Towards Understanding of the Replication and Pathogenesis of Dengue Infection

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PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

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Dengue infection and the unfolded protein response
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Umareddy I and SG Vasudevan
Molecular and functional characterisation of dengue viral NS4B protein
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Role of interferon in dengue infection
Poster presented at the Annual Meeting of the International Society for Interferon and Cytokine Research, Shanghai (2005)

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ABSTRACT

Dengue is the most important human viral disease transmitted by arthropod vectors and over half of the world's population live in areas at risk of infection. Currently there is neither specific treatment nor vaccine to tackle this emerging disease. The work described in this thesis has been conducted at the Novartis Institute for Tropical Diseases (NITD) which aims to find small molecule inhibitors for dengue. In keeping with the goals of the institute, the aim of this thesis was to identify viral and host factors that are important for dengue replication and pathogenesis.

Chapter 1 of this thesis describes the features of dengue disease and reviews the molecular studies of the causative organism- the dengue virus. It also lists out the multifaceted efforts to control dengue and the need to gain comprehensive knowledge of the viral and host factors that influence replication of the dengue virus.

Of the seven non structural proteins described for dengue, the roles of only NS5 and NS3 have been fully explored. *Chapter 2* details the characterisation of NS4B, a small non structural protein of dengue, whose role in dengue replication was previously unexplored. A physical interaction was identified between NS4B and the helicase domain of NS3 using the yeast two-hybrid assay. This interaction was further confirmed by biochemical pull down and immuno-precipitation assays, both with purified proteins and with dengue virus infected cell lysates. Furthermore, NS4B dissociated NS3 from single stranded RNA and consequently enhanced the helicase activity of NS3 in an *in vitro* unwinding assay. A single amino acid mutation in NS4B (Hanley *et al*., 2003) that abolished its interaction with NS3 altered the viral replication. In addition, NS4B co-localized with NS3 in the peri-nuclear region of infected human cells suggesting the *in vivo* significance of this interaction. These results suggest that NS4B modulates dengue replication via its interaction with NS3.

Severe dengue has long been speculated to be a result of a complex combination of viral, epidemiological and host factors in the context of a secondary infection. *Chapter 3* unveils for the first time, a role for viral genomic variations in dengue pathogenesis

via modulation of the response to type I IFN. A strain-dependent difference was detected in gene expression levels of the type I interferon response pathway between two closely related DEN2 strains NGC and TSV01. Activation of type I anti viral responses such as PKR, OAS, ADAR and Mx, were prevalent in infection with TSV01 but not NGC. Biochemical dissection further revealed that NGC but not TSV01 suppressed STAT-1 activation in response to type I IFN but these two strains did not differ in their response to type II IFN. An extension of this study to low passage clinical isolates of various serotypes indicated that this ability to suppress IFN response is independent of serotype as well as viral load. Furthermore, the inability of one such clinical isolate SG 167 (isolated during a recent dengue outbreak which was relatively mild with very few severe dengue cases) to suppress IFN response indicated that type I IFN response could be a prime factor that determines the clinical outcome to dengue infection.

Virus-induced apoptosis mediated by the unfolded protein response (UPR) is hypothesized to be a crucial pathogenic event in dengue infection. *Chapter 4* of this thesis is one of the first reports on the initial events in dengue infection mediated UPR. Phosphorylation of EIF2α was induced in dengue infection but simultaneously, the expression of GADD34 (which dephosphorylates EIF2α) was also enhanced. An inhibitor of GADD34 reduced dengue replication in infected cells indicating that this viral "compensatory" event is required for viral survival. Both the XBP1 and ATF6 pathways of the UPR were also activated by dengue infection. In addition, modulation of the EIF2α and the XBP1 pathways altered dengue replication indicating that UPR pathway components affect the outcome of infection.

Chapter 5 summarizes the conclusions from these studies and discusses some of the future work that can arise from these results. Finally, it is hoped that knowledge gained in this thesis will expedite the quest for an anti-dengue drug.

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Introduction to Dengue

1. INTRODUCTION TO DENGUE

Dengue fever or "break-bone fever" as it was known earlier is a mosquito-transmitted arboviral disease that has resurged as a global public health problem in recent years (Rigau-Perez *et al*., 1998). Dengue fever is an acute febrile illness characterized by fever, headache, muscle and joint pains, rash, and nausea which has a benign and self-limiting course. But a severe form of this disease called the dengue haemorrhagic fever (DHF) can threaten the patient's life primarily through increased vascular permeability and dengue shock syndrome (DSS).

1.1 Dengue Epidemiology: History and Occurrence

The term dengue is derived from a Swahili phrase "ka dinga pepo" (sudden cram-like seizure plague) and has been used as early as in 1801 to describe acute febrile illness with bone pains, haemorrhage and jaundice (Rigau-Perez, 1998). Dengue virus was discovered to be the causative for this disease by Ashburn and colleagues (Sabin, 1952). Serotype 1 and 2 of dengue viruses were described along with the presence of homotypic immunity following infection during the second world war (Sabin, 1952) whereas serotype 3 and 4 dengue viruses were identified in a Manila epidemic of 1956 (Hammon *et al*., 1960).

Today, dengue infection is the most wide spread mosquito-borne human viral infection with around 50-100 million cases reported annually (Halstead, 1988) and about 2.5 billion people at risk (Gubler, 1998, Gubler, 2002a). The WHO estimates that 500,000 cases of dengue (largely children) require hospitalisation each year and that 2.5-5% of these cases are fatal.

Dengue occurs in South and Southeast Asia, Central and South America, Africa, and in the Caribbean and Pacific regions and is endemic in more than 90 tropical countries. Outbreaks are generally restricted to the tropics due to viral transmission by the *Aedes* mosquitoes but contemporary demographic and life-style trends such as population explosion, urbanization and rapid transportation of large numbers of people, have led to the spread of this disease to non-endemic regions (Gubler, 2002a).

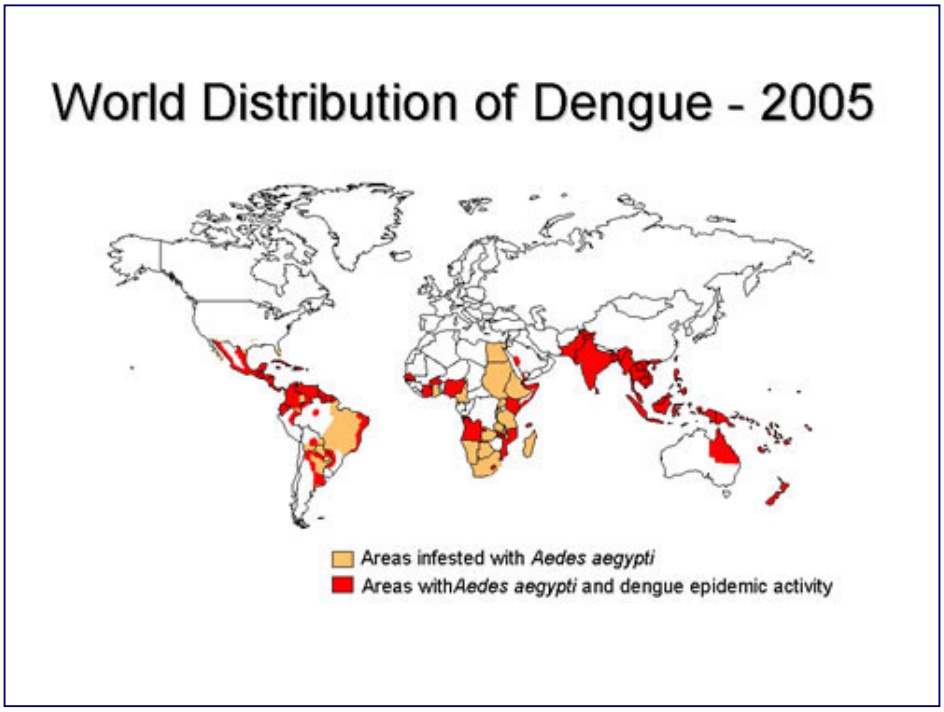


Figure 1: World distribution map of dengue and *Aedes aegypti* in 2005 (adapted from CDC)

1.2 Dengue Viral Transmission Cycle

Dengue viruses are transmitted among human hosts by the peri-domestic mosquito vectors *Aedes aegypti* and *Aedes albopictus*. However, ecological studies of dengue virus in sylvatic habitats of West Africa (Diallo *et al*., 2003) and Malaysia (Rudnick, 1965) have identified transmission cycles involving non-human primates as reservoir hosts and arboreal, tree-hole dwelling *Aedes* species as vectors. This sylvatic cycle seems to be more ancient and host-range changes (from non-human primates to humans and from arboreal *Aedes* species. to *A. aegypti* and *A. albopictus* vectors) are thought to have resulted in the evolution of endemic/epidemic dengue strains separate from sylvatic strains (Weaver & Barrett, 2004).

In the peri-domestic transmission cycle, which is highly efficient and independent of the sylvatic transmission, mosquitoes acquire the dengue virus while feeding on the blood of an infected person. After virus incubation for 8-10 days, an infected mosquito is capable (during probing and blood feeding) of transmitting the virus to susceptible individuals for the rest of its life. The virus circulates in the blood of infected humans

for two to seven days, at approximately the same time as they have fever and *Aedes* mosquitoes may acquire the virus when they feed on an individual during this period.

1.3 Clinical Manifestations

Dengue infections can be asymptomatic (especially in children under 15 years) or lead to a range of clinical presentations, even death (Burke *et al.*, 1988). Population-based studies have shown increasing severity in the clinical features of dengue fever with increasing age of the patient and with repeated infections. After an incubation period of about 3–7 days (range 3–14), the virus can cause classical dengue fever- an acute febrile illness characterized by frontal headache, retroocular pain, muscle and joint pain, nausea, vomiting, and rash. Dengue virus disappears from the blood after an average of 5 days, closely correlated with the disappearance of fever, and no carrier state ensues (Kalayanarooj *et al.*, 1997, Rigau-Perez *et al.*, 1998, Thomas *et al.*, 2003). However, the disease may progress beyond the acute febrile stage to a plasma leakage stage with or without significant hemorrhage that can result in shock or even death (Isarangkura *et al.*, 1987).

1.3.1 Dengue Fever (DF)

DF manifests as an undifferentiated febrile disease with a maculopapular rash in infants and young children. Older children and adults may have either a mild febrile syndrome or the classical and even incapacitating disease. Skin eruptions are common in children and adults with primary infections. There may be a flushing of the face, neck, and chest, a centrifugal maculopapular rash or a confluent petechial rash with round pale areas of normal skin. Laboratory findings include leucopenia and thrombocytopenia. Over one-third of patients with DF test positive in a tourniquet test (more than 20 petechiae in a square patch of skin 2·5×2·5 cm) (Ashburn & Craig, 2004, Kalayanarooj *et al.*, 1997). It is difficult to rule out other febrile illnesses such as the chikungunya, measles, leptospirosis, typhoid, or malaria based on clinical findings alone (Halstead, 1965). But generally, dengue can be ruled out if symptoms start more than two weeks after the patient has left a dengue-endemic area, or if the fever lasts more than two weeks. Dengue infection can progress beyond the acute febrile stage to a severe form

known as dengue hemorrhagic fever (DHF) which can lead to the dengue shock syndrome (DSS).

1.3.2 Dengue Hemorrhagic Fever (DHF)

DHF has been most extensively studied in south-east Asian children (Halstead, 1965) but the basic clinical manifestations are similar throughout the age spectrum in the tropical Americas (Dietz *et al*., 1996). DHF is defined as an acute febrile illness with hemorrhagic manifestations, thrombocytopenia ($\leq 10^5/\mu L$), and evidence of plasma leakage (documented by haemoconcentration, pleural effusions, hypoalbuminaemia or hypoproteinaemia). DHF/DSS commonly begins with a sudden rise in temperature and other symptoms resembling DF but usually develops around day 3–7 of illness at the time of defervescence and continues for 2–7 days. The development of DHF provides warnings of an increased probability of shock. A progressively decreasing platelet count and a rising haematocrit indicate increased probability of impending shock. See *table 1* for the spectrum of DHF classified based on severity of disease (Nimmannitya, 1987). The major patho-physiological change that determines the severity of disease in DHF and differentiates it from DF is the leakage of plasma through endothelial gaps without necrosis or inflammation of the capillary endothelium. However, targeting capillary permeability (using for example, hyaluronidase) does not prevent dengue vascular permeability or shock (Tassniyom *et al*., 1997).

1.3.3 Dengue Shock Syndrome (DSS)

DSS is defined as DHF with signs of circulatory failure, including narrow pulse pressure (≤20 mm Hg), hypotension, or frank shock. Abnormalities in liver functions are common but not jaundice. The four warning signs for impending shock are intense, sustained abdominal pain; persistent vomiting; restlessness or lethargy; and a sudden change from fever to hypothermia with sweating and prostration (Rigau-Perez *et al*., 1998). The prognosis in DHF/DSS depends on prevention or early recognition and treatment of shock. Once shock has set in the fatality rate may be as high as 12-44% (Nimmannitya, 1987).

Classification	Symptoms	Comments
Grade I	Fever, nonspecific constitutional symptoms, evidence of plasma leakage without shock and positive tourniquet test as the only hemorrhagic manifestation	DHF
Grade II	Fever, nonspecific constitutional symptoms, evidence of plasma leakage without shock, positive tourniquet test and spontaneous hemorrhagic manifestations	DHF
Grade III	Circulatory failure as evidenced by rapid and weak pulse, narrowing of pulse pressure, hypotension	DSS
Grade IV	Profound shock, undetectable pulse and blood pressure	DSS

Table 1: Spectrum of dengue hemorrhagic fever as described in (Nimmannitya, 1987).

1.3.4 Other Dengue Symptoms

Some severe but rare manifestations of dengue infection have been described as DF with severe hemorrhage, hepatic damage, cardiomyopathy, encephalopathy and viral encephalitis (Lum *et al.*, 1996, Nimmannitya, 1987, Nimmannitya *et al.*, 1987, Thisyakorn & Thisyakorn, 1994). Vertical transmission of dengue virus has been recorded in a small number of cases, leading to neonatal DF or even DSS (Chye *et al.*, 1997).

1.4 Pathogenesis of Severe Dengue

Pathogenesis of severe dengue appears to be multifactorial and has been attributed to interactions among several factors such as viral virulence, host age, nutritional, genetic and immunological characteristics and inter-current infections (Halstead, 1988, Kalayanarooj *et al.*, 1997, Rothman, 2003, Sakuntabhai *et al.*, 2005). Many studies have however pointed towards immune activation by prior exposure to dengue as one of the most important risk factors for severe dengue (Gubler, 1998, Halstead, 1979, Halstead & O'Rourke, 1977, Kliks *et al.*, 1989, Morens & Halstead, 1990). Infection with one dengue serotype does not provide immunity against the others and sequential heterotypic infection has been shown to increase virus replication and thus the

probability of developing DHF by antibody-dependent enhancement (ADE). ADE has been associated with increased disease severity in a range of viral infections including flaviviruses such as JEV and West Nile (Porterfield, 1986).

Enhancing immunoglobulin G (IgG) antibodies (that can cross react but cannot neutralise the virus) bind to the virus that is attached to the cell surface, bringing the infectious virion into close proximity to the virus receptor. ADE requires the presence of Fcγ receptors on the surface of permissive cells such as phagocytes but viruses enter through normal receptors. Thus virus-specific antibody and the Fcγ receptor together appear to act together as a co-receptor, enhancing the efficiency of virus binding and increasing the number of infected cells (Gollins & Porterfield, 1986). Therefore in an infected patient pre-existing antibody could result in increased viral load, shortened incubation times and increased disease severity. Moreover, as many components of the cell-mediated immune system (CMI) display Fcγ receptors on their cell surface, ADE could act by destroying these cells and further compromising recovery from disease (Stephenson, 2005).

The ADE phenomenon is well documented in *in vitro* studies but there is little evidence that this results in increased disease severity in animal models or even human disease. Some animal studies do show enhanced viremia and physiological signs consistent with DHF upon sequential infection with DENV2 but these results could not be reproduced in any other serotype (Halstead, 2003). Furthermore, other studies have shown that severe dengue can occur even after primary infection and that certain strains of dengue are more virulent (Gubler *et al*., 1978, Leitmeyer *et al*., 1999, Rico-Hesse *et al*., 1998, Rico-Hesse *et al*., 1997, Thein *et al*., 1997). The genetic diversity and co-circulation in nature of virus strains that differ in virulence might explain the severe forms of dengue infections in which viral factors have been implicated. Molecular evolution studies support the role of genetic diversity in disease pathogenesis. For example, despite co circulation of several DENV serotypes in the Americas, it was only in 1981 Cuban epidemic that the first case of DHF was reported. This period coincided with the introduction of a higher pathogenic new genetic variant of a Southeast Asian DENV2 (Damonte *et al*., 2004) indicating that viral factors might play a critical role in disease pathogenesis and severity.

Previous studies have reported some association of polymorphisms in HLA class I, TNF-α and Fcγ receptor IIA genes (Chiewsilp *et al*., 1981, Fernandez-Mestre *et al*., 2004, Loke *et al*., 2001, Stephens *et al*., 2002) with dengue hemorrhagic fever. Notably, some HLA alleles as well as CD209 (Sakuntabhai *et al*., 2005) were specifically associated with the risk of dengue fever rather than dengue hemorrhagic. At present there is no clear molecular evidence that rules out secondary infection, host susceptibility or viral virulence factors as the major molecular factor for dengue pathogenesis. Overall, the risk of infection and disease severity probably results from complex interactions of epidemiological factors: high vector density, high virus circulation, and a susceptible population at risk of a secondary dengue infection, host factors: age, gender, ethnicity, chronic diseases, pre-existence of dengue antibodies, interval between infections, and genetics, and viral factors: serotype, strains, and genotypes (Bravo *et al*., 1987, Kouri *et al*., 1987).

1. 5 Taxonomy of Dengue Virus

The word arbovirus is an ecological term used to describe viruses that require a blood-sucking arthropod to complete their life cycle. Arboviruses are largely zoonoses that depend on animal species other than humans for maintenance. Humans are incidental hosts and do not contribute to their transmission cycle. Most arboviruses of public health importance belong to one of these three families: *Flaviviridae*, *Togaviridae* and *Bunyaviridae* (Gubler, 2002b).

The *Flaviviridae* is a large family of arboviral pathogens responsible for causing severe disease and mortality in humans and animals (Mukhopadhyay *et al*., 2005). This family consists of three genera: *Flavivirus*, *Pestivirus* and *Hepacivirus*. Although pestiviruses and hepaciviruses have genome replication strategies that are similar to those of flaviviruses, they are antigenically distinct, are not arthropod-borne and represent lineages that may have diverged early in evolution of the family. More than 70 species of flavivirus have been described such as Japanese Encephalitis virus (JEV), Murray valley encephalitis virus (MEV), Tick-borne encephalitis virus (TBE), West Nile virus, Kunjin virus and dengue virus (DENV). The four antigenically distinct serotypes

of dengue virus (DENV-1 to DENV-4) belong to the order *Nidovirales* of the *Flaviviradae* family (Calisher & Gould, 2003).

1.5.1 Genome Organisation of the Family Flaviviridae

The *Flaviviridae* family of viruses have single stranded positive sense RNA genomes of around 11 kilo bases (kb). Genome organisation of all flaviviruses is more or less similar but distinctly different from that of pestiviruses and hepaciviruses. The differences in organization of the genomes of these three genera of the flaviviridae family are illustrated in *figure 2*. The genome of flavivirus is capped at the 5'-terminus, but it is not polyadenylated whereas the genomes of hepaci and pestiviruses contain an internal ribosome entry site (IRES) element at their 5'-termini. More than 95% of the RNA genome encodes a single polyprotein that is processed by both viral and host proteases to produce individual proteins (see *figure 7*). Mature gene products of flaviviruses are translated in the order of NH_2-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH. A nucleo-capsid comprising of the 12-14 kDa capsid protein encapsulates the flaviviral genome. This nucleo-capsid is surrounded by a lipid bilayer containing the 7-8 kDa membrane protein and the 55-60 kDa envelope protein (reviewed in (Lindenbach & Rice, 2003)).

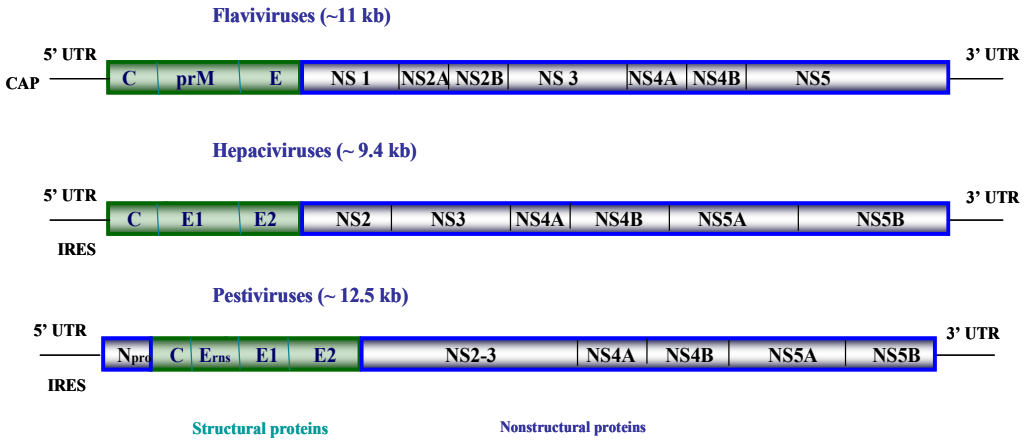


Figure 2: Genomic organization of members of the *Flaviviridae*. The viral genome consists of a single-stranded RNA molecule of positive polarity which is capped in flaviviruses and contains IRES in hepaciviruses and pestiviruses. Untranslated regions (UTR) are present at the 5' and 3' end of the

genomes. Genes encoding the structural proteins are indicated by green boxes and those encoding the non-structural proteins are indicated by blue boxes. Adapted from (Leyssen *et al*., 2000).

1.5.2 Replication Cycle of the Family Flaviviridae

Viral particles bind to cells via interactions between the viral surface glycoprotein and cellular receptors such as heparin sulphate. An unknown co-receptor which has higher affinity and lower specificity to the virus is assumed to trigger internalization of virus into clathrin-coated pits via receptor-mediated endocytosis. It is thought that virions are brought into a pre-lysosomal endocytic compartment where low pH induces conformational changes in the viral envelope and allows it to fuse with the endosomal membrane releasing the nucleocapsid into the cytoplasm. The viral genome is released into cytoplasm by an uncoating mechanism that is not understood yet. After release of the viral genome into the cytoplasm, the 5' untranslated region (5'UTR) directs the RNA to the ribosomes, where the translation of the single open reading frame into a precursor polyprotein occurs. Viruses belonging to the genus *Flavivirus* have a capped 5'UTR whereas those that belong to *Pestivirus* and *Hepacivirus* contain an internal ribosomal entry site (IRES), which directs the ribosome to the first triplet coding for the polyprotein. The viral polyprotein is processed co- and post translationally by cellular proteases (signalases) and viral proteases into individual and functional viral proteins. Viral infection induces rearrangement of cytoplasmic membranes in the perinuclear region into organised structures such as convoluted membranes (CM) or vesicle packets (VP). It has been shown that VPs are sites of RNA replication. RNA-dependent RNA polymerase (NS5) produces minus-strand single-stranded RNA that serves as a template for the production of new plus-strand single-stranded RNA genomes. After replication, the viral genome is encapsulated in the nucleocapsid proteins and directed to the endoplasmic reticulum or other membranous structures induced by viral infection, where the immature virus, surrounded by a lipid envelope containing viral proteins, buds off into the endoplasmic reticular (ER) lumen. Passing through the secretory pathway, the envelope proteins become glycosylated. Finally, mature viruses are released into the extra-cellular space. *Figure 3* is a schematic representation of replicative cycles of the *Flavivirus* compared to *Hepacivirus* and *Pestivirus* (reviewed in (Leyssen *et al*., 2000, Lindenbach & Rice, 2003)).

