surprising; however, the library consisted of only a small subset of genes (only about 5,500 genes from the approximate 30,000 genes encoded by the human genome). Even within this small subset, it is likely that many genes were overlooked due to the limitations of RNAi. The presence of compensatory pathways or the expression of homologues limits the ability of RNAi to detect certain interactions, as knocking down one protein will have no effect on WNV replication if another protein is able to perform the same function. Additionally, although great strides have been made in RNAi technology, not all siRNAs will efficiently silence their target,

although we partially overcame this problem with the use of 3 independent siRNAs. The inefficiency of siRNAs to silence targets could be due to a variety of factors, including the half-life of the siRNA, the level of RNA present in the cells, the half-life and basal level of the target mRNA/protein. Additionally, we would not be able to identify targets that are essential for short-term cell viability, as silencing those genes would result in cytopathology. Our screen was also limited by the methodology we utilized. Since we used WNV VLPs are monitors for WNV infection/replication, we were unable to identify any genes involved in packaging, maturation or egress.

The criteria we chose for our screen were relatively stringent. To be considered a candidate gene, transfection of at least 2 of the 3 individual siRNAs had to reduce WNV infection and/or replication by greater than 60%, without significantly affecting cell viability (less than 40% loss in cell viability as measured by RLuc activity). In addition to the siRNA library screen, these criteria were utilized for all of the confirmatory screens as well. After completion of all confirmatory tests, we identified ten genes which are required for efficient WNV infection and/or replication. The ten genes, ACCN3, ASNS, CEL, MAPK8, PNMT, PRSS8, PSMA1, PSMA2, RRM1 and TNFRSF10B, are shown in Table 4.3 with their known functions. Using microarray data, we were able to confirm the expression of seven of the ten genes in Huh7 cells (results not shown). The other three genes (CEL, PNMT and PRSS8) were either not present on the microarray chip or not found in the Huh7 cells. The identification of these genes which are not expressed is troubling, but not completely unsurprising, as siRNAs have been shown to cause non-specific or off-target effects (242, 243, 318, 492, 543). These data highlight the necessity of careful analysis and alternative, siRNA-independent forms of confirmation when using RNAi technology to identify target genes of interest.

TABLE 4.3. KNOWN FUNCTIONS OF THE 10 CANDIDATE GENES.

Gene	Full and Alternate Names	Gene ID	Known Function(s)
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b; TRAIL-R2; DR5	8795	death receptor signaling; activation of caspase 3, 8, 9 and JNK1
CEL	carboxyl ester lipase	1056	cholesterol and lipid-soluble vitamin ester hydrolysis and absorption; secreted by the pancreas
ASNS	asparagine synthetase	440	synthesis of asparagine
PNMT	phenylethanolamine N-methyltransferase	5409	methylates norepinephrine to form epinephrine
RRM1	ribonucleotide reductase M1	6240	essential for the production of deoxynucleic acids prior to DNA synthesis
ACCN3	amiloride-sensitive cation channel 3; ASIC3	9311	sodium channel; pH sensor;
PSMA1	proteasome (prosome, macropain) subunit, alpha type, 1	5682	protein degradation pathway
PRSS8	protease, serine 8; prostasin	5652	chymotrypsin, peptidase, serine-type peptidase
PSMA2	proteasome (prosome, macropain) subunit, alpha type, 2	5683	protein degradation pathway
MAPK8	mitogen-activated protein kinase 8; c-jun, JNK1	5599	cell signaling; apoptosis

The specific role of these genes during WNV infection/replication is not known. The three genes which do not appear to be expressed in Huh7 cells have unique functions (Table 4.3). CEL, or carboxyl ester lipase, is known as a glycoprotein secreted from the pancreas; PNMT, or phenylethanolamine N-methyltransferase, is responsible for methylating norepinephrine to form epinephrine; and PRSS8, or protease, serine 8, is a chymotrypsin, peptidase, serine-type peptidase. It is possible, however, that these proteins have other, unknown, functions, as well. Two of the other proteins, RRM1 (ribonucleotide reductase M1) and ASNS (asparagine synthestase), appear to have functions involved in maintaining cell growth. RRM1 is one member of ribonucleoside-diphosphate reductase, which is essential for the production of deoxynucleotides prior to DNA synthesis, while ASNS is involved in the synthesis of asparagine. It is possible that the role of these genes during WNV infection is associated more with cell maintenance than viral replication.

The remaining five genes, TNFRSF10B, ACCN3, MAPK8, PSMA1 and PSMA2 are the most intriguing candidate genes. TNFRSF10B (tumor necrosis factor superfamily, member 10B), also known as TRAIL-R2 or DR5, is thought to be involved in FADD-mediated signaling and activates caspase 8, caspase 3, caspase 9 and Jnk to trigger apoptosis. Interestingly, DENV has been shown to induce apoptosis in hepatic cells through Apo2/TRAIL, ligands of TNFRSF10B (369). ACCN3 (amiloride-sensitive cation channel 3) is a sodium channel important for the detection of pH changes. Since the WNV genome is released into the cytoplasm from a lysosome in a pH-dependent fashion, it is possible that ACCN3 plays some role in WNV entry. This is consistent with the ineffectiveness of ACCN3 siRNAs to affect WNV replication in a replicon-bearing cell line. MAPK8 (mitogen-activated protein kinase 8), also known as JNK1 or c-jun, is a MAP kinase involved in a number of signaling pathways. Silencing this gene also had little effect on WNV antigen in replicon-bearing cell lines, suggesting it plays a role in the entry stage of infection. Interestingly, a recent study has indicated that a JNK-like protein in C6/36 cells (mosquito cell line) is involved in the phagocytosis and endocytosis of WNV particles. However, a role of JNK protein in human cells has not been described for WNV infection.

By far the most intriguing candidate genes we identified were PSMA1 and PSMA2, both of which are alpha subunits of the proteasome. Interestingly, through the course of the screen, silencing several other proteasome subunits had an effect on WNV replication; however, these did not meet our criteria of 2 of 3 siRNAs inhibiting WNV infection and/or replication by greater than 60%. This could be due to one or more of the reasons outlined above. The proteasome is involved in a variety of cellular processes, including cell development and differentiation, the immune response, signal transduction

and apoptosis and many different viruses have been shown to require proteasome function for efficient infection, replication and/or release. The availability of proteasome inhibitors was an excellent tool and allowed us to examine the role of the proteasome during WNV infection in even greater detail (see Chapter 5).

CHAPTER 5: THE PROTEASOME AS A CELLULAR CO-FACTOR FOR WNV REPLICATION

Abstract

A large-scale screen of siRNAs targeted against human genes identified the proteasome as a possible co-factor during WNV infection. Specificity of the siRNA knockdown of proteasome subunits was confirmed by using two potent chemical inhibitors of proteasome activity, PS1 and MG132. Pre-treatment of Huh7 and HeLa cells with micromolar concentrations of each of the inhibitors resulted in a dramatic reduction in WNV-dependent FLuc activity and WNV antigen accumulation compared to mock- or DMSO-treated cells. Western blot analysis and IFA clearly show a decrease in levels of WNV NS1 and NS3. In order to begin to tease apart the step or steps at which WNV required proteasome activity, cells were infected with WNV VLPs prior to inhibitor treatment. Interestingly, both inhibitors resulted in impaired WNV replication as late as twelve hours post infection, indicating that proteasome activity is not required at the level of viral entry. Consistent with a decrease in replication, WNV yield from proteasome inhibitor-treated cells was dramatically reduced compared to DMSO-treated cells. Taken together, these data suggest that proteasome activity is a requirement for successful WNV replication (growth) and it likely acts at the level of either genome translation or replication.

Introduction

THE UBIQUITIN-PROTEASOME SYSTEM

Intracellular degradation of proteins is a critical activity for maintaining the health of a cell. Multiple mechanisms are in place to degrade proteins which are targeted for destruction. Calpains, which are calcium-dependent cysteine proteases, are expressed ubiquitously in the cell and are important for degrading proteins involved in adhesion, locomotion, cytoskeletal rearrangement, inflammation and apoptosis (404). Lysosomal degradation is mediated by cathepsins, which belong to the cysteine or aspartate family of proteases (404). These proteases are active at the low pH environment within the lysosomes. The majority of cytosolic protein degradation, however, occurs via the ubiquitin-proteasome system (UPS). Degradation by the UPS involves two distinct steps: the tagging of the substrate with ubiquitin and the degradation of the tagged protein by the proteasome. This process is closely regulated and highly specific.

The UPS system is the principal nonlysosomal, ATP-dependent degradation system within the cell's cytosol and nucleus. It is a key player in a variety of normal cellular processes, including the cellular stress response, cell cycle regulation, cell differentiation and development, intracellular trafficking, DNA repair, transcriptional regulation and silencing, and the cellular immune response (15, 168, 439). Numerous functional proteins are known to be tagged by ubiquitin and ubiquitination is the primary mechanism for removing damaged or abnormal proteins (168). In this way, the UPS is a key player in maintaining cellular homeostasis.

Ubiquitination

Ubiquitination is a highly specific, highly regulated method of targeting proteins for degradation as well as regulating specific cellular processes unassociated with degradation. Ubiquitination results in the formation of a covalent bond between the C-termius of ubiquitin (Ub) and the ε-amino group of a lysine residue on a polypeptide. This occurs by the action of three sequential enzymes: E1, E2 and E3 (89, 211). E1 is an activating enzyme which forms a covalent bond with the carboxyl group of Ub, thus activating the Ub protein. E2, a conjugating enzyme, forms a transient bond with the activated Ub, allowing transfer to the substrate. E3, a ligase, can then catalyze the transfer of the active Ub to a lysine residue on the target substrate (88, 168).

For yeast and humans, a single E1 enzyme is responsible for activating all downstream reactions. The yeast E1, *UBA1,* was shown to be essential for growth and mutations in the mammalian E1 are lethal (216, 373). Specificity of the UPS is achieved by the expression of several different E2 enzymes (and E2 isoforms) (246, 453) and a large number of E3 enzymes. Although E1 serves as an activating enzyme for all E2 enzymes, a single E2 may be responsible for conjugating several different E3 ligases. The E2 enzymes all contain a core domain of approximately 150 amino acids (168); however, certain families of E2 enzymes may exhibit differential N- or C-terminal extensions that help to facilitate specific interactions. Despite their similarities, the E2 enzymes have specific roles in very different biological functions, likely because of the specific interactions between E2 and E3 (168, 217, 439).

Ubiquitination can result in a variety of possible consequences; therefore, the interpretation of the Ub signal is important for modulating the host cell's processes. It is likely that the signal relies on a variety of conditions, including the subcellular

localization of the substrate, the topology of the conjugated Ub and/or the number of Ub present on the substrate. It has been suggested that proteins targeted for degradation by the proteasome contain Ub linked from C-terminal Glycine-76 of one Ub to the internal Lysine-48 of the adjacent Ub (71) while those substrates targeted for nonproteolytic signaling have chains linked by Glycine-76 to Lysine-63 of the protein-bound Ub monomer (219, 528). It is now known that the specificity of the ubiquitination processes is due to different sequence and/or structural motifs present on the substrate that are recognized by specific E3 ligases. Thus, as mentioned previously, the specificity of the UPS is based on the specificity of the E3s.

The E3s are the least defined and most complex of the three enzymes required for ubiquitination. The family of E3s, which are responsible for binding E2 and the substrate, can be subdivided into two subgroups based on the presence of specific domains: those containing HECT (homologous to E6-AP carboxyl terminus) domains (231) and those containing RING finger motif (really interesting new gene) domains (337). HECT domain containing E3s all have an amino acid region consistent with the carboxy-termimus of E6-AP, which is the prototype member of the family. E6-AP is responsible for the ubiquitination of the E6 protein of human papillomavirus types 16 and 18 (231, 232). This domain contains a highly conserved cysteine residue that is essential for the transfer of Ub from E2 to E3 and the subsequent ubiquitination of the substrate (293, 495). RING finger domain E3s, which can be single or multi-subunit, contain a consensus sequence defined by a pattern of Histidine and Cysteine residues which likely serve as zinc binding sites (168, 250, 439). The RING E3s are further divided into two different subgroups based on the presence of either a histidine or a cysteine reside in the fifth coordination site, RING-HC and RING-H2, respectively (95, 168, 250). Unlike the

HECT E3s, there is no evidence to suggest that the RING E3s have an intermediate step. Instead, it appears as though they are able to directly transfer Ub from E2 to the target Lysine on the substrate (622).

Ubiqutin-mediated proteolytic destruction can be regulated at the level of ubiquitination or proteasome activity. Due to the vast number of processes Ub is involved in, the UPS must be highly controlled and maintained. There are multiple methods of maintaining substrate specificity, including specific recognition of residues, the use of ancillary proteins and the modification of substrates and/or ubiquitination enzymes (e.g. phosphorylation) (168). Additionally, recent studies have focused on the roles of deubiquitinating enzymes on both regulating the UPS as well as providing enhancement of Ub signals.

The proteasome

The proteasome is the principal method for the targeted degradation of proteins within the cytosol and nucleus. The 26S proteasome is composed of two major complexes, a 20S catalytic particle (CP) and a 19S regulatory particle (RP) (168). The 20S particle is barrel-shaped and contains 2 different rings, in duplicate. The two outer rings each contain 7 alpha subunits while the two inner rings are each composed of 7 beta subunits. This particle contains the sites of proteolytic activity and will be discussed in more detail later.

In cells, the 20S CP of the proteasome can exist by itself as well as 'linked' to one or two of the 19S RPs at the top and/or bottom of the barrel. The 19S RP contains two multi-subunit complexes referred to as the lid and the base. The base contains six homologous ATPase subunits and three non-ATPase subunits (170). The ATPase subunits, which are found in a variety of cellular machines (36, 417, 427), are very

conserved among eukaryotes. They interact with the alpha subunits on the 20S CP (51, 105, 147) and are important for 'gating' the entrance to the core of the proteasome. In fact, the base of the 19S RP serves as a chaperone for the unwinding and translocation of targeted proteins into the catalytic core (51, 179, 538). The lid is a 400kDa complex composed of eight non-ATPase subunits. It has a disk-like shape and is able to attach and detach as a discrete entity from the RP base (168, 258, 339). Unlike the base, each of the eight subunits of the lid complex contain one of two motifs, a PCI domain (proteasome, COP9, eIF3) or the MPN domain (Mpr1, Pad1, NH_2-terminal) (168, 218). Since all of the subunits are non-ATPases, the exact function of the lid during the enzymatic activities of the proteasome is unclear however, it is required for Ub-mediated protein degradation (169).

As mentioned above, the 20S CP contains the proteolytic activity of the 26S proteasome (43). Solving the crystal structure allowed researchers to better understand both the structure and function of the proteasome (180, 535). The 20S core is a hollow barrel structure composed of 4 stacked rings. The two outer rings, which are identical to each other, are composed of seven alpha subunits, α1-α7. In eukaryotes, the two inner rings each contain seven beta subunits, β1-β7 in eukaryotes. Three of these subunits are catalytically active, β1, β2 and β5, each of which is associated with one of the three major catalytic activities of the proteasome: caspase-like (cleavage after the acidic amino acids), trypsin-like activity (cleavage after hydrophobic amino acids), chymotryspin-like activity (cleavage after the basic amino acids), respectively (112, 278, 414). In archaea, however, all the beta subunits are identical (535). In mammals, following IFN-γ or other immune stimulation, the three catalytically active beta subunits expressed in the 'constiutive' 26S proteasome are replaced by three other subunits, β1i (LMP2), β2i

(LMP10) and β5i (LMP7) (43, 168). This 'new' proteasome is referred to as the immunoproteasome and is thought to be involved in the production of peptides able to be presented by major histocompatability complex class I (MHC I) molecules (280, 612).

The amino-termini of the alpha subunits are important for obstructing access to the catalytic core of the proteasome. For substrates to enter into the core, these amino tails must be moved/rearranged. In fact, deletion of the amino-terminal tail of the α3 subunit resulted in constitutively active proteolytic activity (179). To avoid uncontrolled or unnecessary degradation, the 20S CP is expressed in an inactive or repressed state. Peptidase activity is activated by a variety of stimuli, including the formation of a complex between CP and RP (3, 30), interaction with specific complexes (460, 536, 584, 608) or treatment with mild chemicals (170).

UPS AND VIRAL INFECTIONS

As discussed in Chapter 4, the virus lifecycle is intimately tied to the host cell it inhabits; therefore, it is often necessary for the virus to parasitize host cell machinery during the course of infection. The UPS is a key modulator of a variety of different cellular processes, so it is not surprising that many viruses have evolved mechanisms to manipulate the UPS to their advantage. This manipulation can have a variety of positive outcomes for the virus, including viral evasion of the host's immune response, promotion of viral entry or release, transcriptional regulation of viral or host proteins, enhancing viral replication and/or inhibiting apoptosis (20, 153, 512). Since the first discovery of a papillomavirus protein that directly interacts with a cellular E3 ligase (232, 494), the number of viruses demonstrated to manipulate the UPS has grown dramatically.

Many different viruses are able to promote the degradation of major MHC I molecules or interfere with the generation of viral peptides presented by the MHC I

molecules. Three different proteins expressed by human cytomegalovirus (HCMV; a member of the *Herpesviridae* family), US10, US2 and US11, have been shown to interact with MHC I. US10 is responsible for delaying the trafficking of MHC I from the ER, however, this interaction has not been shown to result in degradation of MHC I (149). On the other hand US2 and US11 are directly involved in the downregulation of MHC I. It is thought that these proteins cause the MHC I molecules to disassociate from the lumen of the ER, resulting in their polyubiquitination and subsequent degradation (275, 515). EBNA-1, a viral protein encoded by Epstein-Barr virus (EBV), contains a glycine-alanine rich domain thought to be involved in inhibiting MHC I presentation, presumably by preventing the degradation of viral antigens (309, 310). It is suggested that this inhibition is a mechanism by which EBV hides from the host's immune response during periods of latency, as EBNA-1 expression is associated with latency in B cells (309, 310). Several herpesviruses express proteins which contain PHD (plant homeodomain) motifs, which are related to the RING-finger motifs common to some E3 ligases. Kaposi's sarcoma-associated herpesvirus (KSHV; human herpesvirus 8) expresses two different membrane-bound proteins, K3 and K5 (also called MIR1 and MIR2, respectively), which are able to downregulate MHC I expression (95, 237). MK3, a ER membrane-associated proteins expressed by murine γ-herpesvirus, also causes the ubiquitination and degradation of MHC I molecules (44).

The downregulation of MHC I molecules is effective for many herpesviruses viruses; however, other viruses have alternative means for avoiding the host's immune response. HIV-1 encodes a membrane-bound protein Vpn which has been shown to downregulate CD4 expression (506). Vpn-induced degradation of CD4 requires a competent UPS and the C-terminal lysines present in CD4, which are thought to be the

sites of ubiquitination (506). A few members of the *Paramyxovirinae* family have evolved mechanisms to evade the host's innate immune response. The V proteins of both mumps virus, simian virus (now parainfluenza virus 5) and human parainfluenza virus inhibit IFN signaling by inducing the degradation of STAT1 or STAT2 (177).

Interestingly, a few viruses are able to manipulate the UPS to promote viral entry or trafficking through the cell. If proteasome activity is impaired or blocked, incoming influenza virus becomes marooned within the endosomes of the cell, suggesting that influenza entry is dependent upon the ubiquitin/vacuolar protein sorting pathway (265). The UPS also appears to be required for the entry of murine coronavirus, as inhibition of the proteasome resulted in an accumulation of viral particles in the endosomes and lysosomes (617). Entry of minute virus (parvovirus) does not require the proteasome; however, its activity is essential for viral translocation into the nucleus (472, 473). The exact mechanism(s) of the UPS involvement in viral trafficking, however, is not yet clear.

Several viruses have mechanisms to avoid apoptosis. This is particularly beneficial, as it allows the virus time to replicate to high levels before the host cell shuts itself down. One common apoptosis-associated target for virus-mediated degradation is p53. The human papillomavirus E6 protein effectively targets p53 for ubiquitination and degradation by forming a complex with the E3 ligase, E6-AP (494) and two adenovirus proteins, E1B 55K and E6 orf6, interact with p53 and target it for degradation (452, 530, 609). Coxsackievirus B3 (CVB3) improves its replication by interfering with the host's cell cycle. This is achieved by target targeting both p53 and cyclin D1, both involved in cell cycle regulation, for degradation (345). As with the other viruses mentioned, the targeted degradation of p53 by CVB3 may also allow the infected cells to avoid apoptosis (24, 153).

In addition to targeting p53 for degradation, CVB3 has other methods of manipulating the UPS. Treatment of cells with proteasome inhibitors resulted in a dramatic decrease in viral RNA transcription and protein translation (344). Recent studies have indicated that the importance of the UPS may reside in the activation of the extracellular signal-regulated kinase (ERK) pathway, which has been shown to be important for CVB3 replication (343). Proteasome inhibition resulted in an inhibition of ERK signaling which appeared to correlate with the proteasome inhibitor-mediated reduction in CVB3 replication (593).

The UPS has also been shown to be involved in the release and maturation of retroviruses. The first indication that the UPS may be involved in retrovirus infection came when researchers discovered the presence of an abundance of free ubiquitin within their particles, although the source of the ubiquitin is not known. Further, depletion of ubiquitin from the host cell by inhibition of the proteasome reduces Rous sarcoma virus, HIV-1 and HIV-2 viral release (362). More in-depth studies indicated that the lysine residues on the Gag polyprotein were ubiquitinated (362, 537). It has been suggested that inhibition of the proteasome impaired the monoubiquitination of Gag on these residues, thereby preventing the release and maturation of viral particles (507).

RATIONALE

Silencing the expression of multiple subunits of the proteasome had a detrimental effect on WNV infection and/or replication. Based on this interesting finding, the goal of these studies was to explore the role of the proteasome in more detail and to confirm its requirement for WNV infection/replication in a siRNA-independent manner. To this end, the effect of chemical inhibitors of proteasome activity was examined for their ability to

impair WNV entry and WNV translation/replication. It was *hypothesized* that inhibiting the proteasome would have a detrimental effect on WNV replication.

Materials and Methods

CELL LINES AND THEIR MAINTENANCE

The cell lines utilized for the following experiments are HeLa rpool cells and Huh7 rpool cells as discussed in Chapter 4. Their maintenance is identical as described in that chapter.

WNV AND WNV VLPS

All of the WNV VLP infections were carried out using WNV hFLuc VLP (See Appendix 1). These VLPs are similar to the WNV FLuc VLP used in Chapter 4; however, the FLuc gene (*Photinus pyralis*) encoded in the replicon has been replaced with a human codon-optimized FLuc, hFLuc (derived from Promega; plasmid PGL2), which gives better signal than the FLuc gene in mammalian cells. These WNV hFLuc VLPs (WNR ChLuc2A NS1-5) produce approximately 5-fold more FLuc signal compared to the *Photinus pyralis* WNV FLuc VLP (data not shown), thus making it easier to detect differences in FLuc expression following proteasome inhibitor treatment. The WNV stock used in this chapter is identical to the one described in Chapter 2 (wild type WNV, not WNV^{HS}).

LUCIFERASE ASSAYS

FLuc assays were utilized to monitor infection/replication of the WNV hFLuc VLPs. To assay FLuc activity, media was removed from cell monolayers and 50µl of FLuc substrate, consisting of 4 parts FLuc lysis buffer (40mM Tricine, 8mM Magnesium acetate, 33mM DTT, 0.1% Triton-X-100) and 1 part FLuc Steady-glo substrate

(Promega), was added to each of the wells. The plates were shaken gently for 30 seconds and read on a Centro XS^3 LB 960 microplate luminometer (Berthold Technologies). To measure RLuc activity, 50µl RLuc substrate (100mM EDTA, 10mM Tris, 5µg/ml CTZ) was added directly to the wells (on top of the FLuc substrate). The plate was shaken for 30 seconds and re-read on the luminometer.

WNV ANTIGEN ACCUMULATION ASSAY (ELISA)

To assay the effect of the proteasome inhibitors on WNV antigen accumulation, HeLa or Huh7 cells were plated onto 96 well plates and treated with the appropriate inhibitor and/or WNV VLPs. Following incubation, an ELISA assay was performed as described in Chapter 4.

CELL VIABILITY (MTT) ASSAY

Cell viability was determined by a standard MTT assay as described in Chapter 4.

VIRUS YIELD ASSAY

Monolayers of cells (plated in 96-well plates) were infected with WNV (MOI = 0.05) for approximately 1hr at 37°C. Following this incubation, the virus was removed and dilutions of two different proteasome inhibitors, MG132 or proteasome inhibitor-1 (PS1) (dissolved in DMSO) diluted in MEM+++ containing, in all dilutions, a final concentration of 1% DMSO were added to the cells. Supernatants from the treated cells were harvested approximately 24 hours post-infection. Titrations of the harvested supernatants were performed on Vero cell monolayers to determine virus yield from the treated cells. In short, Vero cells were infected with dilutions of the harvested cell supernatants for approximately 1 hour. Following infection, the virus was removed and the cells were re-fed with a semisolid overlay containing 0.6% tragacanth (MP

Biomedicals) to prevent virus diffusion and incubated for 36 hours. Viral foci were visualized by immunohistochemistry as described in Chapter 2.

IMMUNOFLUORESCENCE ASSAY

Monolayers of Huh7 and HeLa cells, plated onto chamber slides, were incubated with 4μM MG132 or PS1 for approximately 2 hours and then infected with WNV hFLuc VLP for an additional 24 hours. Following the infection, the cells were assayed by IFA as described in Chapter 2. The fixed cells were probed with the following antibodies: a monoclonal goat anti-NS3 (R&D Systems), a monoclonal mouse anti-NS1 (Eiji Konishi; Kobe University, Kobe, Japan), Alexa Fluor 488 donkey anti-goat IgG (Invitrogen) and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen). Following incubation with the secondary antibody, monolayers were counterstained with DAPI (500ng/ml) and Alexa Fluor 594-conjugated Phalloidin (kindly provide by R. Davey).

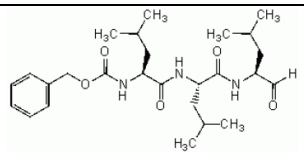
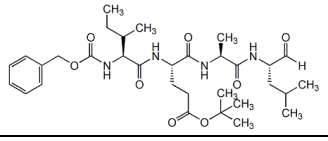
Results

INHIBITION OF PROTEASOME ACTIVITY RESULTS IN AN IMPAIRMENT IN WNV INFECTION AND/OR REPLICATION

As mentioned in Chapter 4, silencing specific subunits of the proteasome reduced the ability of WNV to infect and/or replicate efficiently within the host cell. Although siRNA technology is a powerful tool, the presence of off-target silencing effects (242, 243, 318, 450, 492) can skew or confound interpretations. Therefore, we validated the results derived from the post-translational silencing experiments by examining WNV infection and replication in the presence of two readily available proteasome inhibitors, MG132 and Proteasome Inhibitor-1 (PS1) (Table 5.1). Both of these drugs are highly

specific, potent and reversible inhibitors of the chymotrypsin-like activity of the proteasome (47).

TABLE 5.1: PROTEASOME INHIBITORS.

Compound Name	Structure	Additional information
MG132		Sequence: Z-Leu-Leu-Leu-CHO Cell-permeable, reversible inhibitor of chymotrypsin activity of proteasome
Proteasome Inhibitor-1 (PS1)		Sequence: Z-Ile-Glu(OtBu)-Ala-Leu-CHO Cell-permeable, reversible inhibitor of the chymotrypsin activity of the proteasome

To determine the effect of the inhibitors on WNV infection, Huh7 and HeLa cells, two cell lines in which the original siRNA screen was performed, were pre-treated with the indicated doses of the two drugs prior to infection with WNV VLPs expressing FLuc and maintained on cells through the FLuc accumulation period. Although the initial screen was performed using WNV FLuc VLP, these experiments (and the following experiments), were performed using WNV hFLuc VLP. As described in the Materials section, the replicon encapsulated in this VLP is identical to WNV FLuc VLP with the exception of the FLuc gene, which, in WNV hFLuc VLP, has been codon-optimized for more efficient expression in eukaryotic cells (Promega). To assay WNV replication, we monitored both FLuc expression and WNV antigen accumulation. To measure WNV antigen accumulation, the treated cells were probed, via ELISA (as described in Materials and Methods in Chapter 4) using a WNV polyclonal antibody present in a mouse hyperimmune ascites fluid.

Monolayers of HeLa or Huh7 cells were treated with the indicated doses of each inhibitor for approximately two hours prior to WNV hFLuc VLP infection. Since

inhibiting the proteasome could have some potentially lethal effects on normal cellular functions, thereby confounding our interpretations, the cells were monitored for viability using an MTT assay and RLuc assay, as described in Chapter 4. To simplify presentation of the results, however, only the MTT values are depicted in the following graphs, although RLuc activity correlated well with the MTT values.

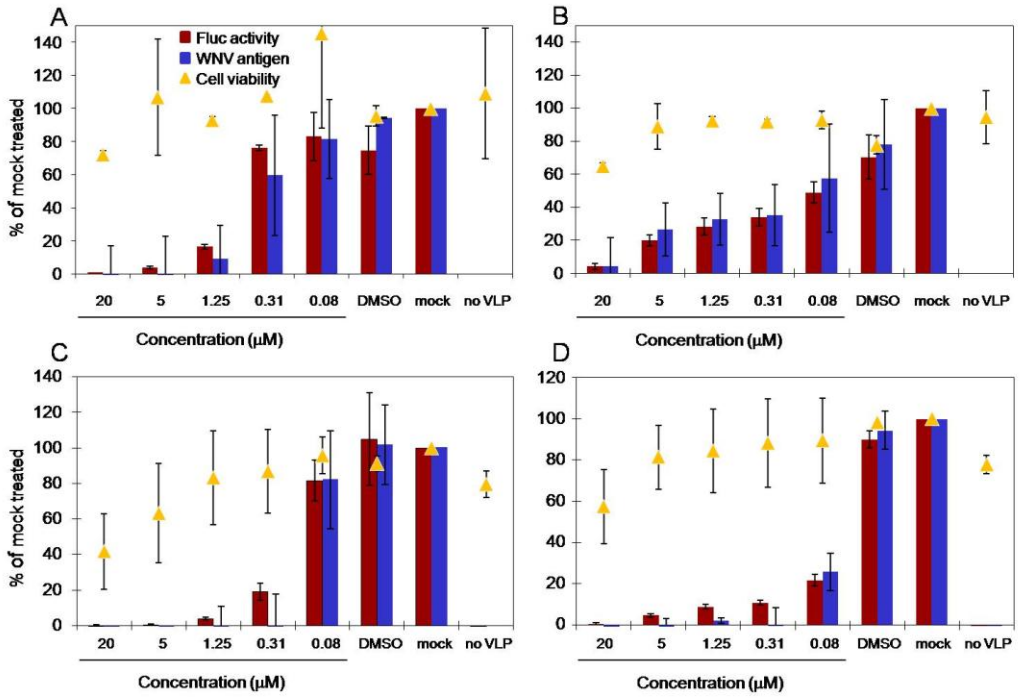


FIGURE 5.1: WNV VLPS ARE SENSITIVE TO PROTEASOME INHIBITORS. Monolayers of Huh7 (Panels A and B) and HeLa (Panels C and D) were treated with the indicated doses of either MG132 (A and C) or PS1 (B and D) for 2 hours prior to WNV VLP infection. Twenty-four hours post infection, the treated cells were assayed for FLuc activity, WNV antigen accumulation or cell viability (See Materials and Methods). Data is presented as the percent of activity compared to WNV VLP-infected, mock-treated cells. Error bars represent the standard deviation from the mean between three independent experiments.