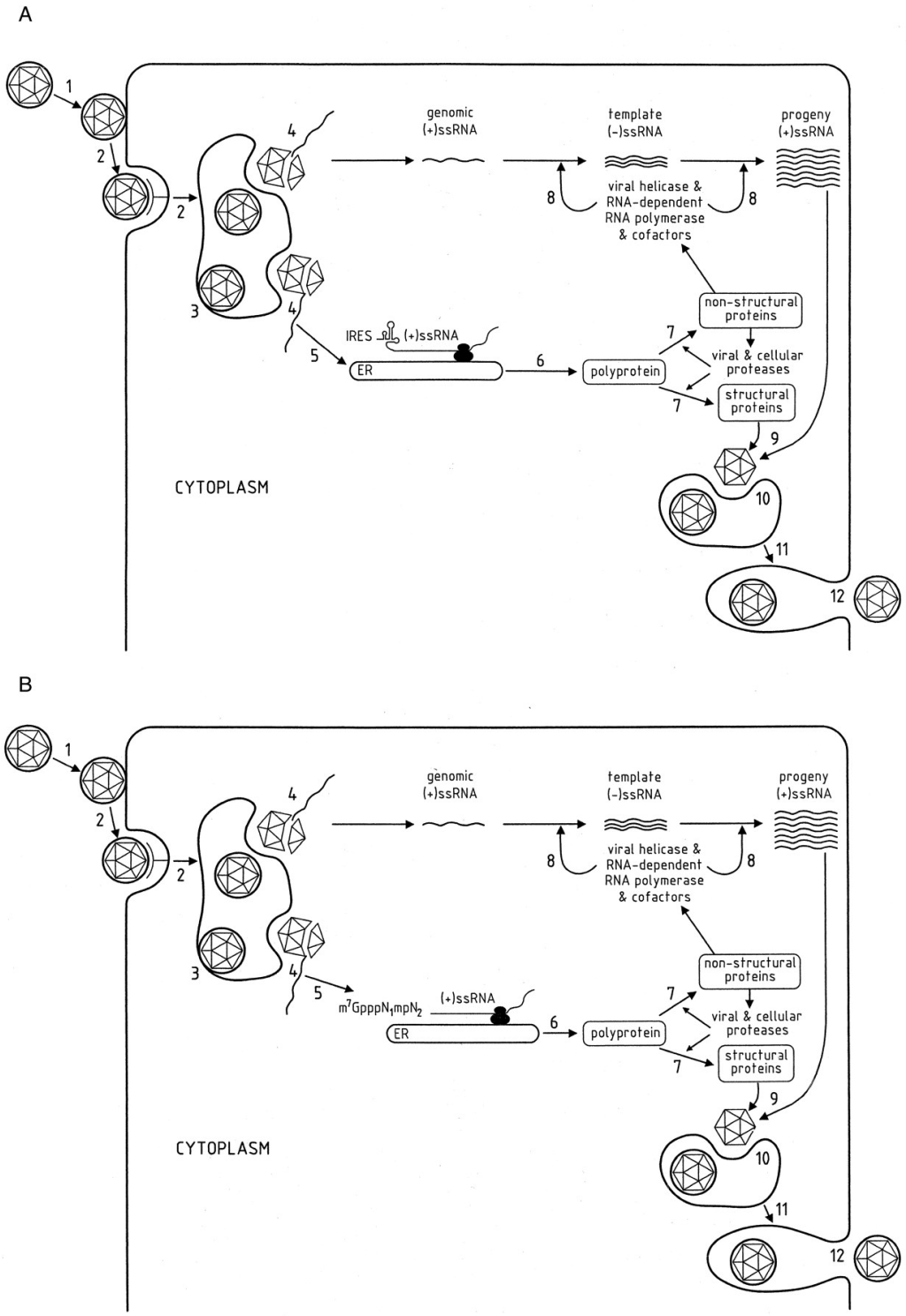


Figure 3: Replicative cycle of members of the *Flaviviridae*. The presumed replication cycles of the hepaciviruses and pestiviruses (A) and of the flaviviruses (B) are shown. 1, adsorption, 2, receptor-mediated endocytosis; 3, low-pH fusion in lysosomes; 4, uncoating; 5, IRES-mediated initiation of translation (A) or cap-mediated initiation of translation (B); 6, translation of the viral RNA into viral

precursor polyprotein; 7, co- and posttranslational proteolytic processing of the viral polyprotein by cellular and viral proteases; 8, membrane-associated synthesis of template minus-strand RNA and progeny plus-strand RNA; 9, assembly of the nucleocapsid; 10, budding of virions in the endoplasmic reticulum; 11, transport and maturation of virions in the endoplasmic reticulum and the Golgi complex; 12, vesicle fusion and release of mature virions. ss, single stranded (taken from (Leyssen *et al.*, 2000)).

1.6 Molecular Biology of Dengue Virus

The molecular biology findings and studies discussed here have largely drawn from studies in other flaviviruses too but have been discussed in context of dengue.

1.6.1 Structure of the Dengue Virus

Electron micrographs of dengue show the virion to be a spherical particle 48 to 50 nm in diameter with an electron dense core of about 30 nm surrounded by lipid envelope. The surface of virus particles contains the envelope (E) and membrane (M) proteins. The E glycoprotein which is the major antigenic determinant on virus particles mediates binding and fusions steps in the viral entry. M protein is a small proteolytic fragment of the precursor prM protein and is produced during maturation of the viral particles in the secretory pathway. The discrete nucleocapsid contains the capsid (C) protein and genomic RNA. In addition to mature virions, smaller (~14 nm) non-infectious particles are also released from infected cells which contain E and M proteins but lack the nucleocapsid (Lindenbach & Rice, 2003, Smith *et al.*, 1970).

Dengue virus surface is composed of 180 copies the E and M proteins. The E protein of dengue virus contains a class II fusion peptide sequence which is important for viral invasion of a host cell. There are remarkable structural deviations between the immature and mature dengue envelopes as revealed by elegant cryo-electron microscopy studies (see *figure 4*). The immature dengue virus particle is covered with 60 asymmetric trimers of prM-E heterodimers that stick out like spikes from its surface. The prM protein protects E against premature fusion while passing through the acidic environment of the trans-Golgi network during morphogenesis. During maturation, the N-terminal amino acids of the prM protein are released by furin cleavage which induces a rearrangement of the E proteins essential for fusion. In the

mature virus, E proteins exist as homodimers that lie on the viral membrane in the form of 30 so-called rafts. Each raft contains three parallel dimers arranged in icosahedral symmetry and organized into a herringbone pattern (Kuhn *et al.*, 2002, Zhang *et al.*, 2003).

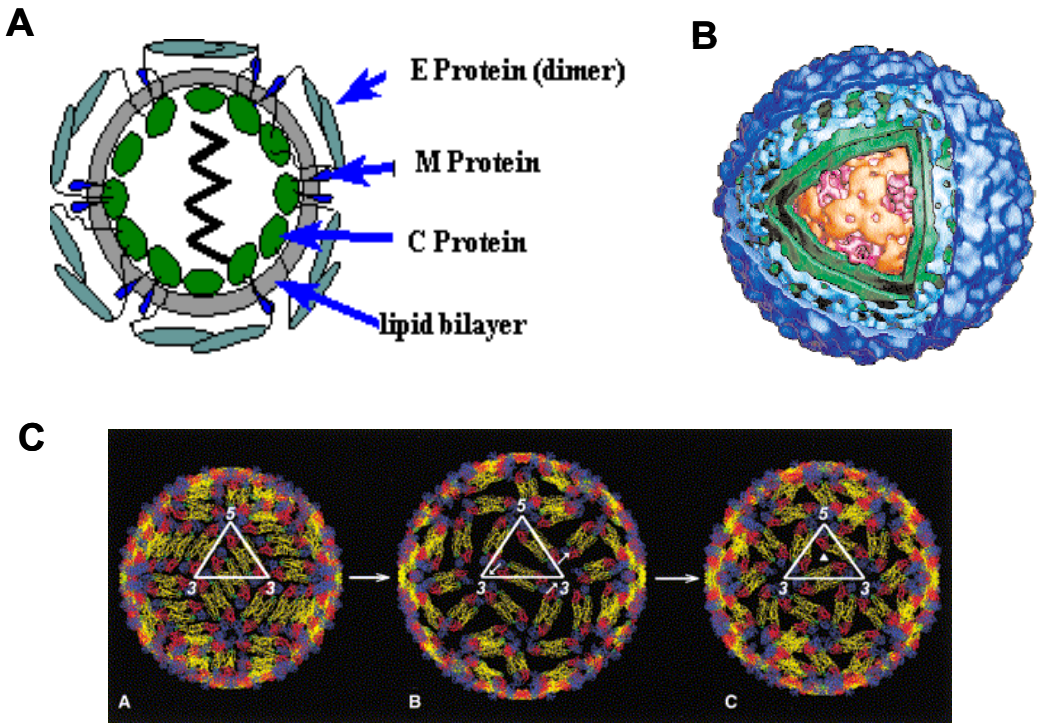


Figure 4: Morphology of the dengue virion. A) The genome is covered by a nucleocapsid which in turn is enveloped by a lipid bilayer and the envelope consisting of the E-protein dimers. B) *In silico* simulation of a cryo-electron micrograph of dengue virions with five distinct sheets visible in the electron density — two outer protein shells (dark and light blue), a lipid bilayer (green), a nucleocapsid shell (orange), and finally, the RNA genome (red) packaged in the centre of the virus C) Proposed acid-catalysed rearrangement of E dimers into the fusogenic state. Adapted from (Kuhn *et al.*, 2002)

1.6.2 Receptor Mediated-Endocytosis of Dengue

Monocytes , macrophages and dendritic cells have been thought to be the major target cells in dengue virus infection (Jessie *et al.*, 2004, Tassaneetrithep *et al.*, 2003) and are responsible for the dissemination of the virus after its initial entry but the primary cell that supports dengue virus replication in severe cases remains unknown. The Fcγ receptor has been shown to bind to dengue virus-neutralising antibody complex (Daughaday *et al.*, 1981) and this mechanism is associated with an increase in infection

and subsequently disease severity (Kliks *et al*., 1989, Littaua *et al*., 1990, Mady *et al*., 1991) but it does not seem to play a role in viral entry (Humphery-Smith & Vincendeau, 1993, Moreno-Altamirano *et al*., 2002).

The first event required for infection by dengue virus is attachment which results from a specific interaction between the E proteins of dengue with cell surface receptors. Two types of molecules have been involved as dengue virus receptors. The first type of molecule is a glycosa-aminoglycan, specifically, heparan sulphate (Chen *et al*., 1997b, Hilgard & Stockert, 2000), but this is thought to serve primarily as initial attachment factors concentrating viral particles at the cell surface for subsequent interaction with other receptors. The second types of molecules are proteins with different molecular masses that have been described as putative dengue virus receptors in several cell lines. Among them, a 45 kDa glycoprotein in C6/36 cells (Salas-Benito & del Angel, 1997), a 74 kDa protein present in Vero cells (Martinez-Barragan & del Angel, 2001), two proteins of approximately 45 kDa and 75 kDa from a myelomonocytic cell line (Bielefeldt-Ohmann, 1998), a 105 kDa protein in erythroleukemia cells (Putnak *et al*., 1997), and proteins of 29 and 43 kDa from an endothelial cell line (Wei *et al*., 2003) have been described (Reyes-Del Valle *et al*., 2005). In monocytes/macrophages, membrane proteins of 27, 45, 67, and 87 kDa were described also as putative receptors for dengue virus (Moreno-Altamirano *et al*., 2002). LPS/CD14-associated binding proteins and other glycoproteins (Chen *et al*., 1999, Marianneau *et al*., 1996) have also been proposed as cellular receptors for dengue. Recently, DC-SIGN that participates in binding large numbers of other viruses such as HIV-1, Ebola and CMV to host cell surfaces has been suggested as a mediator of dengue infection in dendritic cells (Lozach *et al*., 2005, Navarro-Sanchez *et al*., 2003, Tassaneetrithep *et al*., 2003).

These studies show that there is little correlation between the various putative proteinaceous receptors suggesting that dengue may infect different cell types by interaction with distinct receptors that are specific for each cell target. That different serotypes utilise distinct receptors has also been suggested but a recent study showed that all four serotypes of dengue utilise the same receptor at least in mosquito cells (Mercado-Curiel *et al*., 2006). It is also possible that the interaction between the virus

and its cellular receptor(s) is a multi-step process and multiple attachment receptors may be sequentially used for dengue virus to gain entry.

1.6.3 Membrane Fusion

1.6.3.1 Structure of the E protein

Conformational changes in the dengue E protein induced by the acidic pH in the endosome mediate membrane fusion after receptor mediated endocytosis. The structure of soluble E (sE) protein (Kuhn *et al*., 2002, Zhang *et al*., 2003, Zhang *et al*., 2004) consists of three domains; domain I (DI), the N-terminal but structurally central domain, domain II (DII), the fusion domain containing the hydrophobic fusion peptide, and domain III (DIII), the putative receptor binding domain. Cryo-electron microscopy revealed the presence of a C-terminal 'stem' and a two pass transmembrane sequence through which E protein is anchored to the viral surface. The dimeric form of the E protein on viral membrane surface dissociates upon acidification, binds liposomes, and irreversibly trimerizes. These trimers cluster on the liposome surface and induce curvature which might promote fusion. In the E trimer, the fusion peptides of each monomer are at one end and domains DI and DIII are at the other (see *figure 5*). The flexibility of E protein is a function of the movement at the "hinge" region indicated by black arrows in *figure 5*.

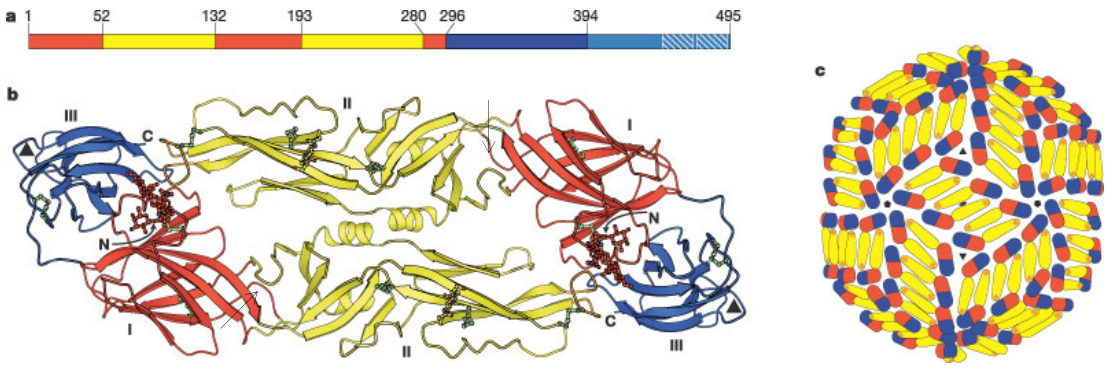


Figure 5: Structure of the dimer of dengue E soluble fragment (sE) in the mature virus particle. A) The three domains of dengue sE. Domain I is red, domain II is yellow, domain III is blue. A 53-residue 'stem' segment links the stably folded sE fragment with the C-terminal transmembrane anchor. B) The sE

dimer. This is the conformation of E in the mature virus particle and in solution above the fusion pH. C) Packing of E on the surface of the virus. Cryo-electron microscopy image reconstructions show that 90 E dimers pack in an icosahedral lattice. Taken from (Modis *et al.*, 2004)

1.6.3.2 Proposed Mechanism of Membrane Fusion

Dengue viral E protein, like that of other flaviviruses (Allison *et al.*, 1995, Ferlenghi *et al.*, 2001, Heinz & Allison, 2000, Heinz *et al.*, 1994) uses a class II fusion mechanism described in (Modis *et al.*, 2004). According to this proposed mechanism (see *figure 6*), E protein associates with a cell surface receptor possibly through the putative receptor binding domain III (blue) and this binding leads to uptake into endosomes. Acidic pH in the endosomes causes dissociation of E dimers which allows domains I (red) and II (yellow) to flex relative to each other. Domain II turns outward, away from the virion surface, and inserts its fusion loop into the target-cell membrane. When domain II turns outward, the tight packing interactions on the outer surface of the virion are lost and E monomers are rearranged laterally to form E trimers. The E protein trimer formation contacts spreads from the fusion loops at the trimer tip to domain I at the base. Domain III shifts and rotates thereby causing the two membranes to bend towards each other, forming apposing 'nipples' and an intermediate 'hemi fusion stalk' is formed which flicks open into narrow fusion pores. Migration of transmembrane segments (light blue) prevents the closure of these pores. With the stems docked along the surface of domains II and with the fusion loops and transmembrane anchors next to each other in the fused membrane, the virus gains entry into the cell.

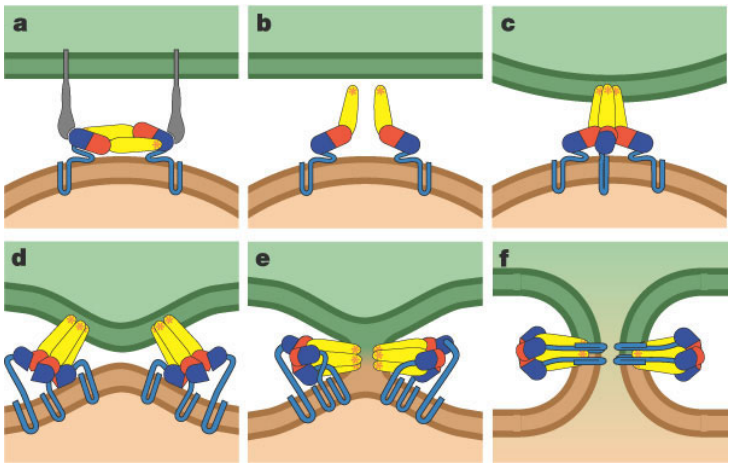


Figure 6: Proposed class II fusion mechanism of dengue E protein. A) E binds to a receptor on the cell surface and the virion is internalized to an endosome. B) Reduced pH in the endosome causes domain II to hinge outward and allows E monomers to rearrange laterally. C) The fusion loop inserts into host-cell membrane, promoting trimer formation. D) Formation of trimer contacts spreads from the fusion loop at the tip of the trimer, to the base of the trimer. Domain III shifts and rotates to create trimer contacts, causing the C-terminal portion of E to fold back towards the fusion loop. Energy release by this refolding bends the apposed membranes. E) Creation of additional trimer contacts between the stem-anchor and domain II leads first to hemi fusion and then F) to formation of a lipid fusion pore (taken from (Modis *et al.*, 2004)).

1.6.4 Translation and Polyprotein Processing

The 5' capped single stranded RNA genome of dengue (like other flaviviruses) that is released after membrane fusion and capsid uncoating acts as mRNA for translation of viral proteins. A non-canonical translation mechanism has also been described for dengue when cap mediated translation is inhibited (Edgil *et al.*, 2006). Because translation occurs in a 5'-3' direction whereas replication occurs in a 3'-5' direction, it is thought that these processes must happen sequentially rather than simultaneously. Structural studies indicate that virions contain 180 copies of E protein and a single copy of the viral genome indicating that translation must be a very efficient process as compared to flaviviral replication. Translation of the single open reading frame produces a large polyprotein that is cleaved co- and post-translationally into 10 proteins (Lindenbach & Rice, 2003). The N-terminal one fourth of the polyprotein encodes structural proteins C-prM-E followed by non structural proteins NS1-NS2A-NS3-NS4A-NS4B-NS5 (Rice *et al.*, 1985). Dengue polyprotein traverses the endoplasmic reticulum (ER) membrane at several positions - prM, E, NS1 and a part of NS4B are thought to localize to the ER lumen via hydrophobic signal sequences while the remaining proteins are thought to be cytoplamic. Membrane association and polyprotein conformation are important for efficient processing (Clum *et al.*, 1997, Zhang & Padmanabhan, 1993).

As illustrated in *figure 7*, viral NS3-2B serine protease is responsible for cleavages among NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/NS4B and NS4B/NS5 (Arias *et al.*, 1993, Cahour *et al.*, 1992, Chambers *et al.*, 1991, Chambers *et al.*, 1990a, Preugschat & Strauss, 1991, Preugschat *et al.*, 1990, Yusof *et al.*, 2000, Zhang *et al.*, 1992)

whereas host signal peptidase is thought to cleave C/prM, prM/E, E/NS1 and the C-terminus of NS4A (Cahour *et al.*, 1992, Despres *et al.*, 1990, Falgout & Markoff, 1995, Gruenberg & Wright, 1992, Markoff, 1989, Stocks & Lobigs, 1995). Furin is required for maturation cleavage of prM into M and the enzyme responsible for NS1/NS2A cleavage is unknown (Elshuber *et al.*, 2003, Falgout & Markoff, 1995, Keelapang *et al.*, 2004, Stadler *et al.*, 1997). At the junction of C and prM there is a short hydrophobic signal peptide that directs the translocation of prM into the ER lumen and anchors C to the ER. This anchored form of C (anchC) is cleaved on the cytoplasmic side of the signal sequence by the NS3-2B protease and is the only structural protein that is cleaved by the viral protease (Amberg *et al.*, 1994, Amberg & Rice, 1999, Lobigs, 1993, Yamshchikov & Compans, 1994). In a coordinated manner, there is a cleavage on the luminal side of the C/prM junction by host signalase. This cleavage is significantly slower than the other signalase cleavages and it is thought that regulated cleavage at this site is important for productive infection (Lee *et al.*, 2000). Another delayed cleavage whose regulation is thought to be important for viral replication is at the NS3/4A junction by the viral protease as evidenced by the NS3-4A precursor reported in flavivirus infected cells (Lindenbach & Rice, 2003, Zhang *et al.*, 1992).

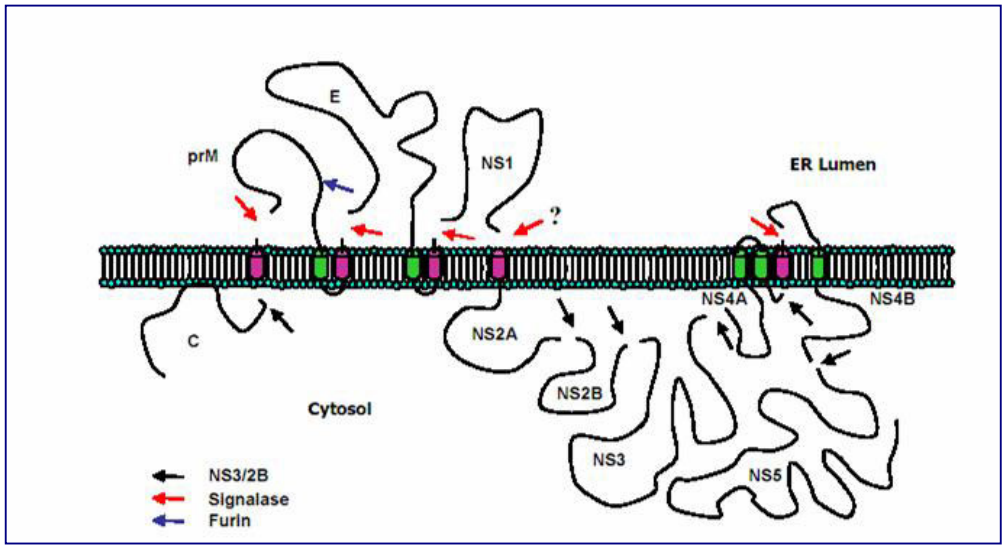


Figure 7: Schematic representation of proteolytic cleavage sites on the dengue polyprotein in order of release of individual proteins. Signal peptides are shown as pink cylinders and transmembrane peptides as green. Peptide cleavage sites are indicates by arrows and the respective proteases are indicated. The NS1/NS2A junction is cleaved by an unknown signalase represented by "?"

1.6.5 Dengue Viral Proteins

Mature gene products of flaviviruses are translated in the order of NH_2-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH and cleaved into individual proteins by both viral and cellular proteases as described earlier. The features of dengue viral proteins are detailed below and summarized in *table 2*.