Each of the inhibitors was dissolved in DMSO. The highest dilution of the inhibitors used in the following studies resulted in a 1% DMSO solution. Therefore, to keep DMSO concentrations equal between wells, subsequent dilutions of each of the

inhibitors were performed in MEM+++ supplemented with 1% DMSO. Additionally, to mimic the conditions the inhibitor-treated cells were exposed to, a set of control wells were also treated with 1% DMSO. For both cell lines, treatment with the highest dose of drug (20μM) resulted in a greater than 50% loss of cell viability; however, the other treatment doses did not appear to have any significant effects on cell viability compared to mock-or DMSO-treated cells (Figure 5.1, A-D). At doses of MG132 and PS1 that were not toxic, there was a reduction in both WNV-encoded FLuc activity and WNV antigen accumulation. In Huh7 cells, as little as 1.25μM of MG132 (Figure 5.1A) resulted in a greater than 80% reduction in both FLuc activity and WNV antigen accumulation compared to media- or DMSO-treated cells. Similar to MG132, treatment with PS1 also dramatically impaired FLuc activity and WNV antigen accumulation in Huh7 cells (Figure 5.1B). PS1 treatment was slightly less effective than MG132 in these cells, with a 60-70% reduction in WNV infection/replication at 1.25μM (compared to the 80% reduction in activity observed following MG132 treatment); however, the inhibitory effect of PS1 appeared to be sustained at lower doses compared to MG132 (a reduction in activity of approximately 60% with 0.31μM PS1) (Figure 5.1B).

Both MG132 (Figure 5.1C) and PS1 (Figure 5.1D) were extremely effective in inhibiting WNV infection/replication in HeLa cells, as well. In fact, both of the inhibitors appeared to be more effective at blocking WNV VLPs than in Huh7 cells. MG132 pretreatment of HeLa cells resulted in almost an 80% impairment of WNV activity, as measured by FLuc activity and antigen accumulation, with a dose as low as 0.31μM (Figure 5.1C). PS1 pretreatment, which appeared to be more effective than MG132, resulted in a reduction in FLuc activity and WNV antigen accumulation of approximately 70% compared to mock- or DMSO-treated cells with as little as 0.08μM (Figure 5.1D).

Since both MG132 and PS1 are reversible inhibitors of the proteasome, we wanted to determine whether removing inhibitor treatment from cells would restore WNV infection/replication to levels observed in mock-treated cells. This would help to ensure that the inhibitor treatment did not result in any permanent damage to normal cellular processes which would prevent WNV VLP infection or replication. Monolayers of Huh7 cells and HeLa were treated with 20, 5, 1.25, 0.31, 0.08, or 0μM of MG132 and PS1, or DMSO alone (diluent control) for 2 hours. Following this incubation, the inhibitor was removed from half of the wells. These wells were washed twice to try to remove as much of the drugs as possible and then allowed to 'recover' for an additional 2 hours. The other wells used in these experiments were maintained with the inhibitor in the incubator over this time period. All of the cells, both with and without the inhibitors, were infected with WNV hFLuc VLPs for 24 hours prior to harvest. As with the previous experiment, monolayers were assayed for FLuc activity, WNV antigen accumulation and cell viability.

Consistent with Figure 5.1, Huh7 (Figure 5.2A) and HeLa (Figure 5.2B) cells that were incubated with either MG132 or PS1 demonstrated a dose-dependent reduction in WNV-dependent FLUC activity and WNV antigen accumulation. However, WNV VLP infection/replication in both Huh7 (Figure 5.2A) and HeLa (Figure 5.2B) cells in which the MG132 and PS1 treatments were washed off was nearly identical to mock- or DMSO-treated cells (between 80-100% of DMSO-treated cells) for all doses of the inhibitors. These data suggested that treatment of the cells did not have a non-specific effect the cell's ability to either be infected by WNV VLPs or to support WNV VLP replication.

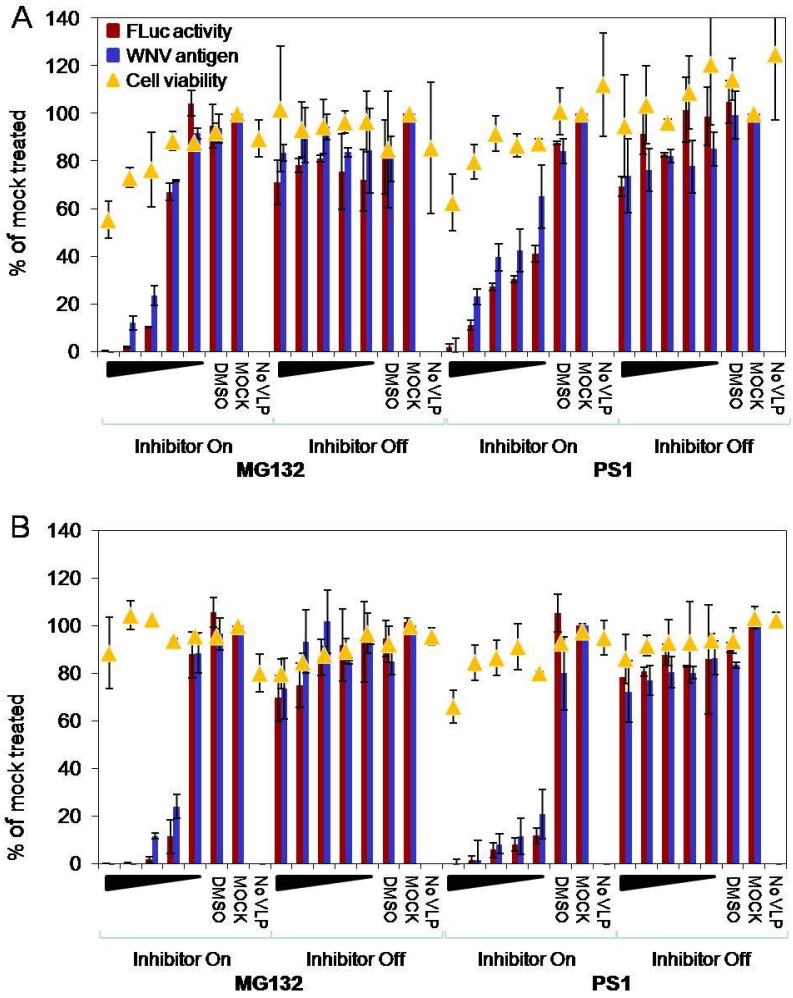


FIGURE 5.2: REMOVING PROTEASOME INHIBITORS RESTORES ABILITY OF CELLS TO SUPPORT WNV INFECTION AND REPLICATION. Monolayers of Huh7 cells (Panel A) and HeLa cells (Panel B) were pretreated with MG132 or PS1 (20, 5, 1.25, 0.31 and 0.08μM) or 1% DMSO for 2 hours. In some wells the inhibitor was removed, washed and allowed to recover for an additional 2 hours (the inhibitor remained on the other wells). Following the 2 hour recovery, all of the recovered and inhibitor-treated cells were infected with WNV VLPs and assayed for FLuc activity, WNV antigen accumulation and cell viability (See Materials and Methods). Data is presented as percent of WNV VLP-infected, mock-treated cells. Error bars represent standard deviations from the mean between two independent experiments.

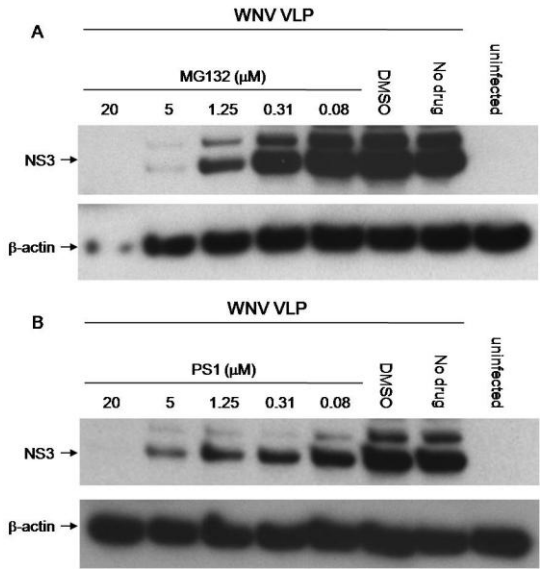


FIGURE 5.3: NS3 PROTEIN EXPRESSION IS IMPAIRED IN PROTEASOME INHIBITOR-TREATED CELLS. Monolayers of HeLa cells were pre-treated with MG132 (Panel A) or PS1 (Panel B) for two hours prior to WNV VLP infection. Twenty-four hours post infection, equal amounts of the treated cell lysates were assayed, by Western blot analysis (See Materials and Methods), for levels of NS3 and β-actin (loading control).

To measure WNV protein expression with a second methodology, WNV protein expression was monitored by Western blot analysis and IFA. For Western blot analysis, cell lysates harvested from monolayers of treated HeLa cells were probed for levels of NS3 protein. Consistent with the previous experiments, monolayers of HeLa cells were pretreated with either MG132 (Figure 5.3A) or PS1 (Figure 5.3B) for 2 hours prior to WNV VLP infection. For comparison, cells treated with DMSO alone or cells not infected with WNV VLPs were assayed alongside. Analysis of the treated cells showed a dose-dependent decrease in NS3 levels compared to media- or DMSO-treated cells. Consistent with the ELISA and FLuc assays, PS1 (Figure 5.3B) was more effective at blocking WNV protein accumulation—as measured by NS3 accumulation—compared to MG132 (Figure 5.3A). The NS3 antibody consistently recognized two bands. The lower band corresponds to the predicted molecular weight of NS3, approximately 70 kd. The

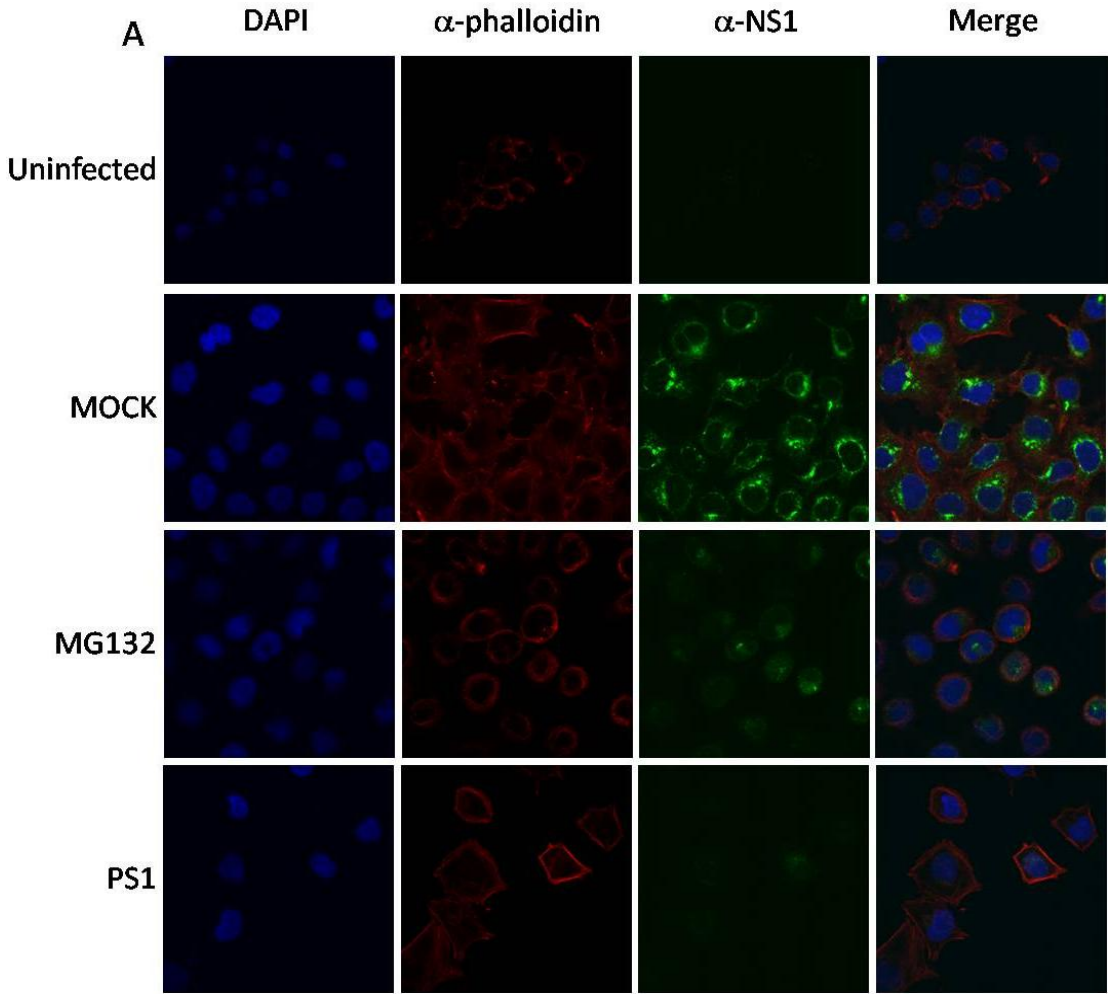
top band may represent uncleaved NS2A-NS3 or NS3-NS4A or, perhaps, the NS2B-NS3 viral protease complex. Levels of β-actin, which served as a loading control, were similar between the treatment groups with the exception of 20μM MG132. This is likely due to cell death, as cytotoxicity has been observed with this dose. The protein expression levels shown in Figure 5.3 are consistent with the WNV VLP antigen accumulation observed following the ELISA and the FLuc expression levels, further supporting the hypothesis that the proteasome is involved in the WNV lifecycle.

IFA analysis of inhibitor-treated HeLa cells was consistent with the Western blot analysis (Figure 5.4). PS1 and MG132 treatment in HeLa cells reduced the detectable levels of both NS1 (Figure 5.4A) and NS3 (Figure 5.4B) compared to DMSO-treated cells. Consistent with our previous studies, PS1 appeared to be more effective at inhibiting WNV protein accumulation than MG132. Interestingly, in HeLa cells, both MG132 and PS1 seemed to be more effective at reducing NS1 protein expression (Figure 5.4A) than NS3. This is a curious finding but is not seen to the same extent in Huh7 cells (results not shown). A phalloidin counterstain of the cells demonstrated that the inhibitor-treated cells did not appear to have any major disruptions or changes in the overall architecture of the cell or any obvious nuclear abnormalities compared to DMSO-treated cells.

THE PROTEASOME IS INVOLVED IN POST-ENTRY STAGES OF WNV INFECTION

Although proteasome inhibition results in a dramatic reduction in both WNV VLP-encoded FLuc activity and WNV antigen accumulation, it is not known at which step(s) in the virus lifecycle the proteasome is involved in. To help separate the early events involved in WNV entry and unpackaging from those involved in WNV translation and replication, HeLa cells were treated with MG132 or PS1 either concurrently or at

various times post-WNV VLP infection. Surprisingly, treatment with either MG132 or PS1 as late as 12 hours post infection resulted in a reduction in WNV-dependent FLUC and WNV antigen expression (Figure 5.5A and 5.5B, respectively).



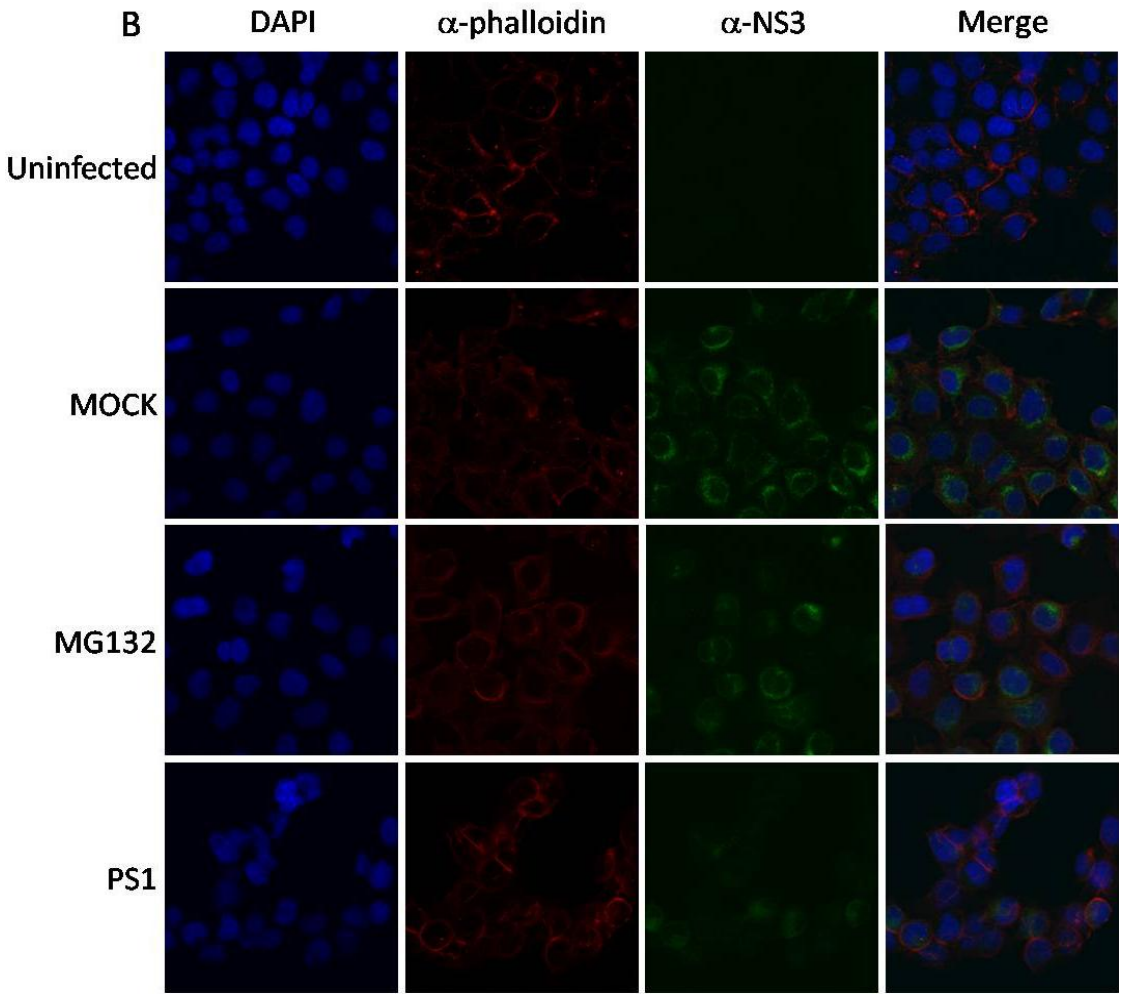


FIGURE 5.4: WNV NS1 AND NS3 EXPRESSION IS REDUCED IN INHIBITOR-TREATED CELLS. IFA on MG132- and PS1-treated HeLa cells. Monolayers of HeLa cells were treated with 4mM MG132 or PS1 and infected with WNV hFLuc VLPs. The cells were fixed and probed for NS1 (Panel A) and NS3 (Panel B). The cells were counterstained with DAPI and phalloidin.

In fact, treatment concurrent with infection and up to 4 hours post-infection resulted in the inhibition of WNV activity to nearly the same extent observed when the drugs were added prior to infection. However, the inhibitory effect of these inhibitors on WNV activity does appear to begin to wane at the later times post-infection with only a 50-60%

reduction in WNV activity at 12 hours post infection with 0.31μM MG132 or 0.08μM PS1 (Figure 5.5). Consistent with the previous studies, PS1 treatment (Figure 5.5B) was more effective than MG132 in these HeLa cells, even at later times post-infection (Figure 5.5). These data suggested that the proteasome may be involved in post-entry stages of WNV infection, likely at the level of translation and/or replication.

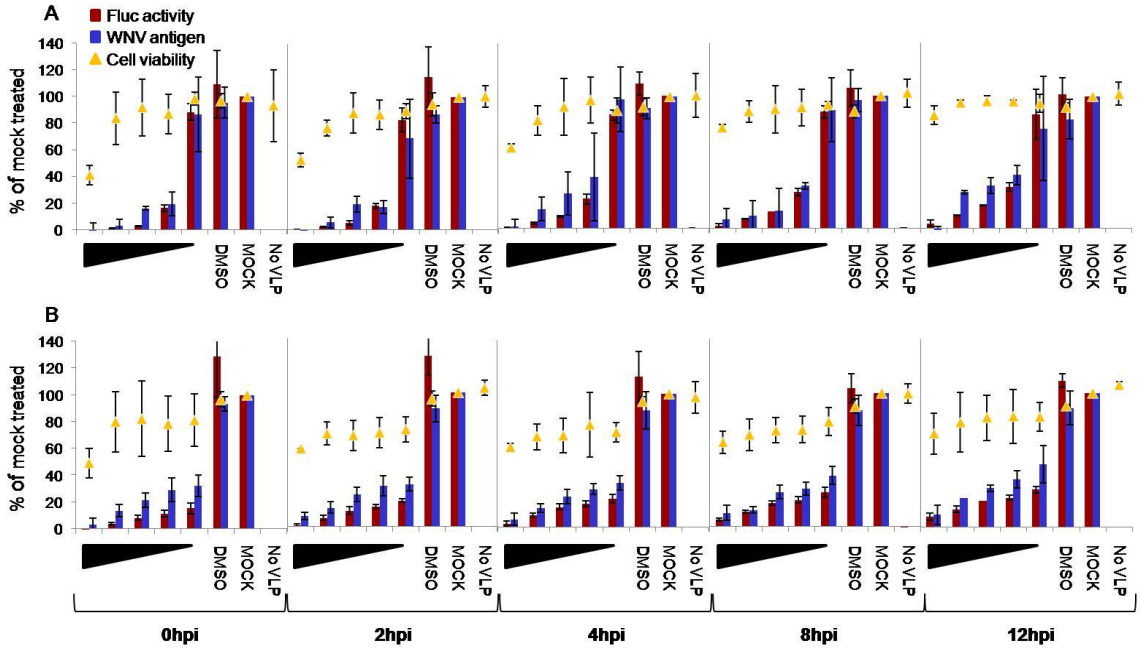


FIGURE 5.5: PROTEASOME INHIBITION IMPAIRS WNV AT POST-ENTRY STAGES OF INFECTION. Time course of proteasome inhibitor treatment and WNV VLP infection. Monolayers of HeLa cells were treated with either MG132 (Panel A) or PS1 (Panel B) concurrent with WNV VLP infection (0hpi) or at the indicated times post-infection and assayed for FLuc activity, WNV antigen accumulation and cell viability (See Materials and Methods). Data is shown as percent of WNV VLP-infected, mock-treated cells and the error bars represent the standard deviations from the mean between two independent experiments.

INHIBITION OF PROTEASOME ACTIVITY IMPAIRS WNV YIELD

WNV VLPs have been shown to mimic WNV infection/replication; however, we wanted to examine the effect of proteasome inhibitor treatment on 'live' WNV infection.

Monolayers of HeLa cells were infected with WNV for 1 hour prior to MG132 or PS1 treatment and assayed for WNV release into the supernatant twenty-four hours later. Titrations performed on supernatants harvested from the treated cells indicated that proteasome inhibitor treatment does, in fact, dramatically reduce the yield of WNV particles (Figure 5.6). MG132 treatment of HeLa cells, as observed in previous experiments, was slightly less effective at impairing WNV production compared to PS1 treatment; however, the treated cells still demonstrated a near 80% loss in secreted infectious particles compared to mock-treated cells with as little as 0.16μM (Figure 5.6).PS1, however, demonstrated inhibition of WNV yield similar to that of MG132 at a concentration of only 0.032μM (Figure 5.6). The inhibition of WNV yield was consistent with the inhibition of WNV VLP-encoded FLuc activity and antigen accumulation. These data indicate that inhibiting proteasome activity in these cell lines severely impairs WNV infection, suggesting that the proteasome is an important cellular co-factor during WNV infection. Since post-treatment of both inhibitors was effective in inhibiting FLuc activity and WNV antigen accumulation, it is likely that the impairment of WNV particle production is due to a decrease in viral translation or replication. Although not tested, it is still possible that inhibition of the proteasome impairs WNV packaging and release in addition to viral translation or replication. However, because our initial studies were performed with WNV VLPs (which are not packaged), it is more likely that the decrease in virus yield is due to a deficit in either proteins available for efficient replication or a decrease in the number of genomes available for packaging.

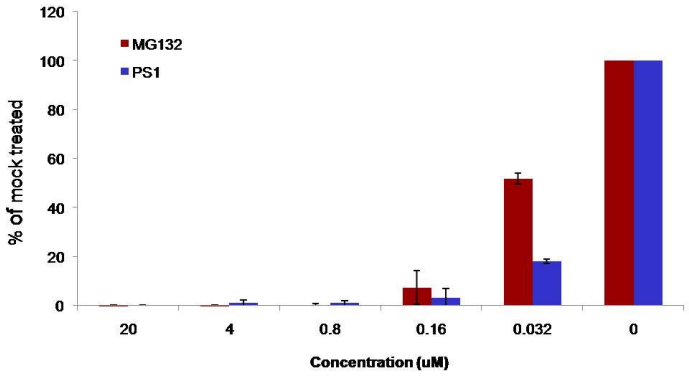


FIGURE 5.6: PROTEASOME INHIBITION IMPAIRS WNV YIELD. Monolayers of HeLa cells were infected with WNV for 1 hour, the virus was removed and the indicated concentrations of MG132 or PS1 were added to the infected cells. Twenty-four hours post infection/treatment the supernatants were removed from the treated cells and the titration of each sample was determined by IHC (See Materials and Methods). Results are shown as percent virus titer from mock-treated cells. Error bars represent the standard deviation from the mean between two independent experiments.

Discussion

The UPS is the primary mechanism for intracellular degradation of short-lived, misfolded or abnormal proteins. It is responsible for the regulation of a variety of cellular processes including pathways involved in cell cycle regulation, antigen processing, transcriptional regulation, apoptosis, signal transduction and transcriptional regulation (88, 168, 171, 404). Because the UPS is involved in regulating or maintaining a vast array of different cellular processes, it is not surprising that viruses have evolved mechanisms to manipulate the UPS to their own advantage. As mentioned in the introduction, viruses have utilized the UPS to promote its replication by several means, including promoting viral entry and intracellular trafficking (265, 472, 473, 617), improving viral replication/translation (344, 593), evading the immune response and

suppressing apoptosis (44, 95, 177, 237, 309, 506) and promoting efficient viral progeny release/budding (507, 537).

As discussed in Chapter 4, a large scale siRNA library screen of human genes indicated that silencing multiple subunits of the proteasome dramatically reduced WNV infection and/or replication. SiRNAs targeted against many of the subunits resulted in varying degrees of WNV infection/replication inhibition; however, silencing two subunits in particular, PSMA1 and PSMA2, consistently reduced WNV infection/replication by greater than 70% compared to non-targeting negative control siRNAs. In this chapter, we used chemical inhibitors of the proteasome to follow up on the observations from Chapter 4. The data presented here clearly demonstrate that chemical inhibition of the proteasome dramatically reduces WNV replication, consistent with the observations from the siRNA library screen.

Pre-treatment with two well-defined proteasome inhibitors, MG132 and PS1, severely impaired WNV infection and/or replication with as little as 0.3 and 0.08μM, respectively, in both HeLa and Huh7 cells. The proteasome is involved in multiple pathways related to normal and essential cellular processes; thus, it is possible that inhibiting the proteasome had a negative impact on the cells. Because the life of an intracellular pathogen is so closely associated with the life of the host cell, a 'sick' cell would not be conducive to efficient WNV replication, thereby confounding the interpretation of our experiments. However, only at the highest dose of each of the drugs (20mM) were any significant changes in cellular viability or overall cellular characteristics observed. This was important, as it indicated that the impairment of WNV infection and/or replication was due to a direct effect of proteasome inhibition and not a by-product of having unhealthy cells.

For many of the experiments, WNV infection and/or replication was monitored indirectly by assaying either FLuc activity or ELISA antigen accumulation. Therefore, to more directly observe the effects of the proteasome inhibitors on WNV infection and/or replication, protein/antigen levels were examined by both IFA and Western blot analysis. Consistent with our indirect assays, total levels of WNV NS3, measured by Western blot, was dramatically reduced following treatment with either MG132 or PS1. Using a polyclonal antibody against WNV, we found that the expression of all other detectable WNV VLP proteins was reduced, as well. NS3, complexed with NS2B, serves as the viral protease and is responsible for the majority of proteolytic cleavages required for proper processing of the viral polyprotein. Because the proteasome is also a protease, it is possible that disrupting its function (or treatment with the inhibitors) has an impact on the ability of NS2B-NS3 to correctly process the viral polyprotein. However, although the expression of the individual viral proteins was reduced following proteasome inhibitor treatment, the banding pattern between the inhibitor-treated and the media-treated cell lysates was identical (results not shown), suggesting that there was no impairment in WNV polyprotein processing. IFA on inhibitor-treated HeLa cells was consistent with the Western blot analysis. Cells which were treated with either PS1 or MG132 demonstrated reduced levels of both NS1 and NS3 compared to DMSO-treated cells.

Interestingly, when inhibitor-treated cell lysates were probed with a polyclonal WNV antibody, the degree of the inhibition of protein accumulation appeared to vary among the different proteins, with a few proteins demonstrating a more pronounced effect (results not shown). Additionally, the IFA clearly showed a decrease in the level of both NS1 and NS3 following treatment with either MG132 or PS1; however, NS3 appeared to be slightly less sensitive to proteasome inhibition compared to NS1.

This is a very curious finding and could be due to a variety of different reasons. It is possible that the antibody against NS3 is more sensitive than the antibody against NS1, particularly with MG132 treatment. It is also possible that viral proteins have different turn-over rates. Because it has critical roles in both replication and translation, perhaps NS3 is more stable than NS1 and, thus, more likely to be detected. Additionally, NS1 is known to be secreted from infected cells, which could reduce intracellular levels of the protein. Regardless, it is an interesting finding and, whether it is a direct effect of the proteasome or not, needs to be investigated further.

WNV VLP infection was impaired following proteasome inhibitor treatment as late as 12 hours post infection. This suggested that WNV VLPs do not require proteasome activity for processes involved in entry. The reduction in antigen levels at late times post-infection also suggests that, unlike influenza virus (265) or mouse hepatitis virus (617), WNV does not utilize the proteasome for early trafficking of the viral particle within endosomes or for the release of the genome into the cytoplasm. Additionally, virus yield of WNV was dramatically reduced following proteasome inhibition; however, it is unlikely that this is caused solely by an inhibition in viral packaging or release, as seen with retroviruses (428, 507, 537), because our initial studies were performed with WNV VLPs, which cannot be packaged. It is likely that the reduction in viral yield is due to a decrease in either (or both) proteins derived from translation or progeny genomes produced during replication. These studies, however, do not *exclude* the possibility that the proteasome is involved in an aspect of WNV packaging and/or release.