1.6.5.1 Structural Proteins

a) Capsid Protein (C)

Capsid is a highly basic protein of about 11 kDa with highly charged residues clustered at the N- and C- termini separated by a short internal hydrophobic domain that mediates membrane association (Boege *et al*., 1983, Khromykh & Westaway, 1996, Rice *et al*., 1985, Trent, 1977). Nascent C also contains a C-terminal hydrophobic anchor that serves as a signal peptide for ER translocation of prM as discussed earlier. The maturation of C from anchC is believed to trigger the assembly of nucleocapsids where in the highly positively charged termini are believed to associate with and neutralize negatively charged viral RNA. A putative bipartite nuclear localization motif is found in C proteins of all flaviviruses and dengue C has been shown to translocate to the nucleus but the significance of this nuclear translocation is unknown (Bulich & Aaskov, 1992, Makino *et al*., 1989, Tadano *et al*., 1989, Wang *et al*., 2002).

b) Membrane Protein (M)

Precursor of membrane (prM) protein has a predicted molecular weight of about 26 kDa and is directed into the ER by the C-terminal hydrophobic residues of the capsid protein. Delayed cleavage of C by NS3-2B regulates the furin (or furin like enzyme) mediated prM to M cleavage (Falgout & Markoff, 1995) and this regulation is important for productive infection (Lee *et al*., 2000). prM is associated with the E protein in its dimer form in immature virions and is thought to stabilize the E protein (Guirakhoo *et al*., 1991, Lorenz *et al*., 2002) and prevent its conformational changes during intracellular transport of immature virions though acidic compartments of the

trans-golgi network. Following cleavage, the M protein is found in mature virions whereas the pr fragment is secreted (Murray *et al*., 1993).

c) Envelope Protein (E)

The ~53 kDa E protein is the major structural protein of the virion and its structure has been discussed earlier (see *section 2.3.1*). E is synthesized as a type I membrane protein containing conserved twelve cysteines that form six disulphide bonds, is N-glycosylated and its proper folding and stabilization depend on prM (Deubel *et al*., 1991, Lorenz *et al*., 2002, Men *et al*., 1991). The E protein is involved in a number of biologic activities including receptor binding, hemagglutination of erythrocytes, induction of the major neutralizing antibody in protective immune response, mediation of virus specific membrane fusion of acid pH endosomes and virus assembly (Chambers *et al*., 1990a, Lindenbach & Rice, 2003, Mukhopadhyay *et al*., 2005).

1.6.5.2 Nonstructural Proteins

a) NS1

NS1 is an approximately 46 kDa glycoprotein that is essential for virus viability but has no established biological activity. During infection *in vitro*, NS1 is synthesised as a hydrophilic water soluble monomer and is translocated into the ER through a hydrophobic signal sequence localized at the carboxyl terminus of the E protein (Falgout & Markoff, 1995). Once inside the ER, NS1 becomes a hydrophobic homodimer which interacts with membranous components (Winkler *et al*., 1989, Winkler *et al*., 1988). The fraction of NS1 that remains associated with intracellular organelles appears to be involved in the early steps of viral replication (Lindenbach & Rice, 2003, Mackenzie *et al*., 1996). Largely the NS1 protein is exported along the secretory pathway to the plasma membrane where it is either anchored via a glycosylphosphatidylinositol (GPI) group (Jacobs *et al*., 2000) or is released as a soluble hexamer from infected mammalian cells (Alcon-LePoder *et al*., 2005, Flamand *et al*., 1999).

In *in vivo* (patient) studies, the soluble form of NS1 was found to be circulating in sera from dengue virus-infected patients (Alcon *et al*., 2002, Young *et al*., 2000). Serum concentrations of soluble NS1 depend on virus serotype, the time course of infection, and the individual host and they appear to be higher in plasma from patients with DHF/DSS during the febrile phase rather than dengue fever (Libraty *et al*., 2002). A recent study has proposed that antibodies elicited by NS1 during infection may play a role in vascular leakage by cross-reacting with and activating cell surface antigens of endothelial cells or platelets and causing their death by apoptosis or complement-mediated lysis (Avirutnan *et al*., 2006).

b) NS2A

NS2A (~22 kDa) is a hydrophobic protein generated by cleavage at the N-terminus by an unknown host signalase (Falgout & Markoff, 1995) and at the C-terminus by the viral protease and is thought to be membrane spanning. In addition a C-terminally truncated form of NS2A has also been reported in yellow fever virus to be important for infectious particle production (Chambers *et al*., 1990b). Studies in other flaviviruses have shown that NS2A interacts with NS3, NS5 and 3' UTR (Mackenzie *et al*., 1998), cannot be complemented in *trans* (Khromykh *et al*., 2000) and that it is important for down regulation of type I interferon response to infection (Liu *et al*., 2004, Liu *et al*., 2006) but the specific function of dengue NS2A has not been established.

(c) NS2B

NS2B protein is an ER resident integral membrane protein of about 14 kDa and has been extensively studied as an essential cofactor for the activity of the NS3 serine protease. Its interaction with NS3 is important for the serine protease activity and the cofactor activity is encoded in a conserved 40 residue hydrophilic region in the centre that is flanked by hydrophobic sequences which mediate membrane insertion. (Arias *et al*., 1993, Clum *et al*., 1997, Falgout *et al*., 1993, Falgout *et al*., 1991, Niyomrattanakit *et al*., 2004, Yon *et al*., 2005, Yusof *et al*., 2000). NS2B of JEV renders bacterial membranes permeable suggesting that it might be involved in modulating membrane permeability during infection (Chang *et al*., 1999).

(d) NS3

NS3 is a multifunctional protein of about 69 kDa. The N-terminal 185 aa residues include a serine catalytic triad which in complex with the NS2B cofactor functions as the viral protease that is required for polyprotein processing (Arias *et al*., 1993, Falgout *et al*., 1991, Zhang *et al*., 1992). The trypsin like catalytic triad of flaviviral NS3 protease is distinct from trypsin due to its preference for dibasic residues at P2 and P1 in the substrate (Chambers *et al*., 1993, Lin *et al*., 1993b, Nestorowicz *et al*., 1994, Yusof *et al*., 2000). Protease can be inhibited by aprotinin and substrate analogues (Leung *et al*., 2001, Murthy *et al*., 2000, Yin *et al*., 2006a, Yin *et al*., 2006b). Crystal structures have been determined for dengue NS3 with and without NS2B cofactor as well as with or without a substrate (Erbel *et al*., 2006, Murthy *et al*., 1999, Murthy *et al*., 2000) and while such studies confirmed the overall similarity to other members of this family, they reinforce an unusually flexible mode of substrate binding.

The C-terminal region of dengue NS3 has conserved domains found in the DEXH family of RNA helicases that utilise energy from ATP hydrolysis for RNA unwinding (Gorbalenya *et al*., 1989, Li *et al*., 1999, Wengler, 1993) and the crystal structure for this domain has been determined recently (Wu *et al*., 2005, Xu *et al*., 2005). The RNA unwinding activity is essential for virus (Matusan *et al*., 2001) but the precise role of helicase in viral replication is not known. It is thought to be important for melting regions of the RNA secondary structure involved in template recognition, increasing polymerase processivity by eliminating secondary structures or resolving duplexes formed during replication. Lending support to this are binding studies that showed an association of NS3 with the 3' stem loop structure and NS5 and a subsequent increase in the NTPase activity of NS3 (Chen *et al*., 1997a, Cui *et al*., 1998, Lindenbach & Rice, 2003). Truncated forms of NS3 have been reported which result from an alternative protease cleavage site in the helicase domain but the role of these forms is not clear (Arias *et al*., 1993, Teo & Wright, 1997). In addition to the NTPase activity, the C-terminal part of NS3 also encodes an RNA triphosphatase (RTPase) activity which is thought to dephosphorylate the 5' end of genomic RNA before capping (Wengler, 1993). Both the NTPase and RTPase activities of the protein could be

inhibited by Mg^{2+}, high ionic strength and a non hydrolysable ATP analogue suggesting that both activities share a common active site (Bartelma & Padmanabhan, 2002).

(e) NS4A

NS4A is a small (~16 kDa) hydrophobic protein and its C-terminus acts as a signal sequence for translocation of NS4B into the ER lumen. However, the processing of the flaviviral NS4A/4B junction by a signal peptidase requires NS2B-NS3 serine protease cleavage at a site just upstream of the signal peptide to yield the so called 2K peptide (Lin *et al*., 1993a, Preugschat & Strauss, 1991) In addition, unprocessed NS3/4A and NS4A/4B forms have been observed (Chambers *et al*., 1990a, Lindenbach & Rice, 2003, Preugschat & Strauss, 1991). Kunjin NS4A localizes to vesicular packets that are sites of RNA replication (Mackenzie *et al*., 1998), and like the other three small hydrophobic proteins NS2A, 2B and NS4B, interferes with the host interferon signaling (Liu *et al*., 2005). Interestingly, NS4A of Hepatitis C virus has been shown to accumulate on mitochondria and render the cells prone to mitochondria-mediated apoptosis (Nomura-Takigawa *et al*., 2006). Recently it has been shown in Kunjin virus that cleavage of the NS4A/4B junction by the viral protease is the key initiation event in the induction of membrane rearrangement and that the NS4A protein intermediate containing the uncleaved C-terminal transmembrane domain plays an essential role in these membrane rearrangements and golgi trafficking (Roosendaal *et al*., 2006).

(f) NS4B

NS4B is an approximately 28 kDa hydrophobic integral membrane protein. Deletion studies and *trans*-complementation experiments of the Bovine viral diarrhoea virus (Grassmann *et al*., 2001) and Kunjin virus (Khromykh *et al*., 2000) indicate an important role of this protein in viral RNA replication. Kunjin NS4B has been shown to localize to the nucleus (Westaway et al., 1997a) but dengue NS4B protein localizes to cytoplasmic foci originating from the endoplasmic reticulum (Miller *et al*., 2006). Mutational analysis show that dengue NS4B could be involved in maintaining the balance between efficient replication in the mosquito vector and the human host (Hanley *et al*., 2003). The best investigated function of flavivirus NS4B is its ability to

block the IFN-α/β-induced signal transduction cascade by interfering with phosphorylation of STAT1 (Munoz-Jordan *et al*., 2005, Munoz-Jordan *et al*., 2003). But the aa residues 54-102 of NS4B that have shown in these studies to be required for IFN antagonism, reside in the ER lumen which raises the question whether these studies (that were done on over expressed proteins) would be relevant to infection setting (Miller *et al*., 2006).

(g) NS5

NS5 is a large (~103 kDa) well conserved multifunctional protein involved in RNA replication. It contains N-terminal RNA cap-processing activity and a C-terminal RNA dependent RNA polymerase (RdRp) activity. NS5 can be phosphorylated by an associated serine/threonine kinase (Kapoor *et al*., 1995, Reed *et al*., 1998). The N-terminal region of NS5 shares homology with S-adenosyl-methionine (SAM)-dependent methyl transferases and it has been shown to transfer methyl groups from SAM for both guanine N-7 and ribose 2'-O methylation events that are required 5' RNA capping (Bartholomeusz & Wright, 1993, Egloff *et al*., 2002, Ray *et al*., 2006). The C-terminal region of NS5 encodes the RdRP activity. Although a self-primed "copy back" RNA has been shown to be the major product of an *in vitro* polymerase activity assays, *de novo*-initiated RNA synthesis is thought to be more important in *in vivo* settings (Ackermann & Padmanabhan, 2001, Rice *et al*., 1985, Tan *et al*., 1996).

The N-terminal region (residues 320-405) also contains two nuclear localization sequences (NLS), a binding site for β-importin (aa 320-368) and an interaction site with NS3 (Brooks *et al*., 2002, Johansson *et al*., 2001, Kapoor *et al*., 1995). The importance of importin binding and nuclear translocation of NS5 has not been understood. However, a recent study demonstrated that NS5 induces IL-8 secretion late in infection suggesting that nuclear translocation of NS5 might modulate the transcription of cytokines such as IL-8 to perhaps counter the anti viral effects of innate immunity (Medin *et al*., 2005). The interaction of NS5 with the NS3 protein has been shown to enhance the RNA triphosphatase (RTPase) and the nucleotide triphosphatase (NTPase) activities of NS3 (Yon *et al*., 2005). Cross linking studies in JEV have shown that the 3'stem loop structure binds to both NS3 and NS5 (Chen *et al*., 1997a). Despite

these associations, NS5 can be physically separated from membrane fractions suggesting that only a fraction of RdRP is required for viral replication (Chu & Westaway, 1992).

Name	Function	Features	Length
C	Nucleocapsid protein	Highly basic, contains bipartite NLS	114aa
prM	Precursor membrane glycoprotein involved in nucleocapsid dissociation. Protects E from acid catalyzed re-arrangement and fusion	Cleaved to form mature M protein prior to virus release	166aa
E	Envelope glycoprotein. crucial for receptor mediated endocytosis, antibody induction, membrane fusion	Dimerizes, contains putative receptor binding site	495aa
NS1	Role in early RNA replication, acts as soluble complement fixing antigen. Putative roles in minus strand replication, virus assembly and release	GPI anchored, secreted after glycosylation, oligomerizes into dimer, possibly hexamer	352aa
NS2A	Unknown function. Possible regulator of NS1 function. Putative role in virus assembly and release	Binds to 3'UTR, NS3 and NS5	218aa
NS2B	Cofactor for NS3 protease	Conserved hydrophobicity as in NS4A/B and NS2A	130aa
NS3	N-terminus has serine protease function while the C terminus has NTPase and helicase activities	Highly conserved, binds to NS5	618aa
NS4A	Unknown function. Speculated to play a role in protein targeting and anchoring.	Binds to most other non structural proteins	150aa
NS4B	Unknown function	Conserved membrane topology across *Flaviviridae*	248aa
NS5	RNA dependent RNA polymerase. Putative methyl transferase domain might function in 5' capping	Contains NLS and exists as both nuclear and cytoplasmic forms which are differentially phosphorylated	900aa

Table 2: Features and functions of flaviviral proteins. The amino acid residues of each protein are given for DENV2. See text for details.

1.6.6. Role of 5' and 3' Untranslated Regions in Dengue Life Cycle

The 5' and 3' untranslated regions (UTRs) of the dengue genome play a very important role in genome stability, viral replication and translation of the polyprotein. Hybridization of the conserved complementary cyclization sequences at the 5' and 3'

ends of the genome result in circularization of the genome. This circularization is considered to provide several advantages for viral replication, including (1) a control mechanism to amplify only full-length templates, (2) coordination of translation and RNA synthesis by overlapping signals at the 5' and 3' ends of the genome, (3) increasing RNA stability, (4) locating the viral polymerase or accessory proteins of the replication complex at the appropriate start site, and (5) controlling the levels of minus strand RNA synthesis (Filomatori *et al*., 2006).

a) 5' UTR

The 100 nucleotide long 5' UTR is important for translation of the genome and also acts as a site for initiation of positive strand synthesis during RNA replication. Although common secondary structures have been found among different flaviviruses, the 5' UTR is not well conserved. However the 5' UTR sequence is conserved almost 100% among the four dengue serotypes (Brinton & Dispoto, 1988, Cahour *et al*., 1995, Filomatori *et al*., 2006, Hahn *et al*., 1987, Lindenbach & Rice, 2003). The predicted 5' dengue viral RNA secondary structure, and consists of a large stem–loop (SLA) that includes a side loop and a second short stem–loop (SLB) terminating in the translation initiator AUG (see *figure 8*). It has been recently shown that the SLA element acts as a promoter for initiation of the viral replication whereas the cyclization motifs at the 3' and 5' ends of the genome are important for circularisation (via long range RNA-RNA interactions) that bring NS5 into proximity with the 3' initiation site (Filomatori *et al*., 2006).

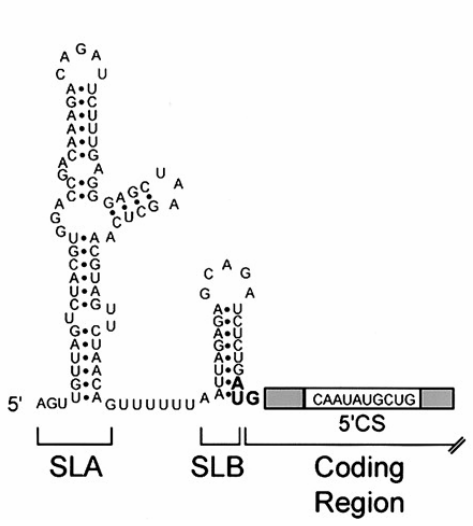


Figure 8: Schematic representation of the predicted secondary structure of an RNA encompassing the first 100 nucleotides of the DV genome obtained by M-fold algorithm. Stem–loop A (SLA), stem–loop B (SLB), and the complementary sequence (5'CS) within the viral coding sequence are indicated. Taken from (Filomatori *et al*., 2006).

b) 3' UTR

The 3' UTR of dengue is about 450 nucleotides long and lacks a poly (A) tail but ends in a very conserved 3' stem–loop (3'SL). A detailed structure–function analysis of the 3'SL in many flaviviruses revealed that it is absolutely required for viral replication (Brinton *et al*., 1986, Elghonemy *et al*., 2005, Men *et al*., 1996, Proutski *et al*., 1997, Rauscher *et al*., 1997, Yu & Markoff, 2005). The 3' stem loop region has been shown to interact with NS3, NS5 and host proteins such as EF1A, PTB and the human La auto antigen although the functional significance of these interactions has not yet been fully explored (Chen *et al*., 1997a, Cui *et al*., 1998, De Nova-Ocampo *et al*., 2002). Another essential RNA element for viral replication, the conserved sequence CS1, is present upstream of the 3'SL (Men *et al*., 1996). This element contains the cyclization sequence CS that is complementary to a sequence at the 5' end present in all mosquito-borne flavivirus genomes (Hahn *et al*., 1987). A synergistic interaction of this element with the 5' UTR is thought to enhance translation initiation as well as RNA stability (Chiu *et al*., 2005).

1.6.7 Dengue RNA Replication

Flaviviral replication complex is assumed to consist of the genomic RNA template together with the NS proteins and presumably some host proteins on cytoplasmic membranes (VPs) (Lindenbach & Rice, 2003, Westaway *et al*., 2003). Replication begins at the 3' end of the genome with the synthesis of a negative-strand RNA that serves as a template for the synthesis of additional positive strand genomic RNA in 10-100 fold excess of the negative strand. Recently it has been shown that a stem loop structure at the 5' UTR acts as a promoter for the synthesis of negative strand. This stem loop element binds NS5 (the viral RdRP) while the hybridisation sequences at the 5' and 3' UTRs mediate the long range RNA-RNA interactions that are required to bring the promoter into proximity with the site of initiation (Filomatori *et al*., 2006).

The negative RNA strands have been isolated exclusively in double-stranded form known as the replicative form (RF) that acts as a recycling template for production of positive strands. As the production of a new positive strand progresses, the previously synthesised positive strand molecule is released at the same rate resulting in a so called replicative intermediate (RI), which is essentially RF with an additional positive strand RNA molecule (Bartholomeusz & Wright, 1993, Chu & Westaway, 1987, Cleaves *et al*., 1981, Nomaguchi *et al*., 2003, Wengler & Gross, 1978, You *et al*., 2001, You & Padmanabhan, 1999). As mentioned earlier, only a fraction of NS5 co-sediments with RdRP activity and polymerase products formed *in vitro* appear to be elongation products of endogenous templates rather than *de novo* synthesis although re-initiation of RNA synthesis has been described in Kunjin virus (Chu & Westaway, 1987). Initiation with exogenous template has been described and the major products of these reactions arise from self primed "copy-back" synthesis and requires templates containing 5' and 3' cyclization sequences (You & Padmanabhan, 1999).

Apart from the 3' and 5' genomic elements non structural proteins NS3 and NS5 play a definitive role in RNA replication. NS5 is essential for the production of RFs (Ackermann & Padmanabhan, 2001, Tan *et al*., 1996) whereas both NS3 and NS5 in addition to unidentified cellular proteins are essential for conversion of RF to RI (Bartholomeusz & Wright, 1993, Kapoor *et al*., 1995, Raviprakash *et al*., 1998). The helicase activity of NS3 is thought to be important for increasing the processivity of RdRP by eliminating secondary structures or resolving duplexes formed during replication. It has also been postulated that differential phosphorylation of NS5 might regulate the NS3 and NS5 interaction and subsequently, the production of RNA (Kapoor *et al*., 1995). In addition localization studies on replication complexes of flaviviruses have identified NS1, NS2A, NS2B and NS4A suggesting their role in RNA replication (Chu & Westaway, 1992, Mackenzie *et al*., 1996, Mackenzie *et al*., 1998). Following replication an $M_7GpppN_{1m}pN_2$ structure caps the 5' end of RNA during maturation. The RTPase (NS3) removes γ and or β phosphates from RNA, a Guanylyl transferase adds guanosine phosphates to dephosphorylated RNA and capped structures are then methylated by the NS5 methyltransferase.

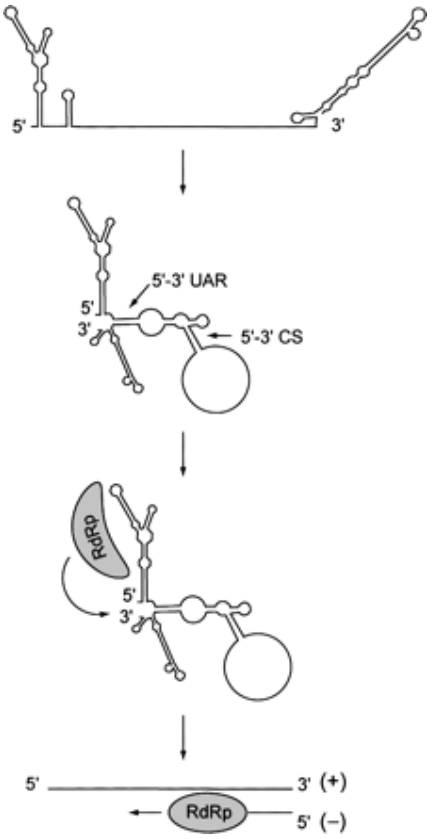


Figure 9: Current model for dengue viral negative strand synthesis. The viral genome circularizes in the absence of proteins mediated by 5'– 3' UAR (upstream AUG region) and 5'–3' CS hybridization. The viral RdRp binds to a 5' stem–loop (SLA), and by long-range RNA–RNA interactions the polymerase is transferred to the site of initiation at the 3' end of the genome. Taken from (Filomatori *et al*., 2006).

1.6.8 Virion Formation and Egress of the Virus

Formation of functional virions of flaviviruses requires the three structural proteins as mentioned before but deletion of structural genes can be complemented by *trans* sequences (Khromykh *et al*., 1998) whereas mutation of polymerase GDD motif prevents virion formation despite active translation and presence of viral ssRNA (Khromykh *et al*., 2001b). Therefore, replication precedes packaging possibly to minimise the transmission of defective RNAs. Virion enclosure involves direct interaction between C protein and RNA. C protein interacts specifically with both 5' and 3' terminal regions of the genome by the highly basic (lys/Arg) N- and C- terminal domains (Ma *et al*., 2004, Wang *et al*., 2002). Molecular switching involving RNA

structures (cyclization sequences, 3'stem loop, pseudoknots) and both viral and cellular proteins are involved in formation of nucleocapsid of enveloped viruses but the mechanism of packaging the flaviviral genome into the nucleocapsid is not well understood. It is thought that that both viral RNA and viral proteins assemble when anchored to the viral-induced smooth membrane structures within the lumen of the rough ER (Barth, 1999, Khromykh *et al*., 2001a). Following the formation of the nucleocapsid, virus particles acquire their envelopes inside the lumen of the rough ER (rER) and associated structures. Electron microscopy studies revealed that a small portion of dengue virus particles are transferred to the Golgi system for maturation and delivered from the cell by exocytosis and a majority of the virus particles enclosed in rER-derived vesicles. Budding at the plasma membrane has also been reported for infections with dengue (Hase *et al*., 1987a), West Nile (Ng *et al*., 1994), and Japanese encephalitis (Hase *et al*., 1987b) viruses but Kunjin virus does not show this mechanism of egress (Mackenzie & Westaway, 2001).

Clearly we do not yet have a complete picture of the vital processes such as translation, and replication in the life cycle of dengue virus as well as the involvement of viral proteins or host factors that direct these processes. Insights into the roles of membrane associated non structural proteins, host factors and the regulatory aspects of translation versus replication of viral genomes are crucial for discovery of therapeutics and antivirals.

1.7 Current Efforts for Control of Dengue

1.7.1 Treatment of Dengue

Currently, there is no specific treatment or vaccine available to tackle dengue and treatment is usually symptomatic. Patients with DF are usually recommended rest, oral fluids to compensate for losses (via diarrhoea or vomiting) and antipyretics for high fever. Acetaminophens but not salicylates are recommended to reduce the risk of acidosis and bleeding complications. The treatment of DHF is a combination of prompt diagnosis, frequent assessment of intra and extra vascular volume status, monitoring for hemorrhagic complications and providing supportive care for the same. Fluid

resuscitation to thwart massive plasma leakage is the core principle of treatment (Thomas *et al*., 2003) and colloid solutions are more efficient than crystalloids in restoring cardiac index and normalising hematocrit (Dung *et al*., 1999).

1.7.2 Vector Control

Given the lack of a dengue vaccines or antivirals, control of *Aedes* mosquitoes is the only means of prevention of DF/DHF. Community-based vector control methods relying on surveillance, treatment (cold aerosol or thermal fogging) and monitoring have been successfully implemented in a large number of countries to counter the vector but re-infestation has emerged as a major problem (Ooi *et al*., 2006, Pinheiro & Corber, 1997). Vector control complemented with laboratory-based surveillance systems would be indispensable for guiding recurrent and emergency interventions. However, accurate diagnosis and prompt treatment have a crucial role in the prevention of suffering and death due to dengue.

1.7.3 Vaccines in Development

Infection with one serotype of dengue virus has been known to induce long lasting protective immunity to that serotype (Sabin, 1952) signifying the feasibility of a vaccine and efforts were initiated more than 70 years ago to produce a vaccine for dengue. Candidate attenuated vaccine viruses and a tetravalent formulation are currently undergoing repeat phase I and II trials. Advances have also been made with chimerical, second generation recombinant and DNA dengue vaccines. *Table 2* summarizes the current status in dengue vaccine development.

However, since enhancing antibody from previous infection and high level of T cell activation during secondary infection contributes to the immunopathology of DHF, an effective dengue vaccine must be able to induce protective response to all four dengue serotypes. Moreover, there are concerns about "immune enhancement" leading to more serious disease following waning of vaccine-elicited immunity. One of the biggest challenges in vaccine development has also been the lack of proper disease models for

testing efficacy. Given these challenges, an effective, safe and affordable vaccine is not an immediate prospect (Chambers *et al.*, 1997).

	Live attenuated	Chimeric Virus	DNA	Inactivated	Subunit recombinant
Number of antigens	10	2	Several	Several	Mainly 1
In vivo replication	Yes	Yes	No	No	No
Immune response	Best	Best	Good	Good	Poor
Memory B and T cells	Best	Best	Good	Fair	Fair
Protection in animals	Yes	Yes	Yes	Yes	Yes
Status of development	Phase I, II Clinical trials	Phase I Clinical trials	Pre-clinical	Pre-clinical	Animal studies

Table 3: Features of candidate dengue vaccines. Adapted from (Chaturvedi *et al.*, 2005).

1.7.4 Prospects for Antiviral Therapy

A specific antiviral treatment for dengue effective in reducing viral load would be able to eliminate disease symptoms and prevent the onset of severe disease and death. A rapid acting compound with low side effect may also be effective as a chemo-prophylactic component, potentially preventing the occurrence of large outbreaks. Several studies have reported potent inhibitors of dengue replication in *in vitro* settings. Concurrently, literature in dengue and other flaviviruses points towards the importance of finding potential targets for antiviral therapy.

1.7.4.1 Inhibitors of Dengue

Salicylates (Liao *et al.*, 2001), entry inhibitors such as heparin and suramin (Chen *et al.*, 1997b), ER α-glucosidase inhibitors (Courageot *et al.*, 2000) and peptide based inhibitors of the dengue viral protese NS3 (Leung *et al.*, 2001, Murthy *et al.*, 2000, Yin *et al.*, 2006a, Yin *et al.*, 2006b) have been shown to inhibit dengue in *in vitro* experiments. While ribavirin (a triazole nucleoside that has been used in treatment of a

number of RNA viral infections including hepatitis C infections) was reported to be very weak against dengue both *in vitro* and *in vivo* (Gabrielsen *et al*., 1992, Huggins, 1989, Koff *et al*., 1983, Leyssen *et al*., 2000, Malinoski *et al*., 1990), a lipophilic analogue of ribavirin -ribavirin-2',3',5'-triacetate- was active in mice (Koff *et al*., 1983). Corticosteroids such as methylprednisolone (Tassniyom *et al*., 1993) or anti-vascular leakage agents such as carbazochrome sodium sulfonate (AC-17) (which decreases capillary permeability) did not reduce mortality in children with severe dengue shock syndrome (Tassniyom *et al*., 1993, Tassniyom *et al*., 1997).

Interferon (IFN) has been long used to treat hepatitis C infections but not flaviviral infections. An unconfirmed report from Cuba described the use of recombinant IFN-α in successful treatment of children with primary dengue infection (Limonta, 1984). In cell culture systems, both IFN-α and IFN-β have been shown to protect cells against dengue infection but IFN-γ has variable effects (Diamond & Harris, 2001, Diamond *et al*., 2000). Interferon inducers such as poly (inosinic acid) - poly (cytidylic acid) [poly(IC)], and 10-carboxymethyl-9-acridanone were reported to cause protective activity against infection of mice with Tick-borne encephalitis virus, Japanese encephalitis virus and West Nile virus. But therapy with IFN or IFN inducers would only be beneficial when treatment is initiated before or very shortly after infection. Especially in case of dengue, by the time severe symptoms appear in the clinical setting, fever has abated in most cases and viremia is undetectable and hence IFN treatment might be too late. Furthermore, interferon might adversely effect the prognosis of severe dengue disease which is thought to develop by largely immune-related mechanisms (Leyssen *et al*., 2003).

While such studies have identified potential inhibitors of dengue replication, most of these experiments have been done under *in vitro* conditions. Furthermore, several questions regarding the mechanism of action as well as efficacy *in vivo* remain unanswered.

1.7.4.2 Targets for Antiviral Therapy of Dengue

Ongoing research has identified possible viral and cellular targets for development of selective antiviral agents for treatment of dengue. Viral enzymatic functions such as the protease (NS3 with cofactor NS2B), helicase/NTPase (NS3), RNA triphophatase, methyl transferase, capping and RNA-dependent RNA polymerases (NS5) have been identified as potential targets for intervention. Alignment of the amino acid sequences indicates that the residues of protease, helicase/NTPase (Kadare & Haenni, 1997, Li *et al*., 1999), methyltransferase (Koonin, 1993) and RNA polymerase enzymes (O'Reilly & Kao, 1998) that are responsible for substrate binding and catalytic function are highly conserved among flaviviruses. Thus a molecule that can selectively inhibit the replication of one flavivirus by targeting one of the viral enzymes involved in viral replication is likely to be effective against other flaviviruses as well.

In addition to these viral targets, cellular enzymes that play a role in viral life cycle may also be valuable targets for therapeutic intervention against dengue. Enzymes such as the inosine monophosphate (IMP) dehydrogenase (Leyssen *et al*., 2005), orotidyleate monophosphate (OMP) decarboxylase and the S-adenosylhomocysteine (SAH) hydrolase (Neyts *et al*., 1996) that provide the key "building blocks" for production of progeny virus and enzymes such as α-glucosidase that are important for viral maturation have been explored as cellular targets (Leyssen *et al*., 2003, Leyssen *et al*., 2000). A recent study documented the role of gelatinolytic metalloproteases (MMP) in vascular leakage of dendritic cells upon dengue infection. This suggests the possibility of a new therapeutic approach for the treatment of viral-induced vascular leakage by specifically targeting MMP (Luplerdlop *et al*., 2006). The major conceivable disadvantage of cellular targets is their toxicity but the trade-off of lower rate of emergence of drug-resistance strains render them worthy of further evaluation.

1.8 Aim of This Thesis

Given the global resurgence of dengue in recent years, there is a clear need to prepare for epidemic surges with antivirals. Knowledge of the dengue disease especially in the molecular terms as well as a through understanding of viral as well as host factors that are essential for dengue replication is crucial to (a) elucidate potential targets of antiviral therapy and (b) obtain key information for rational design of antiviral drugs. Commercial and academic research involved in finding inhibitors for HCV has yielded a lot of information in terms of the flaviviral life cycle and the crucial host factors responsible for that. However, while hepaciviruses share several common features with dengue, there are as many differences in the infection profiles and hence the life cycle of these viruses. A targeted dengue drug would therefore benefit from a comprehensive understanding of the mechanisms of dengue viral life cycle events. The work described here has been conducted at the Novartis Institute for Tropical Diseases (NITD) which aims to find small molecule inhibitors for dengue that could be used for therapy or prophylaxis. In keeping with the goals of the institute, the aim of this thesis is to identify viral and host factors that are important for dengue replication and survival.

Specifically, the goals of this thesis are to:

1) To Characterize the Function of the Small Non Structural Protein NS4B

We set out to understand the function of NS4B in dengue viral life cycle as it was hitherto uncharacterized. Mutation analysis has shown that a single amino acid mutation in NS4B enhanced the replication of DEN4 infectious clone in mammalian cells while decreasing it in human cells suggesting that NS4B might play a role in host tropism of dengue (Hanley et al., 2003). Interestingly, flaviviral NS4B has been shown to antagonise the interferon signaling in response to infection (Munoz-Jordan *et al.*, 2005, Munoz-Jordan *et al.*, 2003). HCV NS4B shares very little amino acid similarity with flaviviral NS4B but the topology of NS4B is surprisingly very well conserved across *Flaviviridae* (Lundin M, 2003). These studies suggest a crucial role for NS4B in viral replication.

2) To Explore a Putative Role for Type I Interferon (IFN) in Dengue Pathogenesis

While NS4B has been shown to antagonise the IFN response to dengue infection suggesting that interferon treatment would be ineffective against dengue (Diamond *et al.*, 2000, Ho *et al.*, 2005, Jones *et al.*, 2005, Munoz-Jordan *et al.*, 2003), at least one study describes the use of recombinant IFN in treating dengue (Limonta, 1984). Severe dengue has been correlated with secondary infection with a different serotype leading to antibody dependent enhancement. A serendipitous discovery of differential regulation of the type I IFN response by different strains of dengue led us to explore the role of viral genomic variations in dengue pathogenesis.

3) To Characterize the Unfolded Protein Response (UPR) to Dengue Infection

Dengue uses the host ER as the primary site of envelope glycoprotein biogenesis, genomic replication, and particle assembly. In the course of productive infection, a large amount of viral proteins accumulate and activate the ER stress response which in turn modulates signaling pathways leading to cell survival or death. Viruses encode functions that inhibit one or more steps in these signaling pathways. For example, replication of hepatitis C virus has been shown to stimulate the ATF6 pathway but attenuate the IRE1–XBP1 pathway. The balance between viral stimulation and inhibition determines in turn viral pathogenesis or replication. The aim of this study is to understand how dengue virus alters the components of the ER stress response and potentially identify modulators of dengue replication in the UPR pathway.

CHAPTER 2

Role of NS4B in Dengue Replication

2. ROLE OF NS4B IN DENGUE REPLICATION

2.1 Introduction

The polymerase, helicase and protease enzymatic activities encoded by the dengue genome ensure viral replication and polyprotein processing. NS3 (618 aa) is a multi-functional protein with protease, helicase, NTPase, and 5'-terminal RNA triphosphatase activities (Arias *et al*., 1993, Benarroch *et al*., 2004, Clum *et al*., 1997, Falgout *et al*., 1991, Li *et al*., 1999, Yusof *et al*., 2000, Zhang *et al*., 1992) while NS5 (900 aa) has RNA dependent RNA polymerase and methyl transferase activities (Ackermann & Padmanabhan, 2001, Chu & Westaway, 1987, Clum *et al*., 1997, Kapoor *et al*., 1995, Tan *et al*., 1996). These two proteins form a functional complex that is vital for dengue replication (Brooks *et al*., 2002, Johansson *et al*., 2001, Yon *et al*., 2005). The role of other flaviviral non-structural proteins is not clear except for NS2B which is a cofactor for the protease activity of NS3 (Clum *et al*., 1997, Falgout *et al*., 1993).

NS4B of *Flaviviridae* is a small (248 aa) hydrophobic protein stably associated with intracellular membranes. Hepatitis C virus NS4B has been characterized to some extent in terms of its biochemical and functional properties (Einav *et al*., 2004, Elazar *et al*., 2004, Gretton *et al*., 2005, Hugle T, 2001, Lundin M, 2003) but the role of flaviviral NS4B in the viral life cycle has not been characterized. NS4B proteins of dengue serotypes share a 78-85% amino acid sequence identity whereas those of Yellow Fever, West Nile and dengue share ≈35% identity. HCV NS4B bears a negligible resemblance. Despite this divergence, the predicted topology of NS4B, containing several endoplasmic reticular and cytoplasmic domains separated by transmembrane regions, is strikingly similar among the *Flaviviridae* suggesting a conserved function of NS4B in the viral life cycle (Lundin M, 2003).

2.1.1 Processing and Topology of NS4B

NS4B is first detected at 6-9 hour post infection in cell lines as a 30 kDa precursor form which is processed in a cell line dependent manner into a 28 kDa form. This

processing does not involve N- or C-terminal truncations and the increase in mobility is thought to be a function of internal modification. In addition, a 39 kDa form has also been observed and this is thought to be the NS4A-4B precursor that escapes normal processing (Preugschat & Strauss, 1991).

The C-terminal region of the NS4A protein preceding NS4B contains a signal sequence that serves to translocate NS4B into the lumen of the ER. This signal peptide is called the 2K fragment owing to its size (2 kDa). In case of flaviviruses (see *figure 10*) after translocation, the 2K fragment is cleaved off the N-terminus of NS4B by host signalase in the ER lumen. The 2K/4B cleavage requires a prior viral protease-mediated cleavage at the so called 4A/2K site (located 23 residues N-terminal of the signalase site) which is conserved among flaviviruses (Cahour *et al*., 1992, Lin *et al*., 1993a, Miller *et al*., 2006). Although precursor forms of NS4A-4B have been reported (Chambers *et al*., 1990a, Lindenbach & Rice, 2003, Preugschat & Strauss, 1991), the viral protease mediated cleavage is important for function of NS4B (Munoz-Jordan *et al*., 2005).

Recently, deletion-mapping studies and proteinase K protection experiments have established a model for membrane topology of dengue NS4B (Miller *et al*., 2006). Computer based predictions of three putative transmembrane domains (pTMDs) in the C-terminal domain located between amino acids 93-146, 146-190, and 190-248 of NS4B were validated experimentally whereas two potential TMDs the N-terminal half of the protein (aa 1-56 and aa 56-93) were not sufficient to mediate membrane targeting. As seen in *figure 10 A*, the 2K fragment and pTMD 4 span the membrane from the cytoplasmic to the luminal site, and pTMDs 3 and 5 from the luminal to the cytoplasmic site. The pTMDs 1 and 2 most probably do not span the membrane. The N- terminus of DENV NS4B is localized in the ER lumen where it is processed by the signalase of the host cell and its C-terminus resides in the cytoplasm where cleavage by the viral protease occurs.

In case of HCV, both the N- and C-terminal parts of NS4B are predicted to be cytoplasmic and both are cleaved by the viral protease (NS3). Following cleavage at the N-terminal part, NS4B translocates into the lumen of the ER. The predicted ER topology of HCV NS4B correlates very well with biochemical data and is represented

in *figure 10 B*. Interestingly, both the N- and C-terminal topology of HCV NS4B as well as its first luminal loop (around 112 aa) have been experimentally shown to be similar to that of dengue virus emphasising their conserved function (Lundin *et al.*, 2006, Lundin M, 2003, Miller *et al.*, 2006).

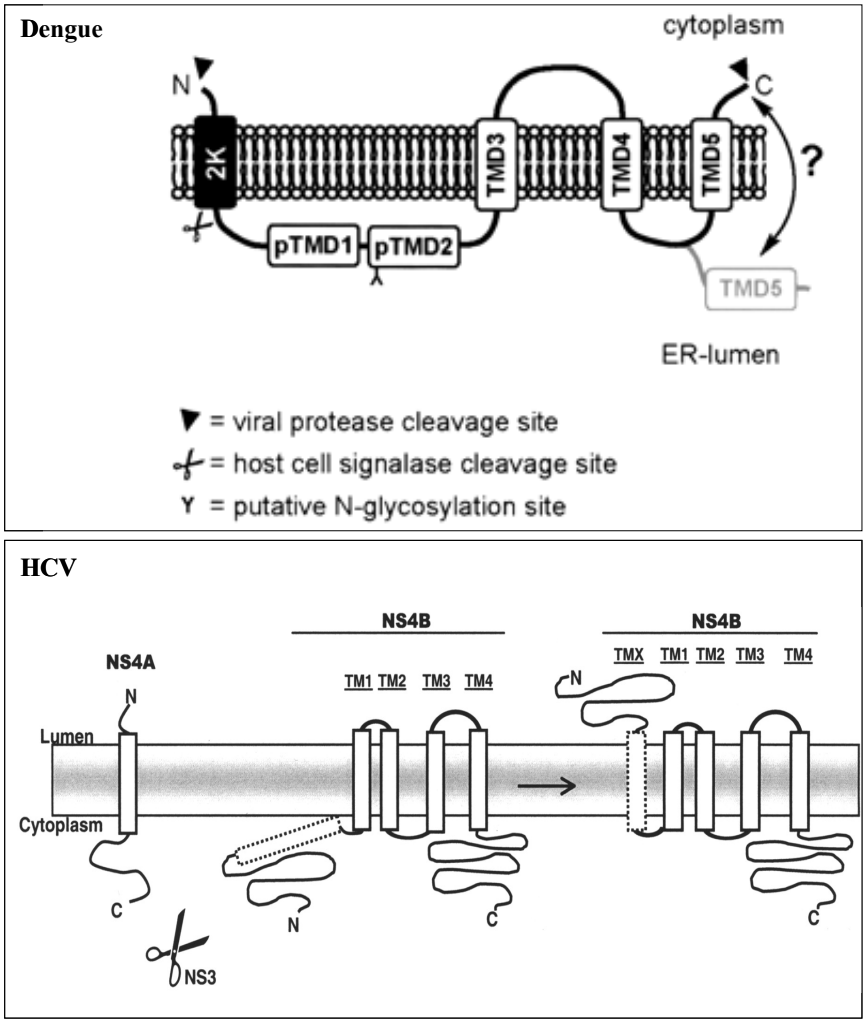


Figure 10: Membrane topology of NS4B of dengue and HCV. A) Dengue 2K serves as a signal sequence for the translocation of the NH_2 terminus of NS4B into the lumen of the ER. Predicted transmembrane domains (TMD) 1 and 2 do not span the membrane but rather reside in the ER lumen. TMDs 3, 4, and 5 span the membrane, and serve as internal signal sequences for membrane association. Glycosylation may occur at an internal *N*-glycosylation site in the predicted TMD 2. B) Topology model of HCV NS4B. The viral protease (NS3 with its cofactor NS4A) residing in the cytoplasm cuts the precursor protein between NS4A and NS4B. After processing, a rearrangement of the NS4B occurs, giving it a fifth transmembrane region, resulting in a luminal orientation of the N-terminal tail of the protein. Although processed differently, the N- and C-terminal domains of both dengue and HCV NS4B achieve similar membrane topologies (Adapted from (Lundin M, 2003, Miller *et al.*, 2006)).

2.1.2 Localization of NS4B

NS4B of Kunjin virus was shown to migrate to the nucleus shortly after infection (*figure 11*). Furthermore, a nuclear export sequence motif in Kunjin NS4B (LTPLLKHLI) bears similarity to that of HIV Rev protein (LPPLERLTL) which has been shown to be actively transported into the nucleus (Westaway et al., 1997a). The significance of this nuclear translocation is not understood and NS4B of no other member of *Flaviviridae* has been shown to translocate to the nucleus. Recent localization studies have shown that dengue NS4B accumulates in the perinuclear region with a staining pattern resembling the ER and in later stages of infection, forms large cytoplasmic foci (*figure 11*). Furthermore, NS4B co-localized with ER markers but not with golgi, *trans*-golgi or intermediate compartment markers (Miller *et al.*, 2006, Munoz-Jordan *et al.*, 2003) suggesting that dengue and HCV NS4B share similar sub-cellular localization (Hugle T, 2001, Kim *et al.*, 1999).

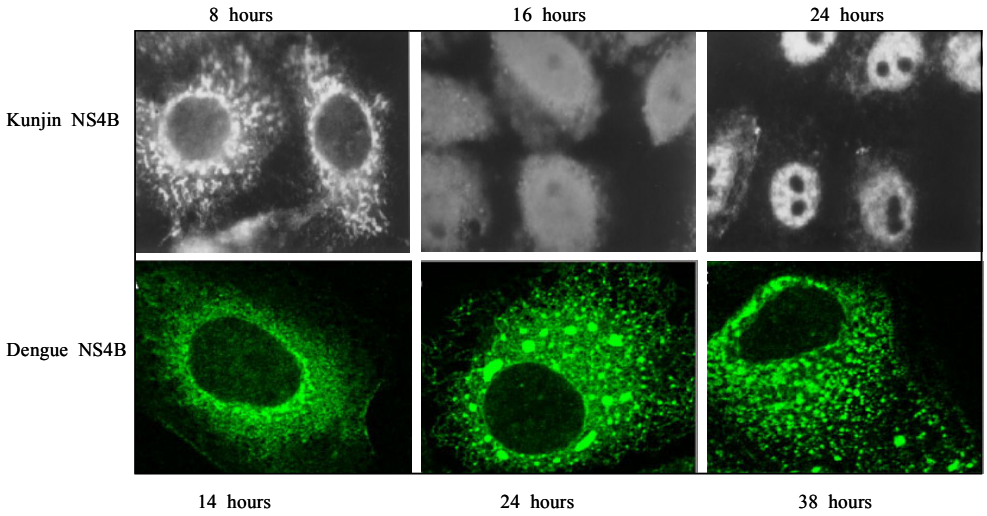


Figure 11: Sub-cellular localization of NS4B. Both dengue and Kunjin NS4B exhibit peri-nuclear localization in the early stages of infection but whereas Kunjin NS4B migrates to the nucleus in later stages of infection, dengue NS4B localizes to cytoplasmic foci. Adapted from (Miller et al., 2006, Westaway et al., 1997a).

2.1.3 Properties of NS4B of Flaviviridae

As with other non structural proteins, the best studies on NS4B are from the field of HCV. An N- terminal amphipathic helix in HCV NS4B has been shown to be important for membrane association, correct localization of replication complex proteins, and RNA replication (Elazar *et al*., 2004). NS4B induces a tight structural, designated membranous web, consisting of vesicles in a membranous matrix, and associates with the viral replication complex in HCV-infected cells (Egger *et al*., 2002, Gretton *et al*., 2005, Konan KV, 2003, Piccininni *et al*., 2002). HCV NS4B is also involved in phosphorylation of NS5A, induction of endoplasmic reticular stress (Zheng *et al*., 2005) and inhibition of cellular protein translation (Egger *et al*., 2002, Kato *et al*., 2002, Koch, 1999). While HCV NS4B has been shown to transform NIH3T3 cells in cooperation with the Ha-ras oncogene (Park *et al*., 2000), it is neither cytopathic nor oncogenic in mice (Wang *et al*., 2006).