Taken together, these data clearly indicate that the proteasome, or the UPS, serves as a co-factor during WNV translation or replication. It is not known whether the

inhibition in WNV activity is due to a direct decrease in the ability of the viral genome to be translated or the ability of the viral proteins to replicate the viral genome. Inhibition of viral translation would impair the formation of replication complexes and reduce the ability to produce new genomes. On the other hand, a reduction in genome replication could also decrease viral protein expression, due to reduced transcripts available for translation. Teasing out these two steps could be the subject of future studies.

Why is the proteasome important? This question is a much more difficult one to answer, as it could be a number of different reasons. The UPS is involved in a multitude of different cellular processes, including signal transduction, transcriptional regulation, immune signaling and apoptosis, any of which could be important for WNV infection. WNV can evade the innate immune response by blocking the phosphorylation of STAT1 (186, 505); however, there is no evidence to suggest that this impairment is achieved by the degradation of a specific protein. Several groups have shown that WNV induces apoptosis (375, 426, 485, 605, 606) (Rossi & Mason, unpublished), providing a possible step at which the proteasome may be involved. One report implicated p53-induced apoptosis as a mechanism by which WNV induced apoptosis (606), however this has not been linked to activities of the UPS.

More recently, a group has demonstrated that WNV induces the unfolded protein response, a pathway involved in ER stress-mediated apoptosis. This appears to be mediated, in part, by CHOP and includes the degradation of ATF6, a transcription factor associated with the ER, by the proteasome (375). An association between the requirement of the proteasome for WNV replication seen in our studies and the degradation of ATF6, however, has not been shown at this time. ATF6 degradation was observed at 36 hours post infection with 'live' WNV; however, all of our studies were performed at 24 hours

post infection with WNV VLPs, when no observable apoptosis was detected. An association between ATF6 degradation and the requirement of proteasome activity for WNV replication would have to be investigated in more detail.

Many viruses have been shown to target specific proteins for degradation. The immune evasion strategies discussed above often result in the degradation of specific proteins, whether it is STAT, MHC I or other molecules. Non-immune molecules can be targeted, as well. Coxsackievirus B3 appears to target cyclin D3 and p53 (345) for degradation; adenovirus also induces the degradation of p53 (452, 530, 609). Therefore, it is a likely possibility that WNV targets specific proteins for degradation and that disrupting that degradation would have a negative impact on WNV replication. Possible targets for degradation are protein(s) involved in translation. A recent report has shown that poliovirus cleaves the host protein poly(rC)-binding protein (PCBP). This cleavage allows the virus to transition from translation to viral replication (432). When the protein is cleaved, it can no longer function in translation, but still has the ability to promote genome replication. Although this specific example is not associated with protein degradation, it is an interesting consideration that a similar mechanism could aid in the differentiation between WNV translation and replication.

The UPS could also be linked to DRiPs, or defective ribosomal products. The process of translation from information encoded on the viral (or cellular) genome to a complete, functional protein is paved with many potential pitfalls. Mistakes can occur at multiple levels, including the addition of incorrect bases or amino acids, early (or even late) termination of the translated product or the improper folding or splicing of proteins. These errors result in the formation of DRiPs (610, 611, 613). It is expected that these incorrect products would be rapidly degraded by the proteasome, as they could

potentially interfere with the function of the normal protein or other cellular processes. It is thought that these DRiPs are important peptides for generating MHC I antigens; however, it seems unlikely that this is important in our model (Huh7 and HeLa cells cannot respond to MHC I presentation of antigens). It is possible, however, that the inhibition of the proteasome results in an accumulation of WNV-generated DRiPs, which interfere with the effective translation and replication of the genome.

CHAPTER 6: CONCLUSIONS

Viral infection of a host or host cell is a dynamic interaction occurring between the viral pathogen and the cell or cells it infects. This interaction, which can be thought of as a battle for dominance between the virus and the host, shapes the outcome of the viral infection and controls viral pathogenesis. The endpoint of the battle is either the elimination of the virus (cell wins) or the destruction of the cell/host and propagation and transmission of the virus (virus wins); although, in some cases, viruses can cause persistent, long-term infections. The interactions that occur between the virus and the cell/host can be one of two basic responses: (*i*) those that result in more efficient replication and virus spread/transmission or (*ii*) those that result in the elimination of the viral pathogen.

The virus must hijack host proteins, often manipulating the cell's own machinery for its own advantage. WNV interacts with two host proteins, TIA-1 and TIAR, preventing the formation of stress granules, a process that normally allows the host to interfere with viral translation (124). Additionally, WNV uses host proteins, such as EF1α (39) and c-yes (214), to promote its own genome replication and virion maturation, respectively. The virus can also employ mechanisms to hide from the host cell's innate immune response (186, 329, 505, 587), particularly by interfering with the ability of the infected cells to respond to IFN treatment. However, despite this, IFN is a powerful weapon utilized by the host to prevent WNV infection. This is highlighted by the discovery that IFN treatment can inhibit WNV replication (12, 397, 475, 486), and that mice lacking IFNAR are more susceptible to WNV infection than WT mice (483). To better understand WNV pathogenesis, we studied aspects of both sides: interactions that

both promote and impede WNV infection/replication. More specifically, we were interested in understanding how the host cell recognizes WNV infection as well as host proteins that are essential for efficient WNV replication. Thus, the overall hypothesis of this dissertation was: *although WNV recruits cellular factors to successfully replicate and spread, the host cell counteracts the virus by recognizing the replicating genome and producing separate factors which function to inhibit WNV replication.*

Summary

CELLULAR RECOGNITION OF VIRAL INFECTION

Consistent with other reports (230, 360, 558, 625), cell lines deficient in PKR or WT cells treated with a chemical inhibitor of PKR showed a near complete abrogation of IFN induction by the transfection of pIC (Figure 2.5, Figure 2.6). This clearly indicated that PKR is a critical component of recognizing intracellular dsRNA and we hypothesized that PKR is also involved in the cellular recognition of WNV. MEF cells deficient in PKR produced nearly 10-fold lower levels of IFN in response to WNV infection compared to WT cells (Figure 2.5). Treatment of mouse or human cell lines with a chemical inhibitor of PKR (Figure 2.7, Figure 2.8) as well as treatment with PKR-specific siRNAs (Figure 2.9, Figure 2.10) caused a significant impairment in WNV-induced IFN production compared to media treatment alone. Treatment did not, however, have an effect on SeV-induced IFN production. This latter result is consistent with reports indicating that the SeV infections are recognized by the PRR RIG-I (261, 377). This also indicated that the PKR inhibitor did not have a non-specific effect on the ability of cells to produce IFN. Surprisingly, silencing RIG-I expression also resulted in a partial, but significant, reduction in WNV-induced IFN production in two of the three cell

lines tested (Figure 2.10). Interestingly, the cell line which showed no response to RIG-I gene silencing was a cell line unable to respond to IFN treatment. This was an intriguing finding, suggesting that perhaps RIG-I is important in a secondary wave of IFN induction or that RIG-I and PKR signaling pathways are connected. However, this is pure speculation and would have to be examined in more detail to fully understand the importance of the observation.

We next began examining the mechanism by which PKR is inducing IFN expression. Several reports have indicated that PKR is able to signal downstream through the IKK complex to activate NFκB (160, 619). Activated NFκB is able to bind to the IFN-β promoter (PRD II) (424); thus, we examined the ability of WNV to induce NFκB in the absence of PKR. Both the degradation of IκBα (Figure 2.11) and the ability of NFκB to translocate into the nucleus (Figure 2.12) following WNV infection were impaired in PKR-null MEF cells, indicating that PKR is important for WNV-induced NFκB activation in these cells. We examined the time course of this activation and found that IκBα degradation was induced as early as 16 hours post WNV infection, corresponding, as well, with an activation of PKR (Figure 2.13). On the other hand, we did not observe an increase in IRF3 activation until later, around 20-24 hours post infection, consistent with previous reports (143, 145, 505).

The *ex vivo* results outlining the importance of PKR correlated well with our *in vitro* results. When BMDCs harvested from WT mice were treated with the PKR inhibitor, there was a near complete abrogation in WNV-induced IFN production (Figure 3.2). At the same time, the inhibitor treatment had no effect on SeV-induced IFN production. When the inhibitor was used to treat CD11c+ DCs derived from IRF-null mice, there was also a near complete abrogation in IFN production following WNV

infection; however, the IRF3-null DCs demonstrated no impairment in WNV-induced IFN production (Figure 3.3), indicating that IRF3 is not essential for WNV-induced IFN induction in these cells. However, as expected, in the absence of IRF3 there was a deficit in SeV-induced IFN production. Consistent with PKR inhibitor treatments, cells derived from PKR-null mice displayed a significant impairment in their ability to produce IFN following WNV infection (Figure 3.4), even up to 48 hours post infection (results not shown); however, the absence of IRF3 had no adverse effect on WNV-induced IFN synthesis in the BMDCs or in mice (48). Interestingly, contrary to our early hypothesis that PKR is involved during early stages of infection and IRF3 during later stages, IFN production was impaired in PKR-null DCs even at times when IRF3 is expected be active, suggesting that IRF3 is not sufficient for IFN production in this cell type.

VIRAL MANIPULATIONS OF HOST FACTORS

In order to identify host factors that WNV utilizes for its own advantage, we screened a library consisting of 3 individual siRNAs for approximately 5,500 human genes. Following two independent runs through the entire screen and multiple confirmatory screens, we identified ten genes which appear to be essential for efficient WNV infection/replication: ACCN3, ASNS, CEL, MAPK8, RRM1, PNMT, PRSS8, PSMA1, PSMA2 and TNFRSF10B (Table 4.2 and Table 4.3). Expression of three of these genes, CEL, PNMT and PRSS8, could not be confirmed in Huh7 cells (using an Affymatrix gene chip). Of the remaining genes, ACCN3, ASNS, CEL and MAPK8 appear to be important for aspects of WNV entry, unpackaging or initial genome translation, as silencing these genes had no effect on the established replication of a WNV genome (Table 4.2). Interestingly, two of these candidate genes, PSMA1 and PSMA2, encode subunits of the proteasome. Further analysis indicated that inhibiting

proteasome activity with chemical inhibitors (MG132 and PS1) in Huh7 and HeLa cells resulted in impaired WNV infection and/or replication (Figure 5.1, Figure 5.2). This corresponded to a decrease in WNV protein/antigen expression, specifically NS1 and NS3 (Figure 5.3, Figure 5.4), as well as a decrease in WNV particle release (Figure 5.6) compared to untreated cells. Interestingly, treatment with MG132 and PS1 up to 12 hours post infection still caused a reduction in WNV replication (Figure 5.5), suggesting that the proteasome is involved in post-entry stages of WNV infection.

The more severe forms of West Nile virus disease, including encephalitis and meningitis, are often associated with elderly populations. This may be due to specific age-related changes in the immune response, termed immunosenescence. This is most often observed in the activation and expansion of T cell populations (381, 410). The generation of MHC class I peptides by the proteasome can also be affected by age (390), indicating that a decrease in proteasome function later in life could contribute to more severe viral infections. The ability of cells to properly generate or present MHC class I peptides could have an effect on the ability to control WNV infection. Recent studies have indicated that macrophages derived from elderly patients show increased levels of TLR3 compared to macrophages derived from younger patients, resulting in increased cytokine production (288). This group suggested that the increase in cytokines could be associated with an increase in the ability of WNV to cross the blood-brain barrier, indicating a possible explaination for increased neurological disease in elderly patients (288).

However, age-related changes or other defects (not associated with age) in the function (or expression) of non-immune-related proteins could also have an impact on the pathogenesis of WNV infection. Although I could find no direct correlation between the

10 identified candidate genes and aging, several genes are associated with diseases which could potentially have an impact on WNV disease progression. Disregulation of many of the genes appears to function in cancer development. An inhibitor of ACCN3 is an approved treatment for essential hypertension and blocking TRAIL, a ligand for TNFRSF10B, resulted in an increase in inflammation in the CNS (213). Thus, we can hypothesize that defects in TNFRSF10B could result in a predisposition to developing neurological diseases; however, an association with TNFRSF10B and aging or in WNV infection has not been determined. A few of the other genes, including MAPK8 and ACCN3 are associated with insulin receptor signaling although, again, a link between insulin and WNV infection has not been investigated. Potential links between these ten genes required for efficient WNV replication and a predisposition for developing severe disease is something that could be investigated further.

Reflections and Future Directions

THE ROLE OF PKR

Our finding that PKR is essential for efficient WNV-induced IFN induction was exciting and, as far as we know, the first demonstration that PKR is utilized for PRR signaling during WNV infection. Whether PKR serves directly as a PRR or as a downstream signaling molecule recruited by a different PRR is not yet clear. In addition to serving as a direct PRR, there are other signaling pathways the PKR may be acting on (Figure 6.1).

One signaling pathway, which we have explored in some detail, involves the activation of NFκB through PKR (Figure 6.1A). PKR, activated by dsRNA, recruits several members of the TRAF family, TRAF2, TRAF5 and/or TRAF6 which, in turn,

activates the IKK complex. The activation of the IKK complex results in the phosphorylation of IκBα, freeing NFκB and allowing it to translocate into the nucleus and bind to the IFN-β promoter. Based on our observation that PKR-null MEF cells show impaired WNV-induced NFκB activation, this seems a likely prospect; however, we did not demonstrate a direct link between NFκB and WNV-induced IFN induction. Examining WNV-induced IFN synthesis in the absence of NFκB activity (using NFκB inhibitors or IκBα dominant negative mutants) may help delineate this signaling pathway.

PKR can also be linked to the TLR3-mediated signaling pathway (Figure 6.1B). In this case, dsRNA interacts with TLR3 which recruits TRIF and signals downstream via TRAF3, IKKε and TBK1 to activate IRF3. PKR has been shown to associate with TRAF3 (416), thus establishing a link between PKR signaling and TLR3 signaling. Alternatively, PKR can also be linked to RIG-I/mda-5 signaling (which also signals downstream to activate IRF3), though its interaction with TRAF3 (Figure 6.1C). However, recent work indicating that WNV has mechanisms in place to block TLR3-mediated signaling (15, 587), suggests that, even if PKR is involved in TLR3-mediated signaling, this pathway is not likely essential for WNV-induced IFN production. The involvement of PKR in RIG-I- or mda-5-mediated signaling is an intriguing prospect, particularly in light of the fact that silencing RIG-I in two different human cell lines resulted in a partial, but significant, reduction in WNV-induced IFN production. However, in mDCs, unlike PKR, IRF3 is not essential for WNV-induced IFN production, arguing against the possibility of PKR being directly involved in RIG-I-mediated IRF3 activation. It is possible that PKR and RIG-I and/or mda-5 signaling pathways are linked,

perhaps through similar downstream signaling molecules; however, more careful dissection of the signaling pathway will have to be done.

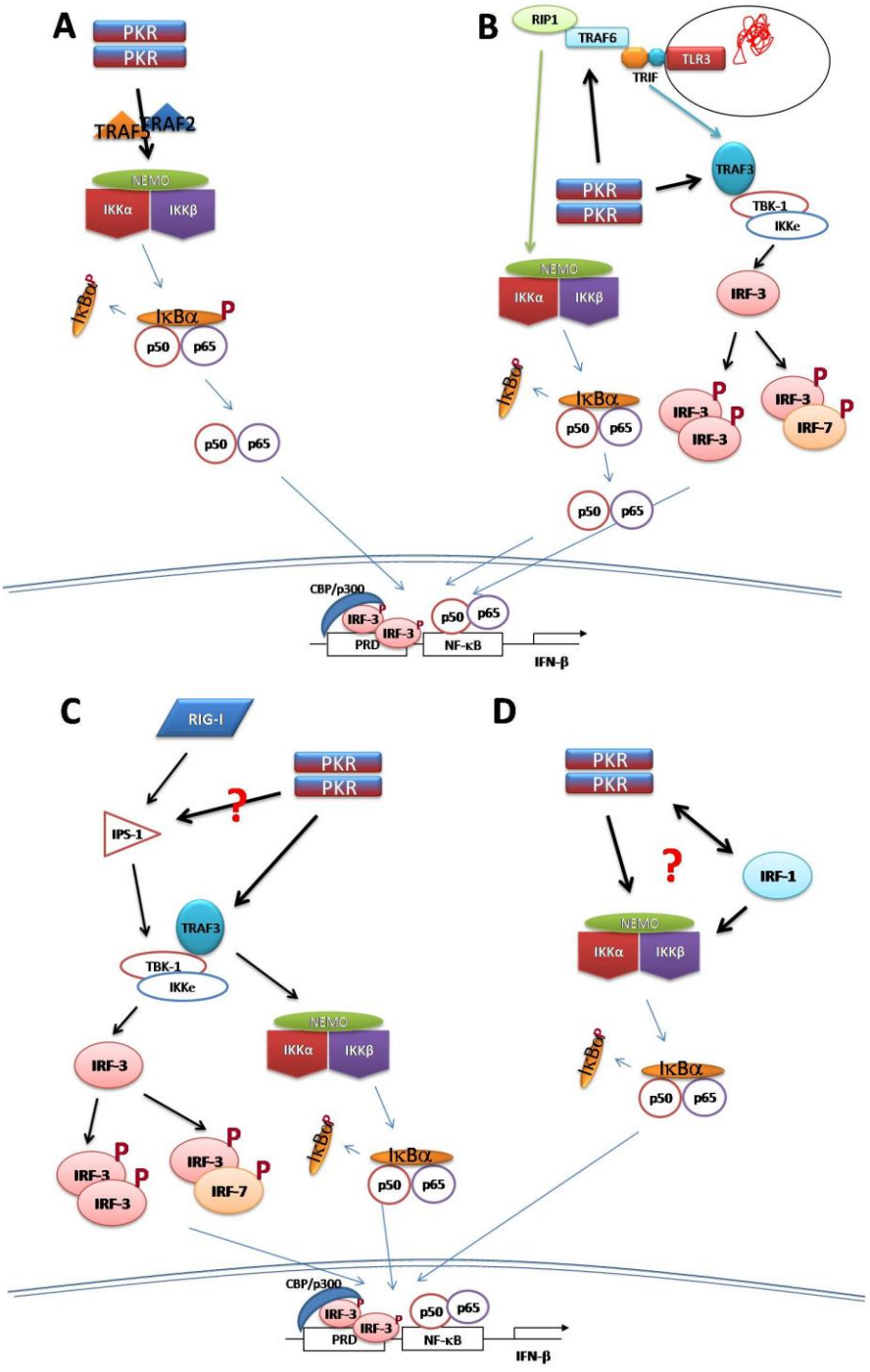


FIGURE 6.1: MECHANISMS OF PKR-INDUCED IFN INDUCTION. PKR can be linked (A) directly to NFkB activation, (B) TLR3-mediated signaling, (C) RIG-I/mda-5-mediated signaling or (D) IRF1-mediated signaling.

One additional mechanism of PKR activation which was not addressed in this dissertation is the role of PKR in IRF1 activation (Figure 6.1D). IRF1, like IRF3, is able to bind to, and activate, the IFN-β promoter (548). IRF1 has been shown to be involved in the activation of NFκB, as well. However, the pathway associated with IRF1-mediated signaling has not been delineated. Additionally, although PKR deficient cells induce IRF1-driven promoters poorly compared to WT cells, the pathway linking PKR and IRF1 is unclear. However, it would be interesting to see whether IRF1 plays any role in WNV-induced IFN production. PKR is involved in all of the four pathways outlined above; however, it may not be as simple as described. Cellular signaling pathways are often interconnected and/or redundant; thus, it is possible that PKR serves in more than just one of these pathways or, maybe more likely, serves in a different, undetermined combination of these signaling pathways.

Following the first observation that PKR may be involved in WNV-induced IFN production, we hypothesized that PKR is involved in IFN production during the early stages of WNV infection, while RIG-I/IRF3 'joins in' during later stages of infection, further promoting an efficient IFN response. We did find that PKR and NFκB are activated as early as 16 hours post infection, while IRF3 is not activated until at least 20 hours post infection, supporting our initial hypothesis. However, our result indicating that IRF3 is not important for WNV-induced IFN production in mDCs as well as the indication that the presence of IRF3 is not sufficient to 'recover' IFN induction in PKR deficient mice, even at 48 hours post infection, argues against this hypothesis. Reports

have clearly demonstrated a role for RIG-I and/or mda-5 in establishing an effective anti-WNV environment.

Two possible scenarios exist: (*i*) the two pathways are interconnected (directly or indirectly) or (*ii*) the two pathways are independent from each other. Our data suggest that PKR, but not IRF3, is required for IFN synthesis, arguing against a direct, downstream connection; however, that does not mean that PKR is not involved in RIG-I/mda-5 signaling. In light of the apparent deficiency in IRF3 phosphorylation in MEF cells lacking PKR, it is possible that IRF3 phosphorylation is mediated, in part, by PKR activity. It is possible that, in the absence of IRF3, another IRF, such as IRF1, can serve as a redundant transcription factor. However, when PKR is absent, the redundant pathway (IRF1, which has been shown to be linked to PKR) and IRF3 are impaired. Abrogation of IRF (1, 3 or 5) and NFκB transcriptional activity would ablate IFN promoter activation. Teasing apart any interaction (or lack of interaction) between PKR and RIG-I is, I believe, critical to fully understanding the role of these two molecules in controlling WNV infection.

THE ROLE OF THE PROTEASOME

During the screening of the siRNA library, silencing multiple subunits of the proteasome caused a reduction in WNV infection and/or replication. In fact, silencing two of the proteasome subunits, PSMA1 and PSMA2, consistently reduced WNV infection and/or replication and treatment of cells with chemical inhibitors of the proteasome had a dramatic effect on the level of WNV replication. Reduction in WNV replication with post-WNV infection proteasome inhibitor treatment indicated that proteasome activity is essential at post-entry stages of WNV infection. Our initial siRNA library screen, as well as subsequent inhibitor experiments, was performed using WNV

VLPs, which are unable to spread beyond the initially infected cells. Thus, it seems unlikely that the proteasome is essential *only* for WNV packaging, maturation and egress, although we did not rule out the possibility that the proteasome can *also* impact these steps. It is likely that the proteasome is required for some aspect of WNV translation or genome replication.

However, the reason why the proteasome is essential for WNV replication is not clear. The proteasome is involved in numerous different cellular functions, including protein degradation, cellular degradation, transcriptional regulation, apoptosis and signal transduction; thus, determining the exact function during WNV infection is difficult. There are, however, a few obvious possibilities. One such possibility is that WNV targets immune system-related proteins for degradation in an attempt to hide from cellular recognition or evade cellular defenses. Although WNV is known to inhibit TLR3 signaling (15, 587) as well as IFN signaling (186, 329, 505), this has not yet been shown to involve the degradation of a specific cellular protein(s).

WNV has also been shown to induce apoptosis in infected cells. Medigeshi et al. recently showed that WNV specifically induces the unfolded protein response through the induction of CHOP and the proteasome-mediated degradation of AFT6 (375). However, we have not tested whether inhibiting the proteasome has an effect on WNV-mediated apoptosis and, more importantly, a link between the degradation of AFT6 seen by Medigeshi et al. and the requirement of the proteasome for efficient WNV replication has not been examined. However, many viruses improve their ability to replicate by targeting specific proteins for degradation. Coxsackievirus B3 targets two proteins, cyclin D3 and p53 (345) and adenovirus also induces the degradation of p53 (452, 530, 609). Thus, it is a likely possibility that WNV requires specific protein(s) to be degraded to

replicate efficiently. Identifying the specific protein(s) targeted for destruction is a difficult task. One intriguing scenario is that WNV requires the degradation of specific protein(s) to efficiently transition between the translation and genome replication phases. One recent report has indicated that poliovirus-mediated cleavage of poly(rC)-binding protein (PCBP) is required for an efficient shift from viral translation to genome replication. Although this is only a cleavage event, and not degradation, the idea that such a mechanism could be involved during WNV replication is one that needs to be examined in detail. Another possibility of proteasome action is through the degradation of defective ribosomal products (DRiPs). These products arise when errors occur during translation and could have an adverse affect on further translation or replication activities. Thus, the inability of the cell to degrade DRiPs could have negative effects on WNV replication.

One additional possibility is that one or more of the WNV proteins are ubiquitinated in order to perform a specific function. Reducing proteasome activity also reduces the level of free ubiquitin in the cell and, thus, could affect viral replication. Although this type of mechanism is seen with HIV (ubiquitination of the HIV structural protein, Gag, is required for viral packaging) (507, 537), it has not been observed for any WNV proteins. In fact, the lack of any apparent changes in the molecular weight of any of the WNV antigens detected in our MHIAF Western blots on proteasome inhibitor-treated cells argues against the ubiquitination of specific WNV nonstructural proteins.If ubiquitin is involved in this way, it must exert its affects by attaching to only a small fraction of a specific WNV protein. I would speculate that the most likely candidates for ubiquitination are nonstructural proteins that have contact with the cytoplasm. However, ubiquitination of a WNV protein is, at this point, highly speculative, as ubiquitination has not been reported for any flavivirus protein.

Final Thoughts

The studies detailed in this dissertation are of particular interest as they open the door for many different avenues of future study. In terms of the role of PKR during WNV-induced IFN production, it would be particularly interesting to examine IFN induction when NFκB activity is impaired. This would formally link the role of PKR-dependent NFkB activation to IFN induction and cement the role of NFkB in WNV-induced IFN production. I believe one important avenue of future research would be to investigate a possible link between PKR and RIG-I and/or mda-5, especially since both appear to be important for mediating an antiviral response to WNV infection. Additionally, since IRF3 is not critical for IFN induction following WNV infection, it will be interesting to determine whether another IRF, such as IRF1, is involved.

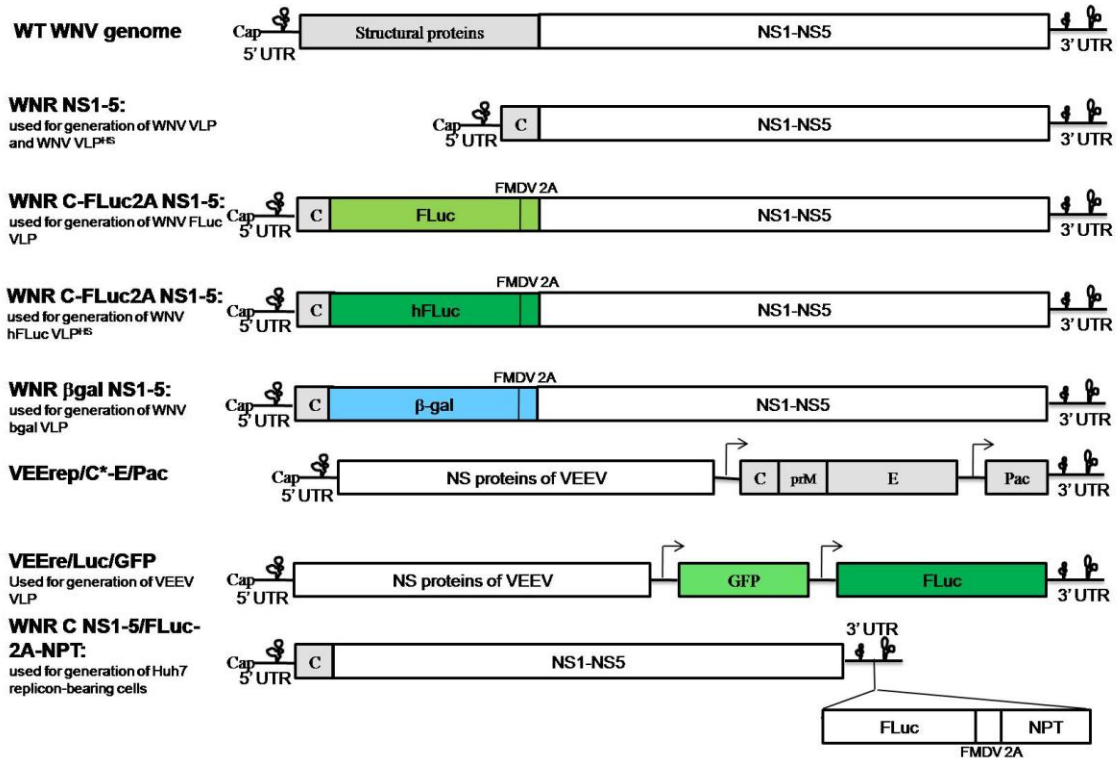
Further studies on the role of the proteasome will be slightly more difficult. The first step is to determine whether the proteasome is involved at the level of translation or genome replication (or both). One interesting avenue would be to explore whether WNV targets a protein(s) (maybe PCBP, as seen with poliovirus infection) for degradation to allow for a transition between translation and genome replication. Identifying specific proteins targeted by WNV for degradation is more complex, given the number of potential targets. However, identifying these proteins could lead to the identification of cellular processes that are important for WNV infection.

Interactions that occur between the virus and the cell/host it infects is critical to pathogenesis and, hence, disease outcome. Understanding all of these interactions, both those involved in the host immune response and those involved in virus replication, is key to developing an understanding of, and ultimately controlling, viral infections. In recent years, much of the focus has been on identifying aspects of the host's innate

immune response and progress has been made. We are also beginning to understand the multi-faceted characteristics of WNV infection and to identify host factors that are important for efficient replication. The results presented here in this dissertation add to the current knowledge and, although provide only a small snapshot of the various, complex WNV-host interactions, help to continue the work forward towards the goal of just about any pathogen study: the development of effective mechanisms to combat disease.

Appendices

APPENDIX A: WNV REPLICONS



This diagram represents the WNV genome and the various replicons utilized for these studies. WNR NS1-5 is the basic WNR. This is the 'starting' block for all of the WNV replicons. WNR CLuc2A NS1-5 is packaged into WNV FLuc VLP (used in Chapter 2 and Chapter 4), WNR ChLuc2A NS1-5 is packaged into WNV hFLuc VLP (used exclusively in Chapter 5). WNR Cβgal2A NS1-5 was packaged into WNV βgal VLP (used exclusively in Chapter 4). VEEVrep/C*-E/Pac was introduced into BHK cells to generate the packaging cell line. This replicon, or a derivative containing a mutation in E conferring heparan-binding capabilities, was used to package all of the WNV VLPs. VEErep/GFP/FLuc was packaged into VEEV VLPs and generated by I. Frolov (UTMB)

and was used exclusively in Chapter 4. The WNR C NS1-5/FLuc-2A-NPT was used to generate the Huh7 replicon bearing cells used in Chapter 4.

APPENDIX B: LIST OF GENES COMPRISING SIRNA LIBRARY

Table shows Accession number, Gene symbol and Gene ID for all genes present in the siRNA library.