Deletion of NS4B as well as insertions in its sequence inhibit replication of HCV, BVDV and Kunjin viruses (Grassmann *et al*., 2001, Khromykh *et al*., 2000, Li & McNally, 2001, Lindstrom *et al*., 2006). BVDV NS4B interacts with NS3 and NS5A (Qu L, 2001) and HCV NS4B interacts with NS3, NS4A and NS5 (Lin *et al*., 1997, Piccininni *et al*., 2002). While all these studies indicate that NS4B is a component of the *Flaviviridae* replication complex, co-immunoprecipitations of cell lysates using antibodies to double stranded RNA failed to reveal the presence of NS4B in the Kunjin virus replication complex (Chu & Westaway, 1992, Westaway *et al*., 2003).

NS4B of the dengue virus, the West Nile virus, and also the yellow fever virus was recently identified as an inhibitor of the IFN-α/β response. Expression of dengue NS4B blocked the IFN-α/β-induced signal transduction cascade by interfering with STAT1 (signal transducer and activator of transcription) phosphorylation. Deletion analyses suggest that the first 125 amino acids of Dengue 2K-NS4B are sufficient for the inhibition of IFN-α/β signaling, and that proper viral polyprotein processing is required for anti-IFN function (Munoz-Jordan *et al*., 2005, Munoz-Jordan *et al*., 2003). But recent topology studies have shown that this region of NS4B that is important for antagonizing the IFN response is in the ER lumen and a direct interaction between

NS4B and cellular cytoplasmic components involved in IFN signaling is therefore not likely. The direct role of NS4B in viral life cycle remains elusive. *Table 4* summarizes the features of *Flaviviridae* NS4B protein and its putative roles in viral life cycle.

Virus	Data	References
Component of replication complex / Interactions with viral proteins		
HCV	Single aa substitution inhibits replication	(Lohmann *et al.*, 1997)
BVDV	Deletions inhibit replication	(Li & McNally, 2001)
Kunjin	Deletions inhibit replication	(Khromykh *et al.*, 2000)
HCV	Interacts with NS4A and NS3	(Lin *et al.*, 1997)
HCV	Modulates RdRP activity of NS5	(Piccininni *et al.*, 2002)
BVDV	Interacts with NS3 and NS5A	(Qu *et al.*, 2001)
HCV	Deletions inhibit NS5A hyper phosphorylation	(Koch, 1999)
Effects on host cell/ Interactions with host cell proteins		
HCV	Suppresses translation	(Kato *et al.*, 2002)
HCV	Activates NF-kB associated signals	(Kato *et al.*, 2000)
HCV	Transforms NIH 3T3 cells	(Park *et al.*, 2000)
Dengue, Yellow fever, West Nile Virus	Antagonises type I IFN response	(Munoz-Jordan *et al.*, 2005, Munoz-Jordan *et al.*, 2003)
HCV	Interacts with PEST domain proteins (p53, RNase L, Waf1 etc)	(Florese *et al.*, 2002)
HCV	Interacts with CREB-RP	(Tong *et al.*, 2002)
Cytopathogenecity/Infectivity		
BVDV	Single aa substitutions cause attenuation	(Qu *et al.*, 2001)
Dengue	Single aa substitution results in decreased replication in mosquito cells and enhanced replication in mammalian cells	(Hanley *et al.*, 2003)

Table 4: Features of the NS4B protein of the *Flaviviridae* family.

2.2 Aim of the Study

The specific aim of this study was to characterize the function of NS4B in dengue viral life cycle. The topology as well as sub-cellular localization of dengue NS4B has been established during the course of this study by another group (Miller *et al*., 2006) and hence this work focused on identifying the function of NS4B. Traditionally deletion, mutation or interaction studies have been used as a means to understand the function of a given protein. Since most non-structural proteins cannot be *trans*-complemented as proven by studies in Kunjin virus, deletion studies could not be used for this purpose. The yeast two hybrid (Y2H) interaction trap method allows for detection of *in vivo* protein-protein interactions in yeast by taking advantage of the modular nature of transcription factors. In an Y2H assay, a "bait" gene is expressed as a fusion to the GAL4 DNA-binding domain (DNA-BD), while a "test" gene or cDNA is expressed as a fusion to the GAL4 activation domain (AD). When "bait" and "test" fusion proteins interact in a yeast reporter strain, the DNA-BD and AD are brought into proximity and activate transcription of the reporter genes (Fields & Song, 1989). This system has been used to identify as well as characterize interactions of several viral proteins including those of *Flaviviridae* (Brooks *et al*., 2002, Dimitrova *et al*., 2003, Hamamoto *et al*., 2005, Johansson *et al*., 2001, Lim *et al*., 2006, Nakai *et al*., 2006, Tellinghuisen & Rice, 2002). The aim of this study is to identify interacting partner(s) of NS4B among the non structural proteins of dengue. Such interactions can be validated using biochemical techniques such as co-immunoprecipitation and mutational analysis. Since non structural proteins are implicated in viral replication, an interacting partner of NS4B among these proteins might provide a clue to its role in viral replication.

2.3 Materials and Methods

Cloning: Non structural protein sequences were amplified from TSV01 cDNA using primers listed and cloned into respective plasmids as denoted in *table 5*. The P104L mutation in NS4B was generated by site directed mutagenesis of C to T at nucleotide position 7136 of TSV01 that corresponds nucleotide position 7129 (P101L mutation) reported in rDEN4-2A-5 (Hanley *et al*., 2003). Yeast expression constructs for NS3 and NS5, NS3-FL, and NS3 pro constructs were described before (Brooks *et al*., 2002, Johansson *et al*., 2001, Li *et al*., 2005a, Xu *et al*., 2005). Other constructs used in this study are described in *table 5*.

Yeast Two-Hybrid Screening: The two-hybrid screens were performed as described in the Matchmaker GAL4 two-hybrid system 3 user manual (Clontech). Briefly, an interaction between two proteins is indicated by the activation of the reporter genes HIS3 and ADE2 which allow growth on media lacking histidine (His) and adenine (Ade) respectively or MEL1 which secretes α-glucosidase that can be assayed on X-α-gal indicator plates. The pGBKT7- and pGADT7-derived constructs (see *Table 5*) encoding dengue NS proteins were co-transformed into AH109 cells and plated onto culture plates lacking tryptophan (Trp) and leucine (Leu) to select for co-transformants. After 72-96 hours, the co-transformants were streaked onto plates lacking Trp, Leu, His and Ade and containing X-α-gal to allow selection of interacting partners.

***In vitro* Translation and Immunoprecipitation:** Radio labelled NS4B was generated from pGBK4B plasmid using TNT T7-coupled reticulocyte lysate system (Promega) and [35S] Met (Amersham Biosciences). 10 µl of *in vitro* translated NS4B was incubated at 4^0C for one hour with or without 5 µg of His tagged NS3 303-618 protein. Ni-NTA agarose beads were added to capture NS4B-NS3 complex and incubated again for one hour. The complex was resolved on a 12% SDS gel and visualized by autoradiography.

Pull Downs: Bacterial cell lysates expressing GST, GST-NS4B and GST-NS4BM were incubated with Glutathione sepharose beads (Amersham) for 2 hours at 4^0C, washed with PBS and these beads were used for pull down experiments. 10 µl each of these

beads were incubated with or without 7 μg of CF NS3 for 3 hours at room temperature, washed thrice with PBS and the proteins eluted by boiling the beads in 40 μl of SDS loading buffer. 10 μl each of these reactions were resolved on a 12% SDS gel and stained by coomassie.

Preparation of Double Stranded (ds) RNA / Single Stranded (ss) RNA Substrate: Plasmid pGEM4Z was linearized by digestion with XbaI and was *in vitro* transcribed in the presence of (α-32P) GTP using Riboprobe kit (Promega). After incubation for 1 h at 37^0C, the reaction mixtures were treated with DNase I and extracted with phenol-CHCl3. Unincorporated NTPs were separated by a Chromaspin-10 spin column, and RNA was precipitated with ethanol. Radio labelled *in vitro* transcription product of pGEM4Z was used as ssRNA substrate for electrophoretic mobility shift assays. Double stranded (ds)RNA substrate preparation has been described elsewhere (Xu *et al*., 2005).

Double Stranded RNA Unwinding Assay: The dsRNA unwinding assay was performed as described in (Xu *et al*., 2005). Briefly, the reaction mixture for this assay contained 25 mM HEPES (pH 7.5), 1 mM ATP, 3 mM MnCl2, 2 mM dithiothreitol, 100 μg of bovine serum albumin , 5 U of RNasin and the 0.25 pmol RNA substrate and 3 μM of NS3 in a final volume of 20 μl. The mixture was incubated for 30 min at 37°C and the reaction was terminated by adding 2.5 μl of termination mix (100 mM Tris-HCl [pH 7.5], 50 mM EDTA, 0.1% Triton X-100, 0.5% SDS, 50% glycerol, 0.1% bromophenol blue). The helicase assay mixtures were resolved on a 10% native gel and analyzed with a Typhoon Phosphorimager (Amersham Biosciences). For each value the background from the negative control was subtracted and the fold variation of ssRNA release from each lane was calculated against ssRNA release by NS3-FL and plotted in a graph. P values were calculated by performing a two tailed T test on raw data.

Plasmid	Region	Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Comments
pGBKT-7	NS4B (aa 1-248)	NS4B	ATATAGAATTCAA CGAGATGGGTTTC CTGGA	ATATAGTCGACTT ACCTTCTTGTGTT GGTCGTGT	Cloning sites Eco RI and SalI and stop codon
pGBKT-7	NS4B (aa 1-135)	NS4BN	ATATAGAATTCAA CGAGATGGGTTTC CTGGA		Cloning sites Eco RI and SalI And stop codon
pGBKT-7	NS4B (aa 136-248)	NS4BC		ATATAGTCGACTT ACCTTCTTGTGTT GGTCGTGT	Cloning sites Eco RI and SalI And stop codon
pGBKT-7	NS4B (aa1-248)	NS4B M (P104 L)	GCTATTCACAAGT CAACCTCATAACT CTCACGGCAGCC	GGCTGCCGTGAGA GTTATGAGGTTGA C TTGTGAATAGC	Using Quick change site directed mutagenesis kit (Stratagene)
pET15b	40aa of the hydrophobic core of NS2B linked via linker GGGGSGGGG to full length NS3	CF NS3			Both the insert pET15b- NS3NS5 (James Cook university) and Vector pET15b-CF40glyNS3pro185 (in-house) were double-digested with *HindIII* and *SalI* followed by ligation of vector (6.2kb) and insert (1.73kb)
pGEX4T-1	NS4B (aa 1-248)	GST-NS4B	ATATAGAATTCAA CGAGATGGGTTTC CTGGA	ATATATCTCGAGT TACCTTCTTGTGTT GGTCGTGT	Cloning sites Eco RI and XhoI and stop codon
pGADT7	NS1	NS1	ATATAGAATTCGA TAGTGGCTGCGTT GTGAG	ATATACTCGAGTA AGGCTGTGACCAA AGAGTTGA	Cloning sites Eco RI and XhoI and stop codon
pGADT7	NS2A	NS2A	ATATAGAATTCGG ACATGGACAGATT GACAA	ATATACTCGAGTA ACCTTTTCTTGCTA GTTCTTG	Cloning sites Eco RI and XhoI and stop codon
pGADT7	NS2B	NS2B	ATATAGAATTCAG CTGGCCACTAAAT GAGGC	ATATACTCGAGTA ATCGTTGTTTTTTC ACTTCCC	Cloning sites Eco RI and XhoI and stop codon
pGADT7	NS4A	NS4A	ATATAGAATTCTC CCTGACTCTGAAT CTAATCAC	ATATACTCGAGTT ATCTTTTCTGAGC CTCTCTAG	Cloning sites Eco RI and XhoI and stop codon
pXJ-40	NS4B (aa 1-248)	pXJ-NS4B	ATATAGGATCCAC ATGAACGAGATGG GTTTCCTGGA	ATATACTCGAGTT AAGCGTAATCTGG TACGTCGTACCTT CTTGTGTTGGTCGT GT	Cloning sites Bam HI and XhoI. Introduction of C-terminal HA tag.
pACT-2	NS3 (aa 1-303)	NS3 1-303			Described in (Brooks et al., 2002, Johansson et al., 2001)1)
pACT-2	NS3 (aa 303-618)	NS3 303-618			Described in (Brooks et al., 2002, Johansson et al., 2001)1)
pACT-2	NS5 (aa 1-405)	NS5 1-405			Described in (Brooks et al., 2002, Johansson et al., 2001)1)
pACT-2	NS5 (aa 405-900)	NS5 405-900			Described in (Brooks et al., 2002, Johansson et al., 2001)1)
pET15b	NS3 (aa 1-618)	NS3-FL			Described in (Xu *et al*., 2005)
pXJ-40	CF40-gly-NS3 FL	pXJ-NS3FL			Kind gift from Dr. Lim Siew Pheng

Table 5: Plasmid constructs used in this report

Electrophoretic Mobility Shift Assay (EMSA): The reaction mixture for this assay (20 µl) contained 20 mM Hepes pH 7.5, 50 mM KCL, 1mM EDTA, 5% glycerol, 1mM DTT, 200 µg/ml BSA along with 32P labelled ssRNA substrate. 1-3 µM of NS3-FL, or CF NS3, and 1-6 µM of GST or GST-NS4B proteins. The mixtures were incubated for 5 minutes at 37°C, 5 µl of loading buffer (20% glycerol) was added and resolved on an 8% native polyacrylamide gel at 4°C. Bands were identified by Typhoon Phosphor imager (Amersham Biosciences).

Cell Culture, Transfection and Immunofluorescence: BHK-21, C6/36 (maintained in RPMI containing 10% FBS) and A549 (maintained in DMEM containing 10% FBS) cell lines were purchased from the ATTC. Medium components were purchased from GIBCO-Invitrogen Corporation. Monolayer of A549 cells were cultured on cover slips in 24 well plates, co-transfected with 1 µg each of pXJ-NS4B and pXJ-NS3FL plasmids using Lipofectamine 2000 (Invitrogen). The cells were fixed in cold methanol 24 hours post transfection. For virus infections, A549 cells were seeded 24 hours before infection with 5 moi of TSV01 and fixed in cold methanol three days post infection. Anti-NS3 and anti-NS4B anti-sera generated in house, were used as primary antibodies. Texas-red conjugated anti-rabbit and FITC conjugated anti-mouse secondary antibodies were used (Jackson Labs). Co-transfection images were captured by Leica fluorescent microscope whereas the images of infected cells which were morphologically slightly different from uninfected cells was captured using a confocal microscope (Olympus).

Antibody Production: NS4B was expressed as a GST fusion protein (56 kDa) and eluted from polyacrylamide gels. Anti-NS4B antiserum was prepared in mice using this eluted NS4B as an antigen. This was purified using E.coli lysate column to remove any non-specific antibodies and was tested on virus infected C6/36, BHK-21 and A549 cell lines as well as transient transfections of NS4B in 293T, Hela and A549 cells by western blotting, immunoprecipitation and immunofluorescence. Polyclonal rabbit anti-DENV-2 NS3 was generated by injecting purified NS3 into rabbits. The serum was collected after four weeks and tested as described earlier.

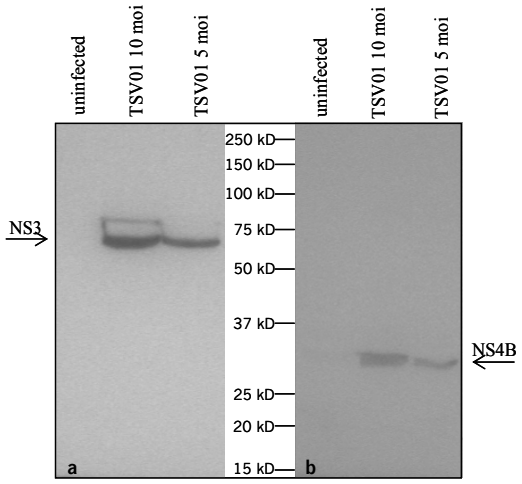


Figure 12: NS3 and NS4B antibodies do not cross react. C6/36 cells were infected with TSV01 strain of DENV-2 and were collected in m-RIPA buffer 2 days post infection. Mock infected cell lysates were used as controls.

Virus Infection and Immunoprecipitation Assays: C6/36 and A549 cell lines were seeded in T75 cell culture flaks 24 hours prior to infection and when about 80% confluent, infected with 10 moi TSV01. Infected and mock infected cells were lysed in 2 mls of cold m-RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1mM EDTA, 1X protease inhibitor cocktail from Sigma) 72 hours post infection. Lysates were pre-cleared with Protein A agarose beads and normalized for protein concentration. About 10 µl of NS4B antibody (roughly 1 µg) is added to 500 µg of extract and incubated overnight at 4^0C with gentle agitation. The complexes are captured by incubation for one hour with 50 µl of Protein A agarose beads. Beads were washed thrice each with m-RIPA buffer and PBS, boiled in 20 µl of loading buffer and western blotting was done with anti-NS3 antibody.

Protein Purification: NS3-FL protein purification has been described elsewhere (Xu *et al*., 2005). Briefly, BL21-RIL expressing NS3-FL and CF NS3 were induced for 16 hours at 16ºC with 10µM of IPTG and lysed in 50mM HEPES pH7.5, 300mM NaCl, 5% glycerol in a cell disrupter. The supernatant was purified using a HiTrap Ni^{2+}-NTA affinity column (Amersham) and proteins were eluted from the column in the same buffer containing 500 mM imidazole, desalted with PD-10 columns (buffer exchanged to 10mM TrisHCl pH 7.5). De-salted fractions were then pooled and concentrated in

an Amicon filter (Millipore). NS3-FL protein was cleaved from Trx with enterokinase, purified using Talon spin columns, and concentrated. Note that NS3-FL described in *figure 5 C* was not cleaved. NS3 pro (the protease domain of NS3 with 40 aminoacids of the NS2B cofactor) and NS5 proteins in the ATPase assay and the EMSA respectively were kind gifts from Dr. Siew Pheng Lim and Dr. Yen Liang Chen.

NS4B is considered a membrane protein. We expressed NS4B and NS4BM as N-terminal fusions of GST in BL21 strain of E. coli cells. Induction with 20 μM IPTG for 20 hours at 16°C greatly enhanced their solubility. Cells were lysed in 20mM Tris-HCl pH 7.5; 0.3M NaCl; 0.25% NP40, 5% Glycerol by sonication for 20 minutes. Clarified supernatant was loaded onto a GST column (5 ml, Amersham Biosciences) pre-equilibrated with 50mM Tris; pH 8.0 and eluted with 10 mM reduced glutathione. Peak fractions were pooled and concentrated by ultra filtration at 3,000 g (Centricon 30 kDa) and passed through gel filtration column (Sephadex-75, Amersham Biosciences) using Tris buffer to obtain pure GST-NS4B and GST-NS4BM.

ATPase Activity Assay: The ATPase assay has been described elsewhere (Xu *et al.*, 2005). Briefly, proteins were pre-incubated in a 96 well plate for 5 min at 37°C in 90 μl reaction buffer after which 10 μl of ATP (Amersham) was added and further incubated for 10 min at 37°C. 100 μl Malachite Green Reagent was added and absorbance was measured at 630 nm to titrate the amount of inorganic phosphate released during the catalysis. The rates were calculated (PO_4 nmol min^{-1}) using Prism software.

2.4 Results

2.4.1 Identification of a Specific Interaction between NS4B and NS3 Using Yeast Two-Hybrid

Yeast two-hybrid interaction study is a powerful tool to detect molecular interactions (Fields & Song, 1989). To elucidate the role of NS4B in viral replication, we carried out yeast two-hybrid screening to find interacting partners of NS4B among the other non structural proteins of dengue. Full length NS4B (NS4B), N-terminal 1-135 aa (NS4B N) and C-terminal 136-248 aa of NS4B (NS4B C), were engineered into the yeast bait vector (*figure 13 A*). A single point mutation of a conserved residue in NS4B (P104L) of DENV-4 has been reported to enhance viral replication in mosquito cells while decreasing its replication in mammalian cells (Hanley *et al*., 2003). We therefore also included this NS4B mutant (NS4BM) in the screen. Full length NS1, NS2A, NS2B, NS4A, NS4B, different domains pertaining to NS3 1-303, NS3 303-618 and NS5 1-405, NS5 405-900 were cloned into the prey vector and tested for expression. Co-transformants were then grown either on low-stringency plates (lacking leucine, tryptophan and adenine) to identify weak interactions or on high-stringency plates (X-α-gal plates lacking leucine, tryptophan, adenine, histidine) to allow identification of strong interactions.

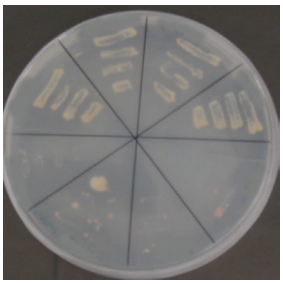
As indicated in *figure 13*, full-length NS4B interacted strongly with the C- terminal region of NS3 (NS3 303-618) which encompasses its helicase motif. The interaction is specific because NS4B interacted very weakly with N-terminal NS3 (NS3 1-303) which contains the protease and NTPase motifs, and with NS2A, while no interaction was detected with NS1, NS2B, NS4A or NS5. Neither NS4B N nor NS4B C interacted with NS3 303-618 suggesting that protein conformation is important for this interaction while NS4BM did not interact with NS3 (*figure 13 A*).

A strong homotypic interaction of NS4B via its N-terminal domain was also identified in this study and this interaction was validated using biochemical immunoprecipitation experiments indicating that NS4B may exist as an oligomer. However, the significance of this interaction is not investigated in this study.

A

	NS1	2A	2B	NS3 1-303	NS3 303-618	4A	4B	NS5 1-405	NS5 405-900
NS4B	-	+	-	+	+++	-	+++	-	-
NS4B N	+	+	+	-	-	-	+++	-	+
NS4B C	+	-	-	+	-	-	-	-	+
NS4B P104L	ND	ND	ND	-	-	ND	ND	ND	ND

B



NS4BM + NS3 $_{303\text{-}618}$

Figure 13: Identification of an interaction between NS4B and NS3 by yeast two-hybrid. A) Screening was done with NS4B, NS4BM, NS4B N and NS4B C as baits (represented in rows) and non structural proteins as preys (represented in columns). The grid shows strong (+++), weak (+) none (-) or not determined (ND) interactions in the yeast two-hybrid screen. NS4B showed a strong homo-association and the region of this interaction was narrowed down to aa 91-136 of its N-terminal domain (data not shown). NS4B interacted strongly with NS3 303-618 and very weakly with NS3 1-303. NS4B N, NS4B C or NS4BM did not interact with NS3 303-618 suggesting that this interaction maybe conformation dependent. B) NS4B but not NS4BM interacted with C-terminus of NS3. Dual transformants of NS4B and NS3 303-618 grew on X-α-gal plates lacking leucine, tryptophan, adenine and histidine proving their interaction wheras NS4BM and NS3 303-618 dual transformants did not grow on these plates.

2.4.2 Verification of Interaction by Pull Down and Immunoprecipitation Assays

In order to corroborate our yeast two-hybrid results, pull down experiments were performed. Equal amounts of S35 labelled *in vitro* translated myc-tagged full length NS4B were incubated with or without His-tagged NS3. Proteins pulled down by Ni-NTA beads were resolved by SDS-PAGE and detected by autoradiography. As shown in *figure 14 A*, NS4B could be specifically pulled down by NS3 303-618. In a reversal of the pull down, GST-NS4B but not GST-NS4BM could pull down CF NS3 (bacterially expressed full length NS3 with 40 aa of NS2B cofactor) supporting the yeast two-hybrid interaction data (*figure 14 B*).

To confirm the interaction between endogenously expressed NS4B and NS3, a co-immunoprecipitation assay was carried out using dengue infected mosquito and mammalian cell lysates. C6/36 and A549 cells were infected with DENV-2 (TSV01 strain) at a multiplicity of infection of 10 and harvested 48 and 60 hours post infection respectively. The presence of both NS3 and NS4B were detected in the infected cells by

immunoblotting with the respective antibodies which did not cross react as shown in *figure 12*. NS4B was immunoprecipitated with a mouse polyclonal anti-NS4B antibody raised in house. The co-immunoprecipitated material was separated by SDS-PAGE and immunoblotted with anti-NS3 antibody. NS3 could be co-immunoprecipitated by anti-NS4B antibody from infected C6/36 (*figure 15 A*) and A549 (*figure 15 B*) cell lysates but not from uninfected lysates. Together, the results from the *in vitro* pull down and the *in vivo* co-immunoprecipitation experiments provide evidence that a specific interaction exists between NS4B and NS3.