RefSeq Accession Number	Gene Symbol	Gene ID	RefSeq Accession Number	Gene Symbol	Gene ID	RefSeq Accession Number	Gene Symbol	Gene ID
NM_052838	38596	1731	NM_021808	GALNT9	50614	NM_004567	PFKFB4	5210
NM_000014	A2M	2	NM_054110	GALNTL2	117248	NM_002626	PFKL	5211
NM_017436	A4GALT	53947	NM_145292	GALNTL5	168391	NM_000289	PFKM	5213
NM_016161	A4GNT	51146	NM_001480	GALR1	2587	NM_002627	PFKP	5214
NM_023928	AACS	65985	NM_003857	GALR2	8811	NM_012395	PFTK1	5218
NM_001086	AADAC	13	NM_003614	GALR3	8484	NM_014224	PGA5	5222
NM_182662	AADAT	51166	NM_147131	GALT	2592	NM_002629	PGAM1	5223
NM_014911	AAK1	22848	NM_000156	GAMT	2593	NM_000290	PGAM2	5224
NM_001088	AANAT	15	NM_198334	GANAB	23193	NM_002630	PGC	5225
NM_001605	AARS	16	NM_198141	GANC	2595	NM_016134	PGCP	10404
NM_015423	AASDHPPT	60496	NM_002046	GAPD	2597	NM_002631	PGD	5226
NM_005763	AASS	10157	NM_014364	GAPDS	26330	NM_014485	PGDS	27306
NM_000663	ABAT	18	NM_002047	GARS	2617	NM_005023	PGGT1B	5229
NM_005502	ABCA1	10347	NM_175085	GART	2618	NM_000291	PGK1	5230
NM_080282	ABCA10	10349	NM_000820	GAS6	2621	NM_138733	PGK2	5232
NM_173076	ABCA12	154091	NM_001482	GATM	2628	NM_012088	PGLS	25796
NM_152701	ABCA13	154664	NM_001005742	GBA	2629	NM_005091	PGLYRP1	8993
NM_212533	ABCA2	6891	NM_020944	GBA2	57704	NM_052890	PGLYRP2	114770
NM_001089	ABCA3	21	NM_020973	GBA3	57733	NM_052891	PGLYRP3	114771
NM_000350	ABCA4	6890	NM_014291	GCAT	23464	NM_020393	PGLYRP4	57115
NM_172232	ABCA5	23461	NM_013976	GCDH	2639	NM_002633	PGM1	5236
NM_172346	ABCA6	81031	NM_000160	GCGR	2642	NM_018290	PGM2	55276
NM_019112	ABCA7	10347	NM_000161	GCH1	2643	NM_015599	PGM3	5238
NM_007168	ABCA8		NM_005258	GCHFR	2644	NM_021965	PGM5	5239
NM_080283	ABCA9	10350	NM_033508	GCK	2645	NM_017712	PGPEP1	54858
NM_000927	ABCB1	10057	NM_001486	GCKR	2646	NM_000926	PGR	5241
NM_012089	ABCB10	23456	NM_001498	GCLC	2729	NM_006667	PGRMC1	10857
NM_003742	ABCB11	85320	NM_002061	GCLM	2730	NM_006320	PGRMC2	10424
NM_018850	ABCB4	5244	NM_021078	GCN5L2	2648	NM_024419	PGS1	9489
NM_178559	ABCB5	7504	NM_001490	GCNT1	2650	NM_177939	PH-4	54681
NM_005689	ABCB6	10058	NM_145655	GCNT2	2651	NM_032592	PHACS	84680
NM_004299	ABCB7	1244	NM_004751	GCNT3	9245	NM_018367	PHCA	55331
NM_007188	ABCB8	11194	NM_006302	GCS1	7841	NM_000444	PHEX	5251
NM_019624	ABCB9	6518	NM_004293	GDA	9615	NM_020889	PHF12	57649
NM_019902	ABCC1	4363	NM_017686	GDAP2	54834	NM_006623	PHGDH	26227
NM_033450	ABCC10	368	NM_001493	GDI1	2664	NM_177967	PHGDHL1	337867
NM_145186	ABCC11	85320	NM_001494	GDI2	2665	NM_002637	PHKA1	5255
NM_145190	ABCC12	5244	NM_017711	GDPD2	54857	NM_000292	PHKA2	5256
NM_172024	ABCC13	150000	NM_181702	GEM	2669	NM_000293	PHKB	5257
NM_000392	ABCC2	23657	NM_018988	GFOD1	54438	NM_006213	PHKG1	5260
NM_020038	ABCC3	8714	NM_002056	GFPT1	2673	NM_000294	PHKG2	5261
NM_005845	ABCC4	56606	NM_005110	GFPT2	9945	NM_178500	PHOSPHO1	162466
NM_005688	ABCC5	10057	NM_145793	GFRA1	2674	NM_006214	PHYH	5264
NM_001171	ABCC6	23461	NM_001495	GFRA2	2675	NM_174933	PHYHD1	254295
NM_000352	ABCC8	6833	NM_001496	GFRA3	2676	NM_014759	PHYHIP	9796
NM_020298	ABCC9	19	NM_145763	GFRA4	64096	NM_002638	PI3	5266
NM_000033	ABCD1	215	NM_000821	GGCX	2677	NM_018323	PI4K2B	55300
NM_005164	ABCD2	6515	NM_003878	GGH	8836	NM_018425	PI4KII	55361
NM_002858	ABCD3	5825	NM_004837	GGPS1	9453	NM_001002837	PIB5PA	27124
NM_005050	ABCD4		NM_013430	GGT1	2678	NM_020472	PIGA	5277
NM_002940	ABCE1	6059	XM_371427	GGT2	2679	NM_153747	PIGC	5279
NM_001090	ABCF1	6517	NM_178026	GGTL3	2686	NM_004569	PIGH	5283
NM_007189	ABCF2	10061	NM_199127	GGTL4	91227	NM_005482	PIGK	10026

NM_018358	ABCF3	89845	NM_004121	GGTLA1	2687	NM_004278	PIGL	9487
NM_207627	ABCG1	9619	NM_080920	GGTLA4	92086	NM_032634	PIGO	84720
NM_004827	ABCG2	23457	NM_000163	GHR	2690	NM_148920	PIGQ	9091
NM_022169	ABCG4	64137	NM_000823	GHRHR	2692	NM_002644	PIGR	5284
NM_022436	ABCG5	5243	NM_004122	GHSR	2693	NM_152309	PIK3AP1	118788
NM_022437	ABCG8	64241	NM_000164	GIPR	2696	NM_002645	PIK3C2A	5286
NM_007011	ABHD2	11057	NM_014030	GIT1	28964	NM_002646	PIK3C2B	5287
NM_138340	ABHD3	171586	NM_057170	GIT2	9815	NM_004570	PIK3C2G	5288
NM_022060	ABHD4	63874	NM_203391	GK	2710	NM_002647	PIK3C3	5289
NM_016006	ABHD5	51099	NM_033214	GK2	2712	NM_006218	PIK3CA	5290
NM_020676	ABHD6	57406	NM_025211	GKAP1	80318	NM_006219	PIK3CB	5291
NM_173567	ABHD7	253152	NM_000169	GLA	2717	NM_005026	PIK3CD	5293
NM_024527	ABHD8	79575	NM_000404	GLB1	2720	NM_002649	PIK3CG	5294
NM_024794	ABHD9	79852	XM_290631	GLCE	26035	NM_181524	PIK3R1	5295
NM_007313	ABL1	25	NM_000170	GLDC	2731	NM_005027	PIK3R2	5296
NM_005158	ABL2	27	NM_005269	GLI	2735	NM_003629	PIK3R3	8503
NM_020469	ABO	28	NM_006708	GLO1	2739	NM_014602	PIK3R4	30849
NM_001091	ABP1	26	NM_002062	GLP1R	2740	NM_002650	PIK4CA	5297
NM_001092	ABR	29	NM_004246	GLP2R	9340	NM_002651	PIK4CB	5298
NM_001607	ACAA1	30	NM_000171	GLRA1	2741	NM_002648	PIM1	5292
NM_006111	ACAA2	10449	NM_002063	GLRA2	2742	NM_006875	PIM2	11040
NM_198838	ACACA	31	NM_006529	GLRA3	8001	NM_006221	PIN1	5300
NM_001093	ACACB	32	NM_000824	GLRB	2743	NM_006222	PIN1L	5301
NM_025247	ACAD10	80724	NM_002064	GLRX	2745	NM_006223	PIN4	5303
NM_014384	ACAD8	27034	NM_014905	GLS	2744	NM_032409	PINK1	65018
NM_014049	ACAD9	28976	NM_005271	GLUD1	2746	NM_003557	PIP5K1A	8394
NM_001608	ACADL	33	NM_012084	GLUD2	2747	NM_003558	PIP5K1B	8395
NM_000016	ACADM	34	NM_002065	GLUL	2752	NM_012398	PIP5K1C	23396
NM_000017	ACADS	35	NM_016571	GLULD1	51557	NM_005028	PIP5K2A	5305
NM_001609	ACADSB	36	NM_005838	GLYAT	10249	NM_003559	PIP5K2B	8396
NM_000018	ACADVL	37	NM_001500	GMDS	2762	NM_024779	PIP5K2C	79837
NM_018677	ACAS2	55902	NM_016573	GMIP	51291	NM_173492	PIP5KL1	138429
NM_032501	ACAS2L	84532	NM_021971	GMPPB	29925	NM_014338	PISD	23761
NM_000019	ACAT1	38	NM_006877	GMPR	2766	NM_006224	PITPNA	5306
NM_005891	ACAT2	39	NM_016576	GMPR2	51292	NM_012399	PITPNB	23760
NM_012332	ACATE2	23597	NM_003875	GMPS	8833	NM_004910	PITPNM1	9600
NM_183377	ACCN1	40	NM_032320	GMRP-1	84280	NM_014968	PITRM1	10531
NM_020039	ACCN2	41	NM_032320	GMRP-1	84280	NM_182740	PKD1L2	114780
NM_020322	ACCN3	9311	NM_002067	GNA11	2767	NM_181536	PKD1L3	342372
NM_018674	ACCN4	55515	NM_007353	GNA12	2768	NM_000297	PKD2	5311
NM_017419	ACCN5	51802	NM_006572	GNA13	10672	NM_016112	PKD2L1	9033
NM_152830	ACE	1636	NM_004297	GNA14	9630	NM_014386	PKD2L2	27039
NM_021804	ACE2	59272	NM_002068	GNA15	2769	NM_006071	PKDREJ	10343
NM_015831	ACHE	43	NM_002069	GNAI1	2770	NM_006823	PKIA	5569
NM_005781	ACK1	10188	NM_002070	GNAI2	2771	NM_181794	PKIB	5570
NM_198830	ACLY	47	NM_006496	GNAI3	2773	NM_181805	PKIG	11142
NM_138326	ACMSD	130013	NM_182978	GNAL	2774	NM_000298	PKLR	5313
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NM_001098	ACO2	50	NM_002072	GNAQ	2776	NM_004203	PKMYT1	9088
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NM_198584	CA13	377677	NM_004983	KCNJ9	3765	NM_002924	RGS7	6000
NM_012113	CA14	23632	NM_138318	KCNK1	3775	NM_033345	RGS8	85397
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NM_023035	CACNA1A	773	NM_016601	KCNK7	10089	NM_175744	RHOC	389
NM_000718	CACNA1B	774	NM_016601	KCNK9	51305	NM_014578	RHOD	29984
NM_000719	CACNA1C	775	NM_004137	KCNMA1	3778	NM_019034	RHOF	54509
NM_000720	CACNA1D	776	NM_004137	KCNMB1	3779	NM_001665	RHOG	391
NM_000721	CACNA1E	777	NM_014407	KCNMB2	10242	NM_004310	RHOH	399
NM_005183	CACNA1F	778	NM_171830	KCNMB3	27094	NM_020663	RHOJ	57381
NM_198376	CACNA1G	8913	NM_002248	KCNMB4	27345	NM_012249	RHOQ	23433
NM_001005407	CACNA1H	8912	NM_002248	KCNN1	3780	NM_018307	RHOT1	55288

NM_021096	CACNA1I	8911	NM_170775	KCNN2	3781	NM_138769	RHOT2	89941
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NM_018398	CACNA2D3	55799	NM_172109	KCNQ1	3784	NM_031430	RILP	83547
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NM_031895	CACNG8	59283	NM_006801	KDELR1	10945	NM_002935	RNASE3	6037
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NM_001743	CALM2	805	NM_003685	KHSRP	8570	NM_014470	RND1	27289
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NM_172172	CAMK2G	818	NM_015229	KIAA0664	23277	NM_003799	RNMT	8731
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NM_015230	CENTD1	116984	NM_138784	LOC116123	116123	NM_148918	SHMT1	6470
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NM_001266	CES1	1066	NM_138798	LOC129531	129531	NM_173216	SIAT1	6480
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NM_014406	CESK1	150160	NM_001008270	LOC135946	135946	NM_006927	SIAT4B	6483
NM_000078	CETP	1071	NM_001008270	LOC136242	136242	NM_006278	SIAT4C	6484
NM_003879	CFLAR	8837	XM_090203	LOC142910	142910	NM_174971	SIAT6	6487
NM_000492	CFTR	1080	XM_090203	LOC144125	144125	NM_018414	SIAT7A	55808
NM_015936	CGI-04	51067	XM_092681	LOC149420	149420	NM_006456	SIAT7B	10610
NM_015944	CGI-14	51005	XM_092681	LOC151575	151575	NM_152996	SIAT7C	256435
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NM_015557	CHD5	26038	NM_145809	LOC203427	203427	NM_175052	SIAT8D	7903
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NM_001274	CHEK1	1111	XM_167134	LOC220686	220686	NM_003896	SIAT9	8869
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NM_001276	CHI3L1	1116	NM_181720	LOC257106	257106	NM_016581	SITPEC	51295
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NM_001328	CTBP1	1487	NM_002409	MGAT3	4248	NM_003043	SLC6A6	6533
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NM_001902	CTH	1491	NM_153329	MGC10204	126133	NM_003045	SLC7A1	6541
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NM_001908	CTSB	1508	NM_153373	MGC15875	85007	NM_004173	SLC7A4	6545
NM_001814	CTSC	1075	NM_032372	MGC16169	93627	NM_003486	SLC7A5	24

NM_001909	CTSD	1509	NM_032372	MGC16186	84332	NM_003982	SLC7A7	9056
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NM_000769	CYP2C19	1557	NM_153827	MINK	50488	NM_030952	SNARK	81788
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NM_000765	CYP3A7	1551	NM_172250	MMAA	166785	NM_003104	SORD	6652
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NM_178134	CYP4Z1	199974	NM_002428	MMP15	4324	NM_182965	SPHK1	8877
NM_000786	CYP51A1	1595	NM_022564	MMP16	4325	NM_020126	SPHK2	56848
NM_000780	CYP7A1	1581	NM_016155	MMP17	4326	NM_003122	SPINK1	6690
NM_004820	CYP7B1	9420	NM_022791	MMP19	4327	NM_021114	SPINK2	6691
NM_004391	CYP8B1	1582	NM_004530	MMP2	4313	NM_014471	SPINK4	27290