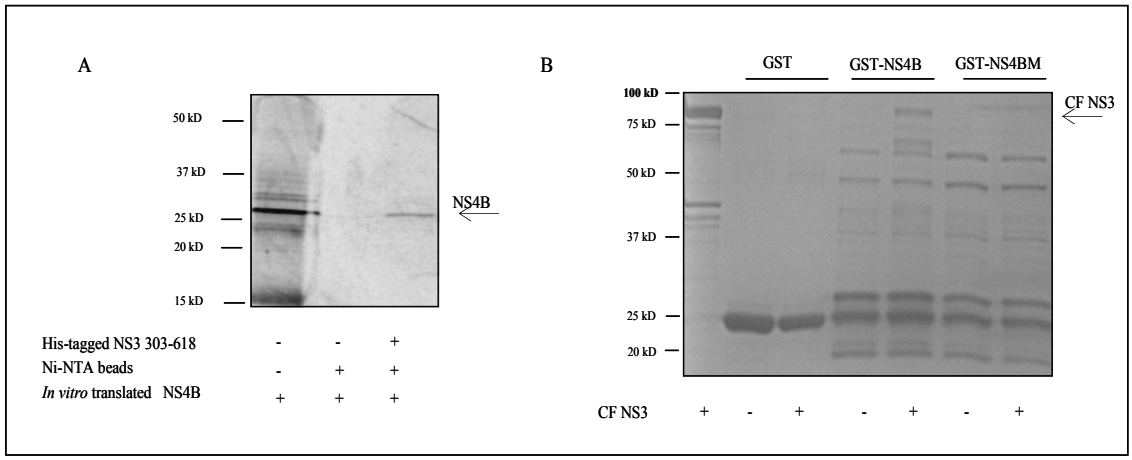


Figure 14: Pull down analyses of NS4B and NS3 interaction. (A) Pull down of NS4B by NS3. Ni-NTA agarose beads were incubated with S35 labelled *in vitro* translated NS4B with or without recombinant His-tagged NS3 303-618 protein. Lane 1 depicts *in vitro* translated NS4B protein as a positive control. Ni-NTA beads did not pull down NS4B in the absence of NS3 303-618 protein (lane 2) and NS3 303-618 specifically pulled down NS4B (lane 3). 1 µl of *in vitro* translated NS4B (from a total volume of 50 µl reaction) was loaded in lane 1 and 1/3rd of the material from the pull downs was loaded in lanes 2 and 3. (B) Pull down of NS3 by NS4B. 10 µl each of Glutathione sepharose beads which were pre-incubated with crude cell lysates of bacteria expressing GST or GST-NS4B or GST-NS4BM were incubated with or without 7 µg of recombinant CF NS3 protein, run on SDS gel and coomassie stained for detection of proteins. Lane 1: 7 µg of CF NS3; Lane 2 and 3: 10 µl of GST beads with and without CF NS3 respectively; Lane 4 and 5: 10 µl of GST-NS4B beads with and without CF NS3 respectively; Lane 6 and 7: 10 µg of GST-NS4BM beads with and without CF NS3 respectively. Note that GST-NS4B but not GST-NS4BM pulled down CF NS3.

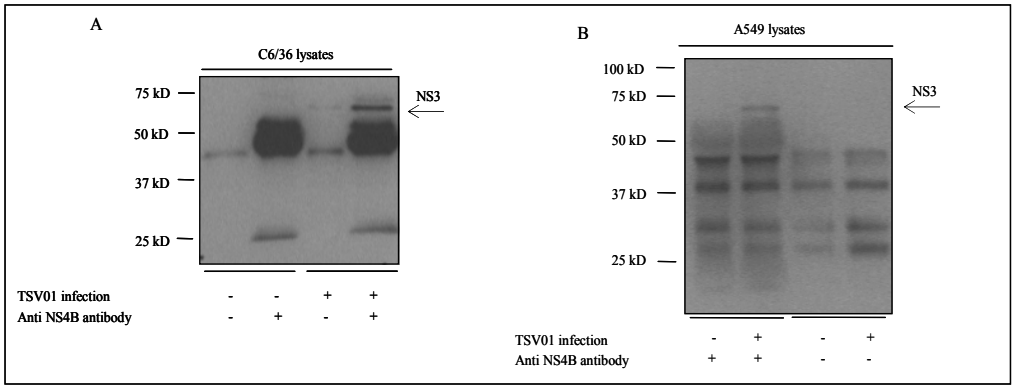


Figure 15: Co-immunoprecipitation studies of NS4B and NS3 interaction. A) Pull down with infected C6/36 cell lysates. Uninfected and infected C6/36 cell lysates were incubated with or without NS4B antibody and protein A beads were added to capture the complex. Western blotting with NS3 antibody showed an approximately 68 kDa band which corresponds to the full length NS3 captured by the anti-NS4B antibody specifically in infected cell lysates (lane 4). Lanes 1 and 2 correspond to uninfected cell lysates (negative control) without and with anti-NS4B antibody respectively while lanes 3 and 4 correspond to infected cell lysates without or with anti-NS4B antibody. B) Pull down with infected A549 cell lysates. Uninfected and infected A549 cell lysates were incubated with or without NS4B antibody and protein A beads were added to capture the complex. Western blotting with NS3 antibody showed that full length NS3 (68 kDa, see arrow) was immuno-precipitated by the NS4B antibody in infected cell lysates (lane 4). Lanes 1 and 3: uninfected cell lysates without and with anti-NS4B antibody respectively; lanes 2 and 4: infected cell lysates without and with anti-NS4B antibody respectively.

2.4.3 NS3 and NS4B Share Similar Sub-Cellular Localization Pattern

To date, the sub-cellular localization of NS4B has not been conclusively demonstrated. While in Kunjin virus, it has been shown that NS4B localizes to the nucleus in the early stages of infection (Westaway *et al.*, 1997a), HCV NS4B has been shown to be an integral ER membrane protein (Hugle *et al.*, 2001). Recently over-expressed dengue NS4B has been shown to localize to the ER compartment (Munoz-Jordan *et al.*, 2005).

We examined the localization of transiently expressed NS4B and NS3 in A549 cells by immunoflorescence. Co-transfected cells were double-labelled with rabbit anti-NS3 and mouse anti-NS4B antibodies and observed under fluorescent microscope. 48 hours after transfection, both NS3 and NS4B showed a reticular staining pattern which surrounded the nucleus and extended through the cytoplasm, typical of the ER

localization and they co-localized with each other when the two labelling were merged (*figure 16 C*). Further confirming the co-localization of the two molecules *in vivo*, A549 cells infected with DENV-2 showed similar co-localization pattern 48 hours post infection (*figure 16 F*). Interestingly, cells infected with dengue showed marked morphological changes in the ER compartment similar to HCV infection (Gretton *et al.*, 2005).

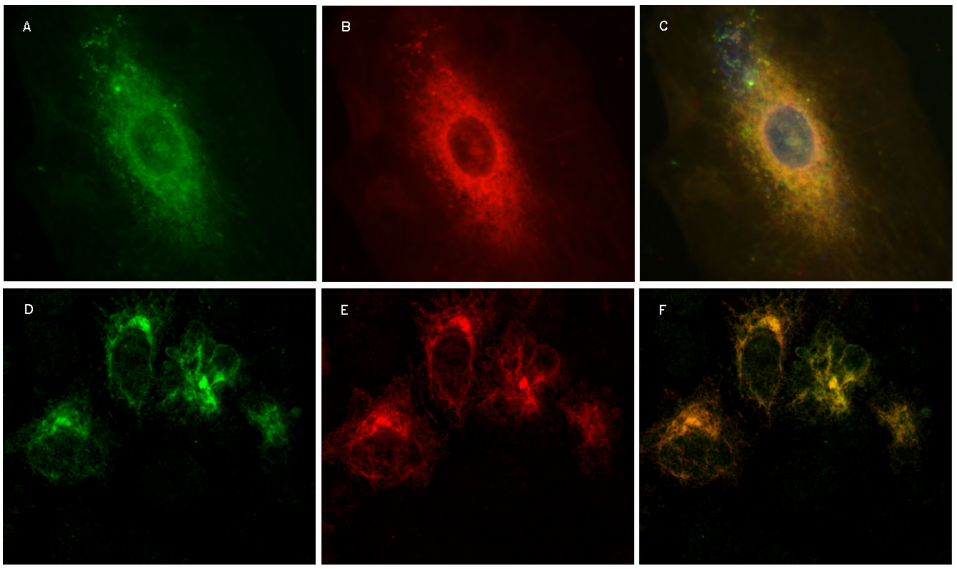


Figure 16: Sub-cellular co-localization of NS3 and NS4B proteins. NS3 and NS4B exhibited similar sub-cellular distribution patterns in A549 cells co-transfected with NS4B and NS3 plasmids (Panels A, B and C) and also in TSV01 virus infected A549 cells (panels D, E and F). Mouse anti-NS4B antibody was detected with FITC labelled anti mouse secondary antibody (green) and rabbit anti-NS3 antibody was detected with Texas-red labelled anti-rabbit secondary antibody (red). The third figures in each of the panels show a merge of the two figures demonstrating co-localization of NS3 and NS4B.

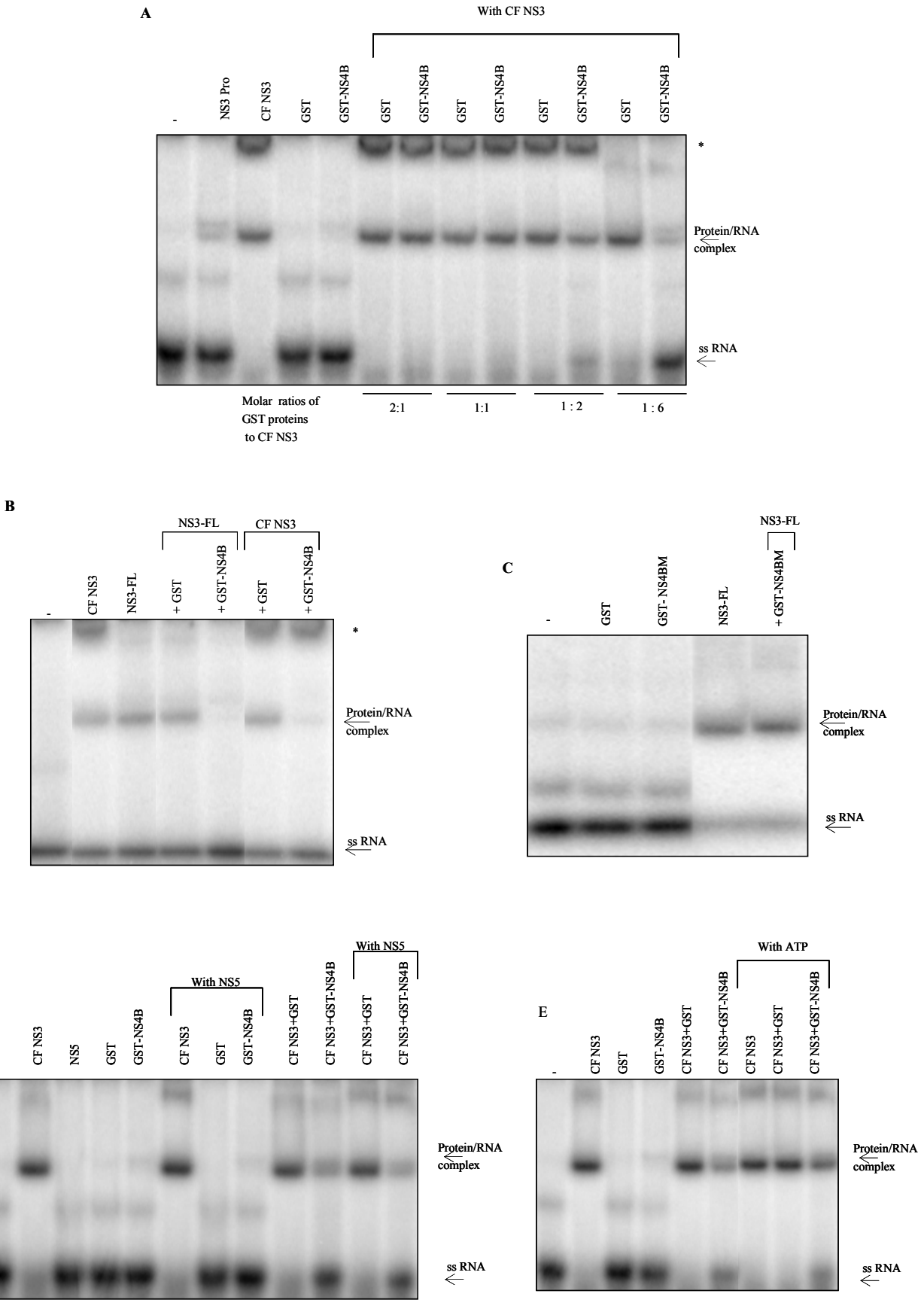
2.4.4 NS4B Dissociates NS3 From ssRNA

The C-terminal domain of flaviviral NS3 has been proposed to function in RNA and protein recognition (Xu *et al.*, 2005, Yon *et al.*, 2005). In order to test if its interaction with NS4B would affect the RNA binding property of NS3, we carried out EMSA. A radio labelled ssRNA probe was generated by incorporation of 32P during *in vitro* transcription and EMSA was performed as described in materials and methods. Briefly, equal amounts of the ssRNA were incubated with proteins at 37°C for 5 minutes to

allow binding and the protein/RNA complexes were resolved on an 8% native gel under non-denaturing conditions.

Increasing amounts of GST-NS4B decreased the binding of NS3-FL (bacterially expressed full length NS3) to ssRNA as shown in *figure 17 A* while GST or GST-NS4B did not bind to ssRNA. The NS2B cofactor is essential for the protease activity of NS3 and crystal structures have indicated that the presence of this cofactor is important for protein conformation. We therefore used NS3 full-length protein which was expressed with the essential cofactor region of NS2B in RNA binding experiments. As seen in *figure 17 B*, NS4B abolished ssRNA binding of both NS3 (NS3-FL) and NS3 with cofactor (CF NS3) verifying that dissociation of NS3 from ssRNA is independent of the NS2B cofactor. In a control experiment, GST-NS4BM did not show any effect on ssRNA binding of NS3 (*figure 17 C*).

Addition of ATP to the reaction had no effect on the RNA binding activity of NS3 independent of NS4B suggesting that the modulation of RNA binding activity of NS3 by NS4B is ATP independent (*figure 17 E*). Like wise, NS5 had no effect on the RNA binding ability of NS3 in the presence of NS4B although it was reported that NS5 modulates the helicase activity of NS3 (Yon *et al.*, 2005) indicating that NS4B does not necessarily compete with NS5 for its interaction with NS3 (*figure 17 D*).


A
With CF NS3
-
NS3 Pro
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Molar ratios of GST proteins to CF NS3
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1 : 2
1 : 6
B
NS3-FL
CF NS3
-
CF NS3
NS3-FL
+ GST
+ GST-NS4B
+ GST
+ GST-NS4B
*
Protein/RNA complex
ss RNA
C
NS3-FL
-
GST
GST- NS4BM
NS3-FL
+ GST-NS4BM
Protein/RNA complex
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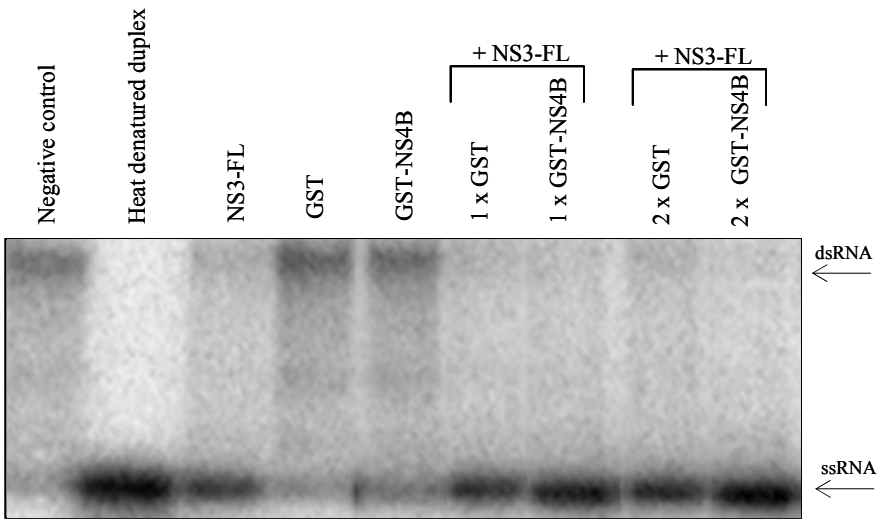
CF NS3
GST
GST-NS4B
CF NS3+GST
CF NS3+GST-NS4B
CF NS3+GST
CF NS3+GST-NS4B
Protein/RNA complex
ss RNA
E
With ATP
-
CF NS3
GST
GST-NS4B
CF NS3+GST
CF NS3+GST-NS4B
CF NS3
CF NS3+GST
CF NS3+GST-NS4B
Protein/RNA complex
ss RNA

Figure 17: NS4B abolishes ssRNA binding of NS3. A) NS4B dissociated NS3 from ssRNA in a dose-dependent manner. Equal amounts of the 32P labelled ssRNA substrate were incubated with proteins at 37^0C for 5 minutes to allow binding and the complexes were resolved on an 8% native gel under non-denaturing conditions. Gels were scanned using a phosphorimager (Amersham). Arrows represent free probe and shifted bands. Lane 1 and 2: negative control and NS3 pro; lane 3: CF NS3; lanes 4 and 5: GST and GST-NS4B; lanes 6-13: increasing molar ratios of GST and GST-NS4B with CF NS3. B) The dissociation of NS3 from ssRNA by NS4B is independent of the NS2B cofactor: Lane 1: Negative control; Lane 2 and 3: 3 μM each of CF NS3 and NS3-FL respectively; Lanes 4 and 6: 6 μM GST with CF NS3 and NS3-FL respectively; Lanes 5 and 7: 6 μM GST-NS4B with CF NS3 and NS3-FL respectively. C) Mutant NS4B does not alter RNA binding of NS3. Lane 1: Negative control; Lanes 2 and 3: GST and GST-NS4BM; Lane 4: NS3-FL; Lane 5; GST-NS4BM with NS3-FL respectively. D) NS5 has no effect on dissociation of NS3 from ss RNA in the presence of NS4B. The lanes 6, 7, 8, 11 and 12 represent EMSA with indicated proteins in the presence of equimolar amount of NS5. Note that there was no effect of addition of NS5 on the NS4B mediated dissociation of NS3 from ssRNA. E) Modulation of RNA binding activity of NS3 by NS4B is ATP independent. Lanes 7-9 represent EMSA in the presence of 1 uM of ATP. Note that GST-NS4B dissociated NS3 from ssRNA independent of the presence of ATP in the reaction.

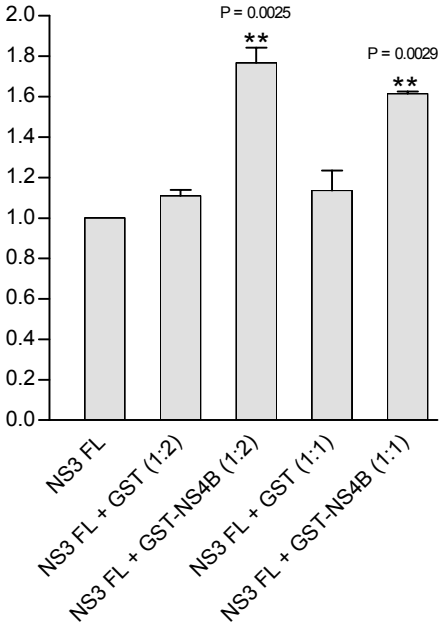
2.4.5 NS4B Modulates the dsRNA Unwinding Activity of NS3

Since NS4B interfered with the RNA binding of NS3, we asked if this affects the helicase activity of NS3 in a dsRNA unwinding/helicase assay (Xu *et al*., 2005). Briefly, a radio labelled dsRNA substrate was incubated with NS3-FL alone or with GST, GST-NS4B or GST-NS4BM at 37°C for 30 min; the mixture was then run on a gel to separate the ssRNA from the dsRNA. Increasing molar ratios of GST or GST-NS4B to NS3 was employed in the assay (2:1, 1:1 and 1:2 molar ratios of NS3 to GST or GST-NS4B) and the ssRNA release was measured by autoradiography. As seen in *figures 18 A* and *18 B*, there was a dose-dependent increase in the helicase activity of NS3 upon addition of GST-NS4B (up to 1.8 fold, $p = 0.005$) as opposed to the addition of GST. GST, GST-NS4B or GST-NS4BM did not exhibit any unwinding activity on their own. GST-NS4BM did not enhance the helicase activity of NS3 (*figure 18 C*). Taken together, these results suggest that NS4B enhanced the overall dsRNA unwinding activity of NS3 by dissociating it from ssRNA and thereby enabling it to bind to a new duplex.

A)


Negative control
Heat denatured duplex
NS3-FL
GST
GST-NS4B
+ NS3-FL
1 x GST
1 x GST-NS4B
+ NS3-FL
2 x GST
2 x GST-NS4B
dsRNA
ssRNA

B)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**
P = 0.0029
**
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1.8
1.6
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1.2
1.0
0.8
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0.2
0.0
NS3 FL
NS3 FL + GST (1:2)
NS3 FL + GST-NS4B (1:2)
NS3 FL + GST (1:1)
NS3 FL + GST-NS4B (1:1)

C)

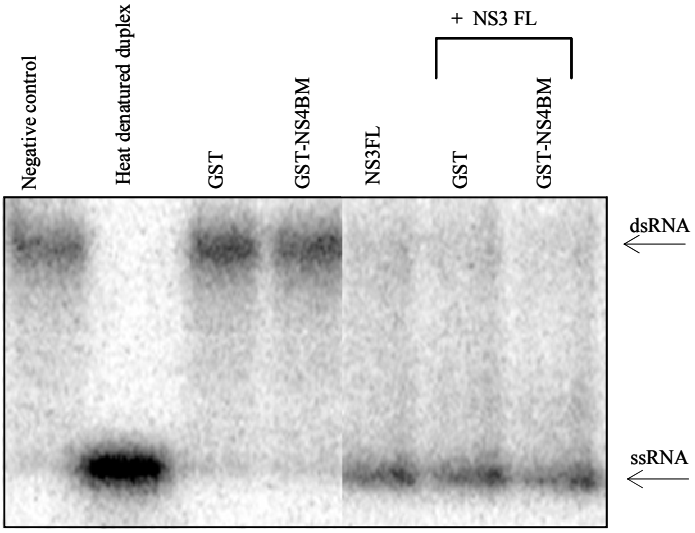
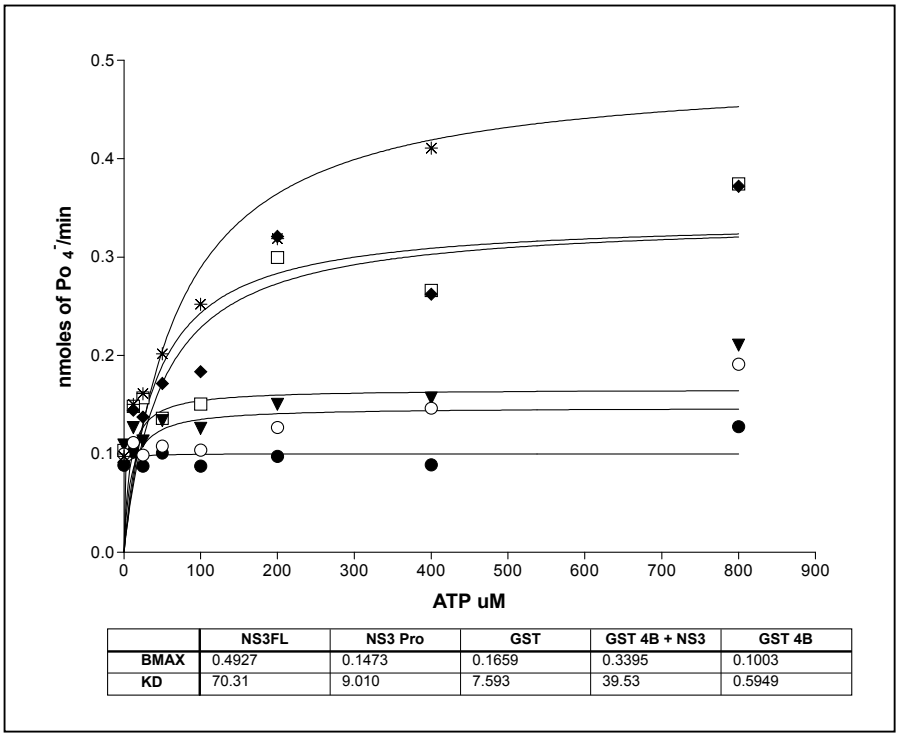

Negative control
Heat denatured duplex
GST
GST-NS4BM
NS3FL
+ NS3 FL
GST
GST-NS4BM
dsRNA
ssRNA

Figure 18: Modulation of RNA unwinding activity of NS3 by NS4B. A) NS4B enhances unwinding activity of NS3 in a dose-dependent manner. Lane 1: negative control; Lane 2: heat denatured duplex; Lane 3: 3 μM NS3-FL; Lanes 4 and 8: 6 μM each GST and GST-NS4B; Lanes 5- 7: 1 μM, 3 μM and 6 μM GST with 3 μM NS3-FL; Lanes 9-11: 1 μM, 3 μM and 6 μM GST-NS4B with 3 μM NS3-FL. In all reactions 0.25 pmoles of dsRNA substrate and 1 mM ATP was used, incubated at 30^0C for 30 min, terminated and resolved on a 10% native gel. B) Quantification of autoradiography signals. Amount of ssRNA in each lane is quantified using Image quant software and represented as a fold variation with respect to the ssRNA released by NS3-FL. GST NS4B enhances the unwinding activity of NS3-FL by 1.8 fold at 6 μM concentration as opposed to GST ($p= 0.005$). The values in the graph represent an average of three staggered experiments. C) NS4BM has no effect on the helicase activity of NS3. Lane 1: negative control; Lane 2: heat denatured duplex; Lanes 3 and 4: 6 μM each GST and GST-NS4BM; Lane 5: 3 μM NS3-FL; Lanes 6 and 7: 6 μM each GST and GST-NS4BM respectively with 3 μM NS3-FL.

2.4.6 NS4B Has No Effect on ATPase Activity of NS3

As the helicase activity of dengue NS3 protein is functionally coupled to its NTPase activity (Reviewed in (Rocak & Linder, 2004, Silverman *et al*., 2003)), we asked whether NS4B modulates the NTPase activity of NS3. ATP hydrolysis activity of NS3 FL was measured in the presence of GST or GST-NS4B by monitoring the amount of inorganic phosphate released in a colorimetric assay described previously (Lanzetta *et al*., 1979, Silverman *et al*., 2003, Xu *et al*., 2005). Briefly, 20 nM NS3 FL and the indicated amount of ATP were used and the reaction carried out in the presence of GST or GST-NS4B. NS3 pro, GST and GST-4B were used as negative controls for ATP hydrolysis. Malachite Green reagent was used to titrate the amount of inorganic phosphate released during the catalysis and the rates were calculated (PO_4 nmol min^{-1}) using Prism software. As seen in *figure 19*, GST and GST-NS4B had similar effects on the ATPase activity of NS3 FL indicating that NS4B had no specific effect on the ATPase function of NS3 FL.



	NS3FL	NS3 Pro	GST	GST 4B + NS3	GST 4B
BMAX	0.4927	0.1473	0.1659	0.3395	0.1003
KD	70.31	9.010	7.593	39.53	0.5949

Figure 19: The ATPase activity of NS3 FL is not altered by NS4B**.** 20 nM NS3 FL and the indicated amounts of ATP were used and the reaction carried out in the presence of GST or GST-NS4B. NS3 pro, GST and GST-NS4B were used as negative controls for ATP hydrolysis. GST and GST-NS4B had similar effects on ATPase activity of NS3 FL indicating that NS4B had no specific effect on the ATPase function of NS3 FL. The panel below the graph indicates changes in the NS3 ATPase kinetics in the presence of GST and GST-NS4B. The values represent an average of two experiments. The symbols are NS3 FL-Asterix (✱), NS3 pro-open circle (✪), GST-closed triangle (▼), GST-NS4B-closed circle (●), GST with NS3 FL- open square (□), GST-NS4B with NS3 FL-closed diamond (◆).

2.5 Discussion

The roles of dengue NS3, NS5 and NS2B in replication have been fairly well characterized but those of the other non structural proteins NS1, NS2A, NS4A and NS4B have remained somewhat elusive. We sought to ascertain the role of NS4B in replication by searching for its interacting partner among the other non structural proteins. In this study, we identified an interaction between NS4B and the C-terminal part of NS3 (aa 303-618) that contains a helicase motif using a variety of molecular genetics and biochemical interaction studies. Full length but neither the N- nor C-terminal truncations of NS4B interacted with NS3 (aa 303-618) in our yeast two-hybrid assay, suggesting that this interaction is dependent on NS4B conformation. We validated this interaction using biochemical pull downs with recombinant proteins and co-immunoprecipitations of endogenously expressed proteins in infected cell lysates and have shown that they co-localize to similar sub-cellular compartments.

Structural analysis of NS3 helicase suggests that it binds to RNA as well as proteins through its C- terminal region (Wu *et al*., 2005, Xu *et al*., 2005). Since NS4B interacted with the C-terminal region of NS3, it seemed likely that RNA binding of NS3 might be affected by this interaction. Our RNA binding experiments have shown that wild type NS4B but not the mutant (NS4BM) dissociates NS3 from ssRNA. Interestingly, while the dissociation is ATP independent, it is dependent on stoichiometry of the molecules. At least two molecules of NS4B per one of NS3 are needed to have a pronounced effect on ssRNA binding of NS3. This data supports our yeast two-hybrid results wherein NS4B interacted with itself and suggests that a functional NS4B molecule may be an oligomer. In this context, it is interesting to note that the N-terminal region of HCV NS4B is important for its polymerisation and subsequent induction of specialised host membranes involved in RNA replication (Yu *et al*., 2006b).

In the case of HCV, NS4A increases the ability of NS3 to bind to RNA and thereby enhances its helicase activity (Gallinari *et al*., 1999, Howe *et al*., 1999, Morgenstern *et al*., 1997, Pang *et al*., 2002). Since dengue NS4B dissociates NS3 from RNA, we hypothesized that NS4B might act as a negative modulator of NS3. Surprisingly, NS4B did not decrease the dsRNA unwinding activity of NS3 but enhanced it in a dose-

dependent manner. The helicase activity of dengue NS3 protein is functionally coupled to its NTPase activity (Reviewed in (Rocak & Linder, 2004) and HCV NS4B has intrinsic RNA binding and NTPase activities (Einav *et al*., 2004). However, dengue NS4B did not exhibit either RNA binding or NTPase activities in our study. Furthermore, it had no effect on the ATP hydrolysis activity of NS3-FL in a colorimetric assay described previously. This suggests that NS4B does not regulate the helicase activity of NS3 via its NTPase function. We hypothesize that NS3 is displaced from ssRNA in the presence of NS4B enabling it to interact with the next duplex and thereby increasing the over-all processivity of the enzyme *in vitro*. In this light, it will be interesting to see if ssRNA and NS4B compete to bind to the same region of NS3.

A single amino acid mutation in NS4B (P104L) that has been reported previously to have pleiotropic effects on dengue replication in mosquito versus human cells (Hanley *et al*., 2003) disrupted the interaction between NS3 and NS4B in both yeast two-hybrid as well as pull down assays. This NS4B mutant had no effect on the RNA binding or helicase activities of NS3. Proline at amino acid position 104 of NS4B is conserved in DEN1-4 but interestingly, Japanese encephalitis virus, Kunjin virus, and West Nile virus each possess a leucine at that position. Therefore, it has been proposed that the substitution of proline by leucine in position 104 of NS4B leads to a change in its structure or conformation (Hanley *et al*., 2003). Taken together with the fact that truncated NS4B did not interact with NS3 in yeast two-hybrid assay, the conformation of NS4B seems to be very important for this interaction.

An *in vivo* implication of the NS4B-NS3 interaction is in the formation of a functional complex that holds the two strands of the RNA apart. It has been proposed that flaviviral NS3 and NS5 act as a functional complex (Brooks *et al*., 2002, Yon *et al*., 2005). The physical interaction of NS3 and NS4B demonstrated in this study might imply that all three molecules (NS3, NS4B and NS5) form a complex that holds the separated strands apart as the helicase moves along the duplex. *In vitro* activity studies show high unwinding activity of the dengue helicase in the absence of other non structural proteins (Xu *et al*., 2005) but little is known about the directionality of the flaviviral NS3 helicase. As the helicase function precedes the RNA dependent RNA polymerase activity of NS5, which progresses in a 3'-5' fashion, it is reasonable to

assume that a factor such as NS4B renders directionality to NS3 helicase. Further *in vivo* studies will be needed to verify these hypotheses and to determine the role of the non structural protein complex in flaviviral replication.

Finally, it is evident that an understanding of the flaviviral replication cycle will require characterization of the physical and functional interactions of the proteins that form the replication complex, including unidentified host proteins. Whereas many studies have indicated how NS3 and NS5 might participate in the replication process, this is the first report of the role of flaviviral NS4B in viral replication. This study implies that NS4B modulates the activity of the viral helicase and mutations in NS4B that affect this ability lead to altered replication efficiency of the virus. Further work on the finely balanced interactions between all these components should ultimately provide a working model for the control of flaviviral replication.

CHAPTER 3

Role of Type I Interferon in Dengue Pathogenesis

3. ROLE OF TYPE I INTERFERON IN DENGUE PATHOGENESIS

3.1 Introduction

3.1.1 Interferon Response to Virus Infection

3.1.1.1 Interferons

Interferons (IFNs) are a heterogeneous family of cytokines, originally identified on the basis of their ability to induce cellular resistance to viral infections. Three major classes of IFNs (type I, type II and type III) have been described and they are classified based on the type of receptor through which they signal. While the biological significance of type III (IFN-λ) is not known, type II (IFN-γ) is produced mainly in haematopoietic cells (NK cells, T cells) upon stimulation by cytokines or antigens and is regarded as a regulator of the adaptive immune system. The type I IFNs (also referred to as IFN-α/β based on the predominant subtypes) are the main cytokines for innate immune responses against viral infections. Type I IFN family consists of many members - IFN-α (alpha), IFN-β (beta), IFN-κ (kappa), IFN-δ (delta), IFN-ε (epsilon), IFN-τ (tau), IFN-ω (omega) and IFN-ζ (limitin). The cellular source of the different type I IFNs varies: IFN-β (1-2 subtypes) is the predominant species produced by various non haematopoietic cells whereas IFN-α (14-20 subtypes) is the predominant species that is produced by haematopoietic cells (reviewed in (Pestka *et al.*, 2004)). Both type I and type II IFNs stimulate an antiviral state in target cells whereby the replication of virus is blocked or impaired due to the synthesis of a number of enzymes that interfere with cellular and virus processes. Both types of IFN can also slow the growth of target cells or make them more susceptible to apoptosis, thereby limiting the extent of virus spread. Finally, both types of IFN have profound immuno-modulatory effects and stimulate the adaptive response. However, while both types influence the properties of immune-effector cells, they show significant differences in signaling patterns which account for different spectrum of antiviral activities of the two types of IFN.

3.1.1.2 Induction of Type I IFNs

The induction of IFN-α/β upon viral infection is regulated primarily at the transcriptional level. Most RNA viruses enter cells by membrane fusion either at the plasma membrane or through endocytotic process and release the nucleic acid into the cytoplasm. It is thought that viral infection is detected through the presence of nucleic acids such as ssRNA (detected by TLR-7) and dsRNA (detected by TLR-3) by Toll-like receptors (TLRs). These receptors signal to downstream transcription factors such as NF-κB and IRF-3 via adaptors such as TRIF or MyD88 leading to IFN induction. Recently, a second pathway for IFN-α/β induction has been characterized. Two RNA helicases - retinoic acid-inducible gene I (RIG-I) or melanoma differentiation-associated gene 5 (MDA-5) have been shown to differentially detect intracellular dsRNA (Hornung *et al*., 2006, Kato *et al*., 2006) and transmit signal though a caspase recruitment domain (CARD)-containing adapter molecule (MAVS/IPS-1/VISA/Cardif). MAVS activates TBK-1 and IKK-ε kinases (which phosphorylate the transcription factors IRF-3 and IRF-7) and leads to IFN induction and regulation. MAVS also leads to immune regulation by NF-κB via activation of IKKα/β/γ complex (reviewed in (Hiscott *et al*., 2006, Honda *et al*., 2005, Levy & Marie, 2004, Seth *et al*., 2006)). The general scheme of induction and action of IFN-β upon virus infection is illustrated in *figure 20*.

3.1.1.3 Type I IFN Signaling and Antiviral Effects

Type I IFN signaling is summarized in *figure 21*. Briefly, a homologous receptor complex consisting of two subunits– IFNAR-1 and IFNAR-2 mediates the IFN-α/β signaling pathway. IFN binds to the receptor complex and cross activates the two receptor associated Janus protein tyrosine kinases (Jaks), Jak1 and Tyk2. This is followed by activation of signal transducers and activators of transcription (STATs) STAT-1 and STAT-2 by tyrosine phosphorylation. Activation of STATs can lead to the formation of IFN-α-activated factor (AAF) which is a homodimer of STAT-1 and IFN-stimulated gene factor 3 (ISG3) which consists of activated STAT-1, STAT-2 and interferon regulatory factor (IRF) 9. These complexes translocate to the nucleus and initiate the transcription regulation of IFN responsive genes. Whereas AAF binds to the

IFN-γ-activated site (GAS) element in the promoters of IFN responsive genes such as IRF-1, the ISGF3 binds to interferon-stimulated response element (ISRE) and regulates the transcription of genes such as IRF-7, IP-10, OAS, Mx, PKR, and ADAR etc which mediate the antiviral action of IFNs (reviewed in (Garcia-Sastre, 2004, Sen, 2001, Stark *et al*., 1998, Taniguchi & Takaoka, 2001)).

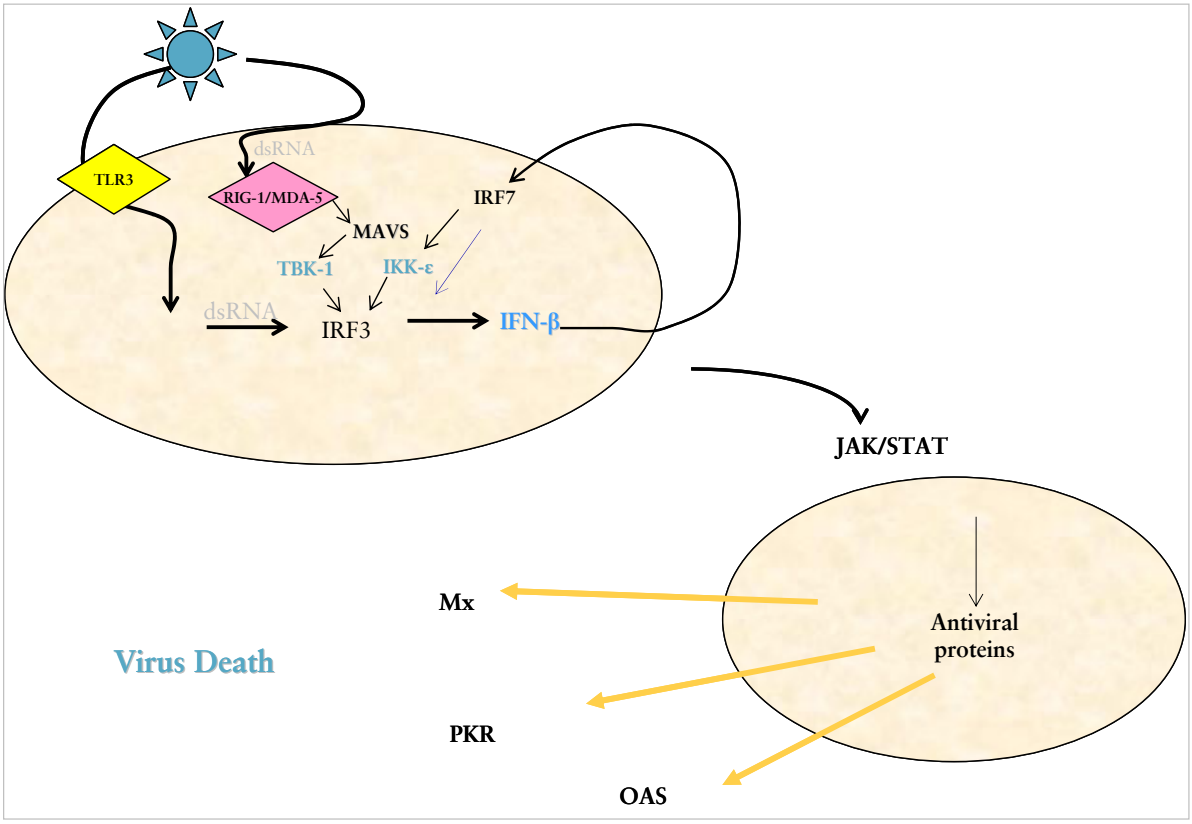


Figure 20: Induction and activity of IFN-α/β. Virus (blue) is recognised either by TLRs (at the plasma membrane or intra-cellular vesicles) or by dsRNA helicases (RIG-I/MDA-5). Both pathways signal via the activation of IRF transcription factors for induction of type I IFNs. The secreted IFNs activate the transcription of antiviral genes via the well characterized JAK/STAT signaling pathway. See text for details.

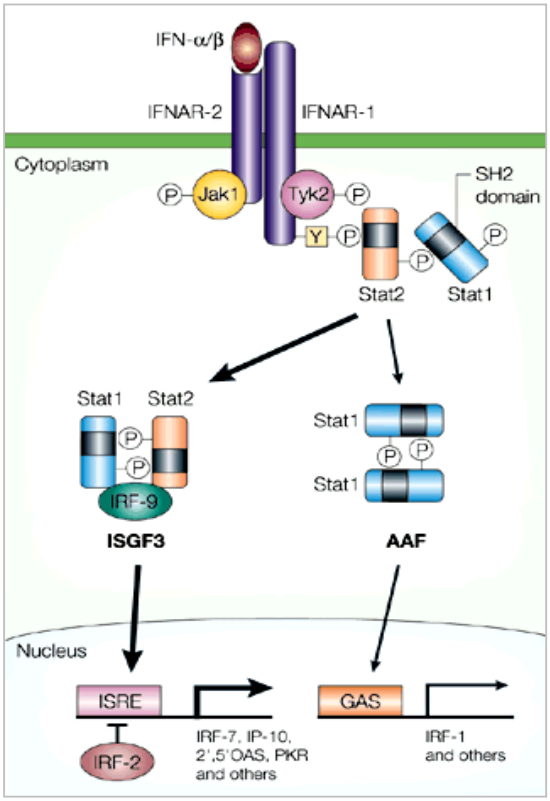


Figure 21: The IFN-α/β signaling pathway. Type I interferon transmits signals through its homologous receptor complex composed of IFNAR-1 and IFNAR-2. Ligand binding results in activation of the receptor-associated Tyk2 and Jak1 and subsequent activation by tyrosine phosphorylation of STAT-1 and STAT-2. A heterotrimeric transcription factor ISGF3 is then formed together with IRF-9. In addition, a STAT-1 homodimer, AAF is also formed. These transcriptional-activator complexes translocate into the nucleus and activate the ISRE or GAS elements in the promoters of interferon responsive genes such as PKR, OAS etc and initiate the antiviral state of the cells. Adapted from (Taniguchi & Takaoka, 2001).

Although more than 100 genes are induced by IFN-α/ β (Der *et al*., 1998), most studies have focused on the antiviral activity of three IFN-inducible genes – Mx, PKR and OAS. The Mx proteins are large GTPases of the dynamin super family which interfere with intracellular trafficking of the viral nucleocapsids and thereby counter viral infections (Haller & Kochs, 2002). The protein kinase PKR is autophosphorylated in the presence of dsRNA and subsequently phosphorylates the elongation factor eIF-2α. Phosphorylation of EIF2α results in inhibition of protein translation and therefore, PKR activation in virus infected cells results in translational block of viral replication (Stark *et al*., 1998, Williams, 2001). The OAS enzymes are 2'5'Oligo A synthetases which activate RNaseL, an enzyme that degrades viral and cellular RNA, stopping replication (Player & Torrence, 1998, Zhou *et al*., 1997). In addition to these well characterized proteins, there are other IFN inducible genes that participate in the inhibition of viral replication such as ADAR (Saunders & Barber, 2003), viperin (Chin & Cresswell, 2001), MAVS (Seth *et al*., 2006) etc (Samuel, 2001).

3.1.2 Evasion of the IFN System by Viruses

The long co-evolutionary history of viruses with their hosts has seen the development of a variety of evasive adaptations that allow viruses to circumvent or inactivate host antiviral mechanisms. Viruses have evolved strategies to either inhibit IFN synthesis, bind and inactivate secreted IFN molecules, block IFN-activated signaling, or disturb the action of IFN-induced antiviral proteins (reviewed in (Goodbourn *et al.*, 2000, Levy & Garcia-Sastre, 2001)). *Table 6* summarizes various examples of the viral interference with the IFN system (Best *et al.*, 2005, Garcia-Sastre, 2004, Levy & Garcia-Sastre, 2001, Lin *et al.*, 2006b, Munoz-Jordan *et al.*, 2005, Sen, 2001).

Virus	*Mechanism of action/inhibition*
Inhibition of IFN binding to cognate receptors	
Poxviruses	Encode type I and type II IFN receptor decoys
Inhibition of IFN production	
Human herpesvirus-8	Encodes IRF homologue that blocks IFN production
Human papillomavirus	Encodes E6 protein that binds to IRF-3 and blocks IFN production
Hepatitis C	Blocks activation of IRF-3 by inhibiting MAVS and IRF-3
West Nile virus	Blocks activation of IRF-3
Measles virus, hepatitis B virus, Ebola virus, Bunyamwera and Rift valley fever viruses	Block IFN production by unknown mechanisms
Inhibition of Jak/STAT pathway	
Adenovirus	E1A decreases STAT1 and p48 levels, interacts with STAT1
Ebola virus	Blocks IFN signaling, mechanism unknown
Epstein-Barr virus	EBNA-2 blocks IFN signal transduction, mechanism unknown
Murine polyoma virus	T antigen binds to and inactivates Jak1
Human cytomegalovirus	Reduces levels of Jak1 and p48
Human papillomavirus	E7 protein binds to p48
Human parainfluenza virus	Targets STAT2 for degradation
Simian virus *5*	V protein targets STAT1 for proteasome-mediated degradation
Sendai virus	Blocks STAT1 phosphorylation
Japanese encephalitis virus, Tick borne encephalitis virus,	Encode NS5 that prevents phosphorylation of Tyk2 and Stat1
Dengue, West Nile and yellow fever viruses	Encode NS4B that prevents activation of STAT-1, dengue virus also degrades STAT-2
Inhibition of IFN-induced antiviral enzymes	
Hepatitis B virus	Capsid protein inhibits MxA gene expression
Encephalomyocarditis virus	Induces RNase L inhibitor
Herpes simplex virus	Encodes 2´5´OAS antagonists

Human immunodeficiency virus	Induces RNase L inhibitor and down regulates PKR
Reovirus, Rotavirus, Pox virus, influenza virus	Encode proteins that bind dsRNA and inhibits PKR
Poliovirus	Induces the degradation of PKR
Hepatitis C virus	NS5A and E2 bind to and inhibit PKR;
Influenza virus	Induces cellular inhibitor of PKR (p58IPK)
Adenoviruises	Produces VA RNA that binds to but fails to activate PKR
Herpes simplex virus	Encodes US11 that blocks PKR activity
Baculovirus	Encodes PK2 that binds and inactivates PKR

Table 6: Viral interference with the IFN system (Best *et al.*, 2005, Garcia-Sastre, 2004, Levy & Garcia-Sastre, 2001, Lin *et al.*, 2006b, Munoz-Jordan *et al.*, 2005, Sen, 2001).