NM_006639	CYSLTR1	10800	NM_004771	MMP20	9313	NM_006846	SPINK5	11005
NM_020377	CYSLTR2	57105	NM_147191	MMP21	118856	NM_181642	SPINT1	6692
NM_130468	D4ST1	113189	NM_004659	MMP23A	8511	NM_021102	SPINT2	10653
NM_021080	DAB1	1600	NM_006983	MMP23B	8510	NM_032802	SPPL2A	84888
NM_001343	DAB2	1601	NM_006690	MMP24	10893	NM_152988	SPPL2B	56928
NM_138709	DAB2IP	153090	NM_022718	MMP25	64386	NM_003124	SPR	6697
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NM_014326	DAPK2	23604	NM_002422	MMP3	4314	NM_021199	SQRDL	58472
NM_001348	DAPK3	1613	NM_002423	MMP7	4316	NM_198291	SRC	6714
NM_001349	DARS	1615	NM_002424	MMP8	4317	NM_080744	SRCRB4D	136853
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NM_001918	DBT	1629	NM_058165	MOGAT1	116255	NM_080823	SRMS	6725
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NM_001919	DCI	1632	NM_178176	MOGAT3	346606	NM_182691	SRPK2	6733
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NM_022779	DDX31	64794	NM_017705	MPRG	54852	NM_005990	STK10	6793
NM_005804	DDX39	10212	NM_145243	MPRP-1	115209	NM_000455	STK11	6794
NM_024415	DDX4	54514	NM_021126	MPST	4357	NM_052902	STK11IP	114790
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NM_020664	DECR2	26063	NM_006039	MRC2	9902	NM_004226	STK17B	9262
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NM_152879	DGKD	8527	NM_032046	MSP	84000	NM_145001	STK32A	202374
NM_003647	DGKE	8526	NM_138716	MSR1	4481	NM_018401	STK32B	55351
NM_001346	DGKG	1608	NM_012331	MSRA	4482	NM_173575	STK32C	282974
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NM_004717	DGKI	9162	NM_002447	MST1R	4486	NM_080836	STK35	140901
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NM_152430	D-GPCR	143503	NM_002451	MTAP	4507	NM_015000	STK38L	23012
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NM_013406	DHPS	1725	NM_006636	MTHFD2	10797	NM_001001434	STX16	8675
NM_138452	DHRS1	115817	NM_005957	MTHFR	4524	NM_016930	STX18	53407
NM_016246	DHRS10	51171	NM_006441	MTHFS	10588	NM_004603	STX1A	6804
NM_182908	DHRS2	10202	NM_000252	MTM1	4534	NM_004177	STX3A	6809
NM_004753	DHRS3	9249	NM_176789	MTMR1	8776	NM_004604	STX4A	6810
NM_021004	DHRS4	10901	NM_201281	MTMR2	8898	NM_003164	STX5A	6811
NM_198083	DHRS4L2	317749	NM_153050	MTMR3	8897	NM_003569	STX7	8417
NM_020139	DHRS6	56898	NM_004687	MTMR4	9110	NM_004853	STX8	9482
NM_016029	DHRS7	51635	NM_004685	MTMR6	9107	NM_018423	STYK1	55359
NM_016245	DHRS8	51170	XM_044727	MTMR7	9108	NM_145251	STYX	6815

NM_199204	DHRS9	10170	NM_017677	MTMR8	55613	NM_003850	SUCLA2	8803
NM_145177	DHRSX	207063	NM_173708	MTND1	4535	NM_003849	SUCLG1	8802
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NM_003587	DHX16	8449	NM_173710	MTND3	4537	NM_033050	SUCNR1	56670
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NM_000398	DIA1	1727	NM_173713	MTND5	4540	NM_177534	SULT1A1	6817
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			NM_005958	MTNR1A	4543	NM_177552	SULT1A3	6818
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NM_001007023	DIO2	1734	NM_133645	MTO1	25821	NM_001056	SULT1C1	6819
NM_001362	DIO3	1735	NM_000253	MTP	4547	NM_006588	SULT1C2	27233
NM_145173	DIRAS1	148252	NM_000254	MTR	4548	NM_005420	SULT1E1	6783
NM_017594	DIRAS2	54769	NM_002454	MTRR	4552	NM_003167	SULT2A1	6822
NM_015518	DKFZP434C131	25989	NM_005592	MUSK	4593	NM_004605	SULT2B1	6820
NM_173655	DKFZp434C1418	285220	NM_000255	MUT	4594	NM_176874	SULT4A1	25830
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NM_032136	DKFZP434L1717	84076	NM_000431	MVK	4598	NM_007192	SUPT16H	11198
NM_031291	DKFZP434N1235	83447	NM_031300	MXD3	83463	NM_003173	SUV39H1	6839
NM_031306	DKFZP564B1023	83479	NM_206819	MYBPC1	4604	NM_024670	SUV39H2	79723
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NM_014033	DKFZP586A0522	25840	NM_000256	MYBPC3	4607	NM_003895	SYNJ1	8867
NM_015533	DKFZP586B1621	26007	NM_004997	MYBPH	4608	NM_003898	SYNJ2	8871
NM_015430	DKFZP586H2123	25891	NM_005963	MYH1	4619	NM_025228	T3JAM	80342
NM_182502	DKFZp686L1818	132724	NM_022844	MYH11	4629	NM_152787	TAB3	257397
NM_032039	DKFZP761D0211	83986	NM_003802	MYH13	8735	NM_001006667	TAC3	6866
XM_290615	DKFZp762F0713	283554	NM_002470	MYH3	4621	NM_001057	TACR2	6865
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NM_001931	DLAT	1737	NM_002471	MYH6	4624	NM_138923	TAF1	6872
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NM_144776	FTHFD	10840	NM_015148	PASK	23178	NM_017944	USP47	55031
NM_015440	FTHFSDC1	25902	NM_138294	PATE	160065	NM_032236	USP48	84196
NM_022476	FTS	64400	NM_000920	PC	5091	NM_004275	USP49	9477
NM_000147	FUCA1	2517	NM_003884	PCAF	8850	NM_003481	USP5	8078
NM_032020	FUCA2	2519	NM_000281	PCBD	5092	NM_201286	USP51	158880
NM_145059	FUK	197258	NM_000282	PCCA	5095	NM_014871	USP52	9924
NM_002569	FURIN	5045	NM_000532	PCCB	5096	NM_004505	USP6	9098
NM_000148	FUT1	2523	NM_002589	PCDH7	5099	NM_003470	USP7	7874
NM_032664	FUT10	84750	NM_002591	PCK1	5105	NM_005154	USP8	9101
NM_173540	FUT11	170384	NM_004563	PCK2	5106	NM_021906	USP9X	8239
NM_000511	FUT2	2524	NM_006197	PCM1	5108	NM_004654	USP9Y	8287
NM_000149	FUT3	2525	NM_005389	PCMT1	5110	NM_005715	UST	10090
NM_002033	FUT4	2526	NM_013363	PCOLCE2	26577	NM_018949	UTS2R	2837
NM_002034	FUT5	2527	NM_002768	PCOLN3	5119	NM_025076	UXS1	80146
NM_000150	FUT6	2528	NM_000439	PCSK1	5122	NM_006295	VARS2	7407
NM_004479	FUT7	2529	NM_002594	PCSK2	5126	NM_006373	VAT1	10493
NM_178154	FUT8	2530	NM_017573	PCSK4	54760	NM_005428	VAV1	7409
NM_006581	FUT9	10690	NM_006200	PCSK5	5125	NM_003371	VAV2	7410
NM_002035	FVT1	2531	NM_138324	PCSK6	5046	NM_006113	VAV3	10451
NM_012192	FXC1	26515	NM_004716	PCSK7	9159	NM_014000	VCL	7414
NM_021902	FXYD1	5348	NM_174936	PCSK9	255738	NM_007126	VCP	7415
NM_021603	FXYD2	486	NM_033019	PCTK1	5127	NM_003374	VDAC1	7416
NM_021910	FXYD3	5349	NM_002595	PCTK2	5128	NM_003375	VDAC2	7417
NM_014164	FXYD5	53827	NM_212502	PCTK3	5129	NM_005662	VDAC3	7419
NM_022006	FXYD7	53822	NM_021213	PCTP	58488	NM_000376	VDR	7421
NM_002036	FY	2532	NM_005017	PCYT1A	5130	NM_052867	VGCNL1	259232
NM_002037	FYN	2534	NM_004845	PCYT1B	9468	NM_080552	VIAAT	140679
NM_003505	FZD1	8321	NM_002861	PCYT2	5833	NM_004624	VIPR1	7433
NM_007197	FZD10	11211	NM_004208	PDCD8	9131	NM_003382	VIPR2	7434
NM_001466	FZD2	2535	NM_006661	PDE10A	10846	NM_003383	VLDLR	7436
NM_017412	FZD3	7976	NM_016953	PDE11A	50940	NM_020633	VN1R1	57191
NM_012193	FZD4	8322	NM_005019	PDE1A	5136	NM_173856	VN1R2	317701
NM_003468	FZD5	7855	NM_000924	PDE1B	5153	NM_174980	VN1R3	317702
NM_003506	FZD6	8323	NM_005020	PDE1C	5137	NM_173857	VN1R4	317703
NM_003507	FZD7	8324	NM_002599	PDE2A	5138	NM_173858	VN1R5	317705
NM_031866	FZD8	8325	NM_000921	PDE3A	5139	NM_004666	VNN1	8876
NM_003508	FZD9	8326	NM_000922	PDE3B	5140	NM_078488	VNN2	8875
NM_000151	G6PC	2538	NM_006202	PDE4A	5141	NM_018399	VNN3	55350
NM_021176	G6PC2	57818	NM_002600	PDE4B	5142	NM_057180	VPS29	51699
NM_138387	G6PC3	92579	NM_000923	PDE4C	5143	NM_003384	VRK1	7443
NM_000402	G6PD	2539	NM_006203	PDE4D	5144	NM_006296	VRK2	7444
NM_000152	GAA	2548	NM_033430	PDE5A	8654	NM_016440	VRK3	51231
NM_007285	GABARAPL2	11345	NM_000440	PDE6A	5145	NM_000552	VWF	7450
NM_001470	GABBR1	2550	NM_000283	PDE6B	5158	NM_213646	WARS	7453
NM_000806	GABRA1	2554	NM_006204	PDE6C	5146	NM_201263	WARS2	10352
NM_000807	GABRA2	2555	NM_002601	PDE6D	5147	NM_148913	WBSCR21	83451
NM_000808	GABRA3	2556	NM_002602	PDE6G	5148	NM_017528	WBSCR22	114049
NM_000809	GABRA4	2557	NM_006205	PDE6H	5149	NM_145241	WDR31	114987
NM_000810	GABRA5	2558	NM_002604	PDE7A	5150	NM_003390	WEE1	7465
NM_000811	GABRA6	2559	NM_018945	PDE7B	27115	NM_000553	WRN	7486
NM_000812	GABRB1	2560	NM_173454	PDE8A	5151	NM_020135	WRNIP1	56897
NM_000813	GABRB2	2561	NM_003719	PDE8B	8622	NM_130792	WWOX	51741
NM_021912	GABRB3	2562	NM_001001583	PDE9A	5152	NM_005283	XCR1	2829
NM_000815	GABRD	2563	NM_022341	PDF	64146	NM_000379	XDH	7498
NM_021987	GABRE	2564	NM_033135	PDGFD	80310	NM_021083	XK	7504

NM_173536	GABRG1	2565	NM_006206	PDGFRA	5156	NM_020383	XPNPEP1	7511
NM_198904	GABRG2	2566	NM_002609	PDGFRB	5159	NM_003399	XPNPEP2	7512
NM_033223	GABRG3	2567	NM_006207	PDGFRL	5157	NM_003400	XPO1	7514
NM_014211	GABRP	2568	NM_000284	PDHA1	5160	NM_007235	XPOT	11260
NM_018558	GABRQ	55879	NM_005390	PDHA2	5161	NM_004736	XPR1	9213
NM_002042	GABRR1	2569	NM_000925	PDHB	5162	NM_012255	XRN2	22803
NM_002043	GABRR2	2570	NM_003477	PDHX	8050	NM_005108	XYLB	9942
NM_000817	GAD1	2571	NM_006849	PDIP	64714	NM_022166	XYLT1	64131
NM_000818	GAD2	2572	NM_006810	PDIR	10954	NM_022167	XYLT2	64132
NM_005255	GAK	2580	NM_002610	PDK1	5163	NM_003680	YARS	8565
NM_005255	GAK	2580	NM_002611	PDK2	5164	NM_005433	YES1	7525
NM_004861	GAL3ST1	9514	NM_005391	PDK3	5165	NM_139313	YME1L1	10730
NM_000153	GALC	2581	NM_002612	PDK4	5166	NM_139323	YWHAB	7529
NM_000403	GALE	2582	NM_020786	PDP2	57546	NM_006761	YWHAE	7531
NM_001478	GALGT	2583	NM_031268	PDPK1	5170	NM_012479	YWHAG	7532
NM_153446	GALGT2	124872	NM_017990	PDPR	55066	NM_003405	YWHAH	7533
NM_000154	GALK1	2584	NM_003681	PDXK	8566	NM_006826	YWHAQ	10971
NM_002044	GALK2	2585	NM_206836	PECI	10455	NM_145690	YWHAZ	7534
NM_138801	GALM	130589	NM_018441	PECR	55825	NM_020205	ZA20D1	56957
NM_000512	GALNS	2588	NM_148172	PEMT	10400	NM_152444	ZADH1	145482
NM_020474	GALNT1	2589	NM_000285	PEPD	5184	NM_175907	ZADH2	284273
NM_198321	GALNT10	55568	NM_000466	PEX1	5189	NM_133646	ZAK	51776
NM_022087	GALNT11	63917	NM_000319	PEX5	5830	NM_006821	ZAP128	10965
NM_024642	GALNT12	79695	NM_000287	PEX6	5190	NM_207519	ZAP70	7535
NM_024572	GALNT14	79623	NM_012393	PFAS	5198	NM_024657	ZCWCC2	79710
NM_004481	GALNT2	2590	NM_002625	PFKFB1	5207	NM_016598	ZDHHC3	51304
NM_004482	GALNT3	2591	NM_002625	PFKFB1	5207	NM_007324	ZFYVE9	9372
NM_003774	GALNT4	8693	NM_006212	PFKFB2	5208	NM_005857	ZMPSTE24	10269
NM_014568	GALNT5	11227	NM_006212	PFKFB2	5208	NM_005857	ZMPSTE24	10269
NM_007210	GALNT6	11226	NM_004566	PFKFB3	5209	NM_001004433	ZNT8	55532
NM_017423	GALNT7	51809	NM_004567	PFKFB4	5210	NM_017580	ZRANB1	54764
NM_017417	GALNT8	26290				NM_032143	ZRANB3	84083

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Vita

Felicia Gilfoy Santa Maria Guerra, (born Felicia Dawn Gilfoy), was born in Springfield Missouri on May 1, 1980. She was the second child of Carlton D Gilfoy and Karen L Gilfoy. She grew up in Independence, MO and graduated from Fort Osage Senior High School in May 1998. She matriculated into Southwest Missouri State University (now Missouri State University) where she majored in Biology (emphasis in Microbiology) with a minor in Biochemistry and earned her Bachelor of Science in May 2002. During her undergraduate career, she worked on varied projects in microbiology and neurology. She began her graduate education at The University of Texas Medical Branch in Galveston Texas in August 2002 and joined the laboratory of Peter W. Mason in August 2003. Her dissertation research in this lab focused on identifying host factors that mediate West Nile virus infection.

Education

B.S. May 2002, Southwest Missouri State University, Springfield, Missouri

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Steiert, John G and **Gilfoy, Felicia D.** 2002. Infection Rates of *Amblyomma americanum* and *Dermacentor variabilis* by *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in Southwest Missouri. Vector Borne and Zoonotic Diseases. 2:53-60

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Abstracts

F D Gilfoy, F Scholle, P W Mason

West Nile virus suppression of the type I interferon response in murine cells. Poster presented at the 24th annual American Society of Virology, State College, PA; June 18-22, 2005

F D Gilfoy, R Fayzulin, P W Mason

Utilizing siRNAs to identify host proteins essential for West Nile virus replication. Poster presented at the Texas-United Kingdom Collaborative Research Initiative:

Infectious Diseases: Old Challenges-New Solutions, London, United Kingdom; December 7-7, 2005

F D Gilfoy, R Fayzulin, N Dewsbury, P W Mason

Utilizing siRNAs to identify host proteins essential for West Nile virus replication. Oral presentation at the American Society of Virology, Madison, WI; July 15-19, 2006

F Gilfoy and P W Mason

The double-stranded RNA-dependent protein kinase (PKR) mediates West Nile virus-induced IFN production. Oral presentation at the American Society of Virology, Corvalis, OR; July 14-18, 2007

F Gilfoy, G Milligan and P W Mason

PKR activity is important for WNV-induced IFN synthesis in vitro and ex vivo, and correlates with activation of NFκB. Oral presentation at the American Society of Virology, Ithica, NY; July 12-16, 2008