3.1.3 IFN and Flaviviruses

IFNs have been shown to be crucial for recovery from infection by various flaviviruses (Crance *et al.*, 2003, Diamond *et al.*, 2000, Lin *et al.*, 2006b, Sumpter *et al.*, 2004). However, use of IFN *in vivo* for treatment of flavivirus infection have not been uniformly successful (Ajariyakhajorn *et al.*, 2005, Solomon *et al.*, 2003). Moreover it was known that IFN can protect cells from *de-novo* infections but had no effect on established infection indicating that flaviviral replication yields IFN antagonists. This is supported by the ability of some flaviviruses, including dengue (Munoz-Jordan *et al.*, 2005, Munoz-Jordan *et al.*, 2003), Japanese encephalitis virus (Lin *et al.*, 2006b), West Nile virus (Guo *et al.*, 2005), Tick borne encephalitis virus (Best *et al.*, 2005) and Kunjin virus (Liu *et al.*, 2006, Liu *et al.*, 2005) to inhibit the JAK/STAT signaling in response to IFNs. The non-structural proteins of flaviviruses have been implicated to be responsible for IFN antagonism. Japanese encephalitis (Lin *et al.*, 2006b) and Tick borne encephalitis (Best *et al.*, 2005) viruses seem to employ the NS5 protein for interference with the IFN system whereas recent studies of KUN suggested that NS2A, NS2B, NS3, NS4A, and NS4B were all capable of inhibiting JAK-STAT signaling (Liu *et al.*, 2005). Interestingly, a recent study showed that a single mutation in NS2A of West Nile disables its ability to inhibit interferon induction (Liu *et al.*, 2006).

Dengue, West Nile and yellow fever viruses seem to inhibit type I IFN signaling via NS4B which prevents activation of STAT-1 (Munoz-Jordan *et al.*, 2005, Munoz-Jordan *et al.*, 2003). Furthermore, two independent studies that showed respectively that

STAT2 expression is down regulated in human myeloblastoma cell line K562 stably transfected with dengue replicon (Jones *et al*., 2005) and that dendritic cell lines infected with dengue have reduced Tyk2 activation (Ho *et al*., 2005) advocate that dengue antagonises type I but not type II IFN response. While these studies show that dengue infection can overcome IFN signaling and subsequent antiviral effects, there have been *in vivo* studies that show that IFN can counter established dengue infection (Ajariyakhajorn *et al*., 2005, Limonta, 1984).

3.2 Aim of the Study

NS4B has been shown to antagonise the IFN signaling in response to dengue infection (Munoz-Jordan *et al*., 2003) suggesting that IFN therapy would not be relavent to dengue infection. However, one study describes the use of recombinant IFN in successfully treating dengue patients (Limonta, 1984). In light of such controversies, the aim of this study was to first characterize the type I IFN response to dengue infection before investigating the role of NS4B in this process. A serendipitous discovery of differential regulation of the type I IFN response by different strains of dengue subsequently led us to explore the role of viral genomic variations in determining host responses and clinical outcome to infection. While it has been known that different strains of the same dengue serotype exhibit marked differences in clinical outcome (Edgil *et al*., 2003, Rosen, 1977), previous studies have largely concentrated on phylogenetic or epidemiological perspectives (Gubler *et al*., 1978, Gubler *et al*., 1981, Leitmeyer *et al*., 1999). In this study, we sought to identify the specific mechanisms through which the antiviral effects of IFN are countered by different strains of dengue. We also explored the possibility of identifying a virulence factor such as viral load or a virus encoded factor that might potentially be used as a marker for severity.

3.3 Materials and Methods

Cells, Viruses and Plasmids: Cell lines were obtained from ATCC and maintained in RPMI 1640 (BHK-21, C6/36), Minimal Essential Medium (HepG2), Hams F12K, (A549) cell culture medium with 10% fetal bovine serum (FBS) and penicillin/streptomycin (GibcoBRL). All cells were cultured at 37ºC in a humidified incubator with 5% (v/v) CO2, except for C6/36 cells which were cultured at 28ºC. Infection was done with 2% FBS for all cell lines except C6/36 in which case 5% FBS was used. TSV01 (GenBank number AY037116) and NGC (GenBank number M29095) and clinical isolates were propagated in C6/36. The Malaysian strains [MY 2569, MY 10245 (DEN1), MY 22563, MY 10340 (DEN2), MY 22713 (DEN4)] used in this study were kind gifts from Dr. Shyamala Devi from the University Malaya, Kula Lumpur, Malaysia. SG 167 strain was obtained from a prospective longitudinal study - the Early Dengue (EDEN) study in Singapore. Virus stocks were prepared by single passage in C6/36 for 5 to 7 days. The virus-infected supernatants were harvested; titrated and same viral stock was used throughout this study. Heat-inactivated virus was prepared by incubating virus samples at 55°C for 1 hr. FLAG-MAVS plasmid was from Dr. Zhijian J. Chen, Howard Hughes Medical Institute, Texas (Seth *et al*., 2005). Please see *supplementary table ST2* for standard nomenclature of viral strains.

Plaque Assay: BHK-21 cells were infected with serial dilutions of infected cell culture supernatants or viral stocks for 1 hr. The cells were then incubated for 5 days in 0.8% methyl-cellulose medium (with 2% FBS), fixed in 4% formaldehyde for 20 minutes and stained with crystal violet. Plaques were counted manually and concentrations of plaque forming units per ml (pfu/ml) of the sample cell culture supernatant calculated. All plaque assays were performed in triplicate and an average is presented.

Interferons: Hu-IFN-α-2a, Hu-IFNβ 1A (Chemicon) were used at 500 U/ml whereas Hu IFN-γ (BD pharmingen) was used at 500 ng/ml concentration in IFN treatment assays of A549 and HepG2 for 30 min. For TLDA analysis, cells were infected for 18 hours and treated for 24 hours with 1000 U/ml IFN-α. Both IFN-α and IFN-β were used inter-changeably in the type I IFN signaling studies as they signal through the same receptors.

TaqMan Low Density Array (TLDA) and RNA Quantification: 35 genes from the innate IFN response pathway were picked for TaqMan Micro Fluidic Cards (3M Company, ABI). 100 ng of total RNA was reverse transcribed using High-Capacity cDNA Archive Kit (ABI) and processed according to manufacturers instructions together with data analysis using SDS2.2 software (ABI). Each sample had four biological repeats and the differentially expressed genes were detected by Significance Analysis of Microarray (SAM) described in (Tusher *et al*., 2001). Cut-offs were employed at fold change=2 and q value=0.05. Negative values represent down regulation while positive values represent up regulation of gene expression. These experiments were done in collaboration with Dr. Martin Hibberd, Genome Institute of Singapore, Singapore.

Immunoblotting and Antibodies: Cell lysates were harvested in modified RIPA buffer (50mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150mM Nacl, 1mM EDTA, with protease and phosphatase inhibitors from sigma) and protein concentrations were normalized for immunoblotting. The following primary antibodies were used: rabbit anti-phosphotyrosine 701 STAT1 (Cell Signaling), rabbit anti-phosphotyrosine 689 STAT2 (Upstate), rabbit anti-STAT1 (Santacruz), rabbit anti-STAT-2 (Santa Cruz), mouse anti-FLAG (M2; Sigma), rabbit anti-IRF-3 (Santa Cruz). Antibodies against NS5 and NS4B were generated in house.

IRF-3 Studies: For detection of IRF-3 activation, A549 cells were infected with TSV01 or NGC and lysed at indicated times. RNA extraction was done with Qiagen RNA extraction kit and primers sequences were adopted from (Izaguirre *et al*., 2003) for RT PCR analysis. Sense primer 5'-CGGAAGCTTCTGAAGCGGCTGTTGGTG-3' and antisense primer 5'-GTGCTCGAGACCATGAGGAGCGAGGGC-3' IRF-3 dimerization assay has been described elsewhere (Lin *et al*., 2006a). Briefly, cells were suspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% NP-40 supplemented with protease and phosphatase inhibitors from Sigma). Whole-cell extracts were fractionated by 7.5% native PAGE before immunoblotting with IRF-3 antibody.

3.4 Results

3.4.1 Identification of a Strain-Dependent Regulation of Type I IFN Signaling in Dengue Infection

3.4.1.1 TSV01 Does Not Inhibit STAT-1 Activation

The IFN response to dengue infection has not been well characterized. While dengue NS4B has been shown to inhibit both type I and type II IFN signaling in monkey cell lines (Munoz-Jordan *et al*., 2003), other studies have shown that dengue infection down regulates type I but not type II IFN signaling (Ho *et al*., 2005, Jones *et al*., 2005). We sought to characterize the IFN response to dengue infection. For this, NS4B (cloned from TSV01 into pXJ vector as described in *table 5*) was transfected into A549 cells. 24 hours after transfection, the cells were serum starved for 12 hours and treated with 500 U/ml of IFN-β for 30 minutes. Activation of STAT-1 in IFN treated cells was monitored by immunoblotting with phospho-STAT-1 antibody. Surprisingly, NS4B did not inhibit phosphorylation of STAT-1 upon stimulation with IFN-β contradicting previous reports (*figure 22 A*). We also looked in A549 cells transfected with NS4B for activation of STAT-1 via its nuclear localization upon stimulation with IFN-β. As seen in *figure 23*, STAT-1 nuclear translocation was normal in cells treated with IFN in the presence of NS4B.

The IFN suppression studies were done with over-expressed NS4B which might not represent physiological situation. Furthermore while a recent publication pointed out the importance of the N-terminal 2K fragment in the suppression of IFN signaling (Munoz-Jordan *et al*., 2005), topology studies indicated that the region of NS4B that is required for this suppression is luminal (Miller *et al*., 2006) which presents a logistical challenge to the idea of a direct interaction of NS4B with the host defence machinery. We therefore repeated these studies in a virus infection setting to mimic closely the physiological situation. A549 cells were infected with TSV01 with multiplicity of infection (moi) of 10 and 72 hours post infection, were treated for 30 minutes with 500 U/ml of IFN-β. As seen in *figure 22 B*, TSV01 infection did not inhibit STAT-1 phosphorylation in IFN treated cells.

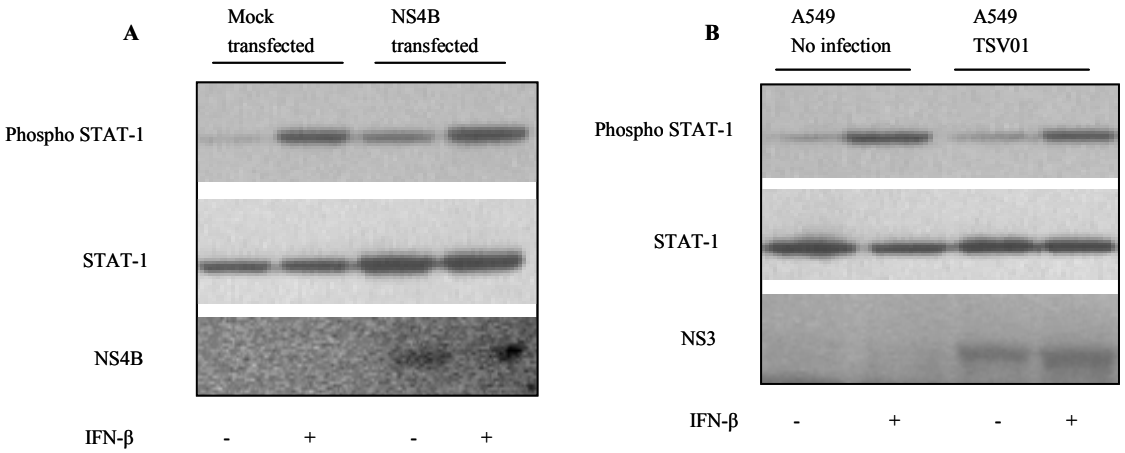
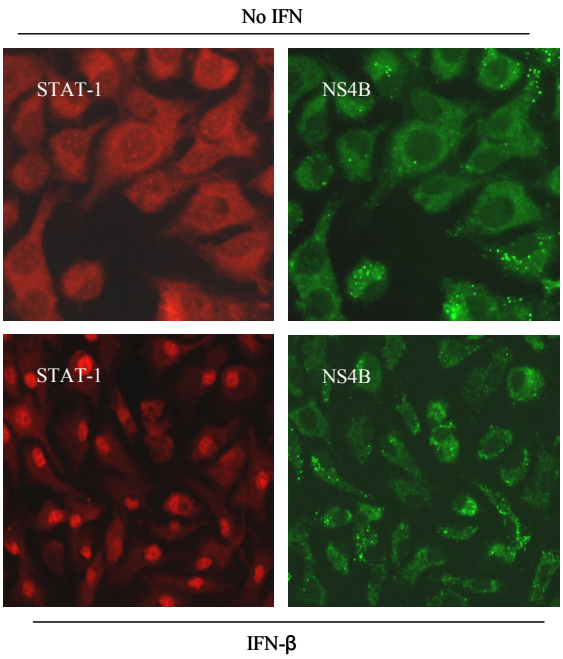


Figure 22: A) NS4B of TSV01 does not inhibit phosphorylation of STAT-1 upon IFN treatment. A549 cells were either mock transfected or transfected with NS4B and 36 hours post transfection, 500 U/ml of IFN-β was added for 25 minutes as represented in the figure. Lysates were collected in m-RIPA buffer and western blotting was done with phospho-STAT-1, STAT-1 and NS4B antibodies. STAT-1 phosphorylation occured in the presence of NS4B. B) TSV01 infection does not inhibit IFN signaling. A549 cells were infected with 10 moi of TSV01 and 72 hours post infection, treated with IFN as described before. Immunoblotting with NS3 showed the presence of infection. STAT-1 phosphorylation was similar in uninfected and infected cells showing that TSV01 did not inhibit type I IFN signaling.

Figure 23: NS4B of TSV01 does not inhibit nuclear translocation of STAT-1 upon IFN treatment. A549 cells were either mock transfected or transfected with NS4B and 36 hours post transfection, 500 U/ml of IFN-β was added for 25 minutes as indicated in the figure, fixed with cold methanol and immunoflorescence was done with STAT-1 and NS4B antibodies. STAT-1 translocated to the nucleus even in the presence of NS4B showing that NS4B did not antagonise nuclear translocation of STAT-1.



3.4.1.2 Interferon Response to Infection Is Different Between NGC and TSV01 Strains

Ongoing differential gene expression studies in our laboratory hinted at the role of genomic variations of dengue strains on host response. For example, Significance Analysis of Micro arrays (SAM) on HepG2 cells infected for 48 hours with 10 moi of either TSV01 or NGC revealed that while 88 genes were differentially expressed between cells infected with these two stains. The most important difference was in the genes of the interferon mediated innate immunity pathway (Hibberd M, personal communication). However, comparison of the genomic sequences of two closely related DEN-2 stains- the prototype strain New Guinea C (NGC) and the recent clinical isolate TSV01 revealed very few variations leading to non-conserved amino acid substitutions. Most of the other elements of host response are very similar to these strains suggesting that viral sequence variations might manipulate specific arms of the host response.

To validate that NGC did not activate the interferon pathway genes as efficiently as TSV01, we picked 35 type I IFN specific genes and performed a customised TaqMan Low-Density Array (TLDA) experiment. A549 cells were infected for 48 hours with 10 moi each of live and heat inactivated NGC and TSV01 and infection was confirmed with plaque assays on culture supernatants. The gene expression values of infected samples were compared individually to samples infected with heat inactivated virus. Four biological repeats were used in this experiment and cut offs were employed at q=0.05 and fold change=2. The results are represented as fold change in samples with live virus versus those with heat inactivated virus in *table 7*. Whereas some of the host response genes are similar in both, TSV01 elucidated higher levels of expression of most interferon related genes (such as STAT1, STAT2, MX1, OAS 2 etc) as opposed to NGC. These results implied that viral genomic variations generate significant differences in innate immune responses to infection.

Pathway/Function	Gene	NGC	TSV01
IFN	IFNB1	251.9	159.8
IFN Induction	RIG-I	13.7	68.3
IFN Induction	IFIH1	66.7	129.4
IFN Induction	IRF-7	-	39.2
IFN Receptor	IFNAR2	-	2.4
IFN Receptor	IFNGR1	-	2.3
IFN Receptor	IFNGR2	-	2.3
IFN Signaling	JAK2	2.3	2.7
IFN Signaling	NMI	-	9.7
IFN Signaling	PIAS1	-	2.2
IFN Signaling	SOCS1	4.3	10
IFN Signaling	SOCS3	2.9	4.8
IFN Signaling	STAT1	-	7.4
IFN Signaling	STAT2	3.5	17.4
IFN Signaling	STAT5A	4.2	2.9
IFN Signaling	TNFAIP3	20.7	17.4
IFN induced	EIF2AK2	-	10.2
IFN induced	G1P2	87.7	113.7
IFN induced	G1P3	31.2	105.3
IFN induced	IFI35	2.4	43.4
IFN induced	IFI44	90.1	119.8
IFN induced	IFIT1	105.9	121.5
IFN induced	IFITM1	78.1	105.4
IFN induced	IL6	129.5	47.8
IFN induced	ISG20	5	11.3
IFN induced	ISGF3G	-	13.4
IFN induced	MX1	61.7	108.2
IFN induced	OAS1	9.2	71.2
IFN induced	OAS2	78.1	120.9
IFN induced	OAS3	5.2	45.7
IFN induced	OASL	119.6	143
IFN induced	RSAD2	192.3	132.8
IFN induced	ADAR	-	2.9
IFN induced	B2M	3.9	10.5
Proteosome	PSMB9	5.8	20.6
Chemokine	CXCL10	229.4	215.8

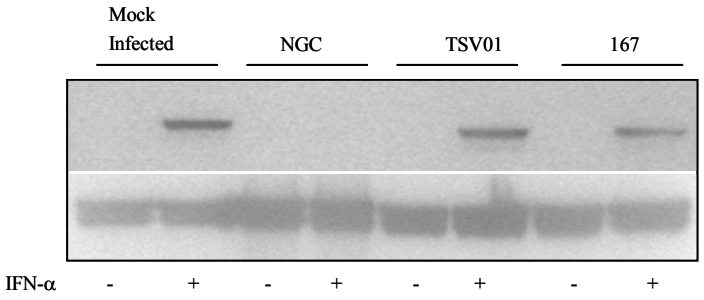
Table 7: Type I interferon response to infection is different between NGC and TSV01 strains. The gene expression values of A549 cells infected live viruses compared to those with heat inactivated virus are represented in the table as fold change. While infection with TSV01 elicited a strong IFN response as evidenced by higher expression levels of IFN signaling (such as STAT1, STAT2 etc) and IFN induced genes (MX1, OAS 2 etc), NGC did not elicit such a response suggesting that NGC might encode an IFN countering mechanism.

3.4.1.3 Differential Ability of Dengue Viral Strains to Suppress Type I IFN Signaling

NGC elicited very little IFN response as opposed to TSV01 and the IFN antagonism studies earlier (Munoz-Jordan *et al*., 2003) used a DEN2 strain 16681 which is more similar to NGC than TSV01 in its sequence. We therefore hypothesized that NGC but not TSV01 would inhibit STAT-1 activation upon stimulation with type I IFN. We also included a clinical isolate SG 167 (DENV1) from a recent outbreak of dengue in Singapore as a control in this experiment since both TSV01 and NGC are high-passage laboratory strains and might have acquired mutant phenotypes that are not physiologically relevant. HepG2 cells were infected at 5 moi with NGC, TSV01 and SG 167. Twenty four hours post infection, the cells were serum starved and treated with 500 U/ml of IFN-α for 30 minutes. Cell lysates were normalized for protein concentration and western blotting was done with phospho STAT-1 and STAT-1 antibodies. *Figure 24 A* shows that treating mock infected cells with IFN-α induced STAT-1 phosphorylation and this is the same with cells infected with both TSV01 and SG 167. However NGC infection inhibited STAT-1 phosphorylation upon IFN stimulation.

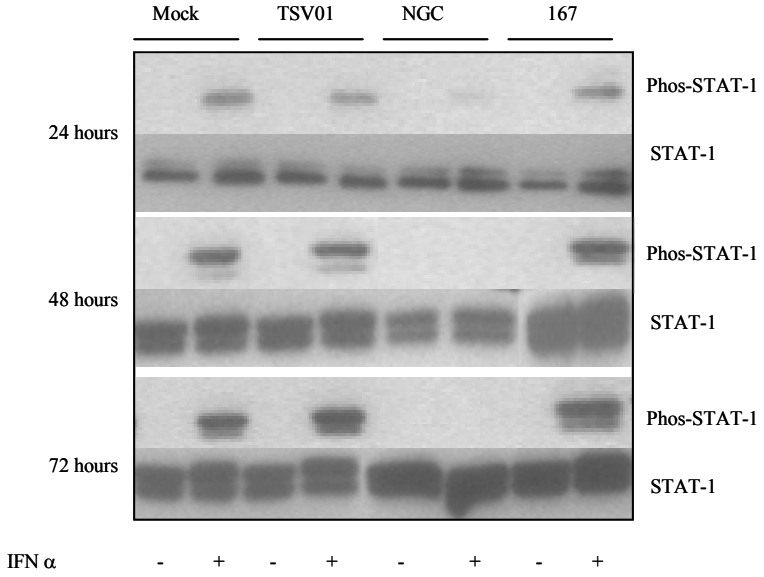
We repeated this experiment in A549 cells in which TLDA studies were done. This time, the infection was allowed to proceed for 24, 48 or 72 hours to see if TSV01 and SG 167 inhibit signaling at an advanced time point. IFN-α treatment were done as described for HepG2 cells and samples were collected at specified time points, protein concentration normalized and western blotting was done with phospho STAT-1 and STAT-1 antibodies. As seen in *figure 24 B*, NGC inhibited STAT-1 phosphorylation as early as 24 hours post infection but TSV01 and SG 167 did not inhibit it even after 72 hours of infection.

A)



Virus	pfu/ml
TSV01	5500
NGC	23500
SG 167	7000

B)



	pfu/ml		
	TSV01	**NGC**	**167**
24 hrs	1100	50000	4000
48 hrs	15000	1150000	84000
72 hrs	13800	165000	ND

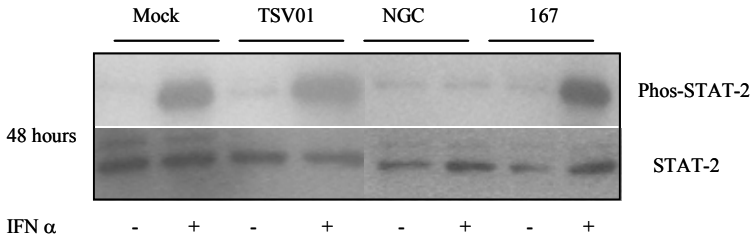
Figure 24: Differential ability of dengue viral strains to suppress type I IFN signaling. A) HepG2 cells were infected with 5 moi each of NGC, TSV01 and SG 167. Thirty six hours post infection cells (in indicated lanes) were treated with 500 U/ml of IFN-α for 30 minutes. Cell lysates were run on an 8% SDS gel and immunoblotted with phospho STAT-1 and STAT-1 antibodies. Plaque assays (n=3) were done with culture supernatants to control for infection and are represented as an average pfu/ml of indicated viral strains. B) A549 cells were infected with 5 moi each of NGC, TSV01 and SG 167 and treated with IFN-α at 24, 48 and 72 hour post infection and immunoblotted with phospho STAT-1 and STAT-1 antibodies. In both cell lines NGC infection inhibited activation of STAT-1 as opposed to TSV01 and SG 167. Plaque assays (n=3) were done with culture supernatants to control for infection and are represented as an average pfu/ml of indicated viral strains. (ND=not determined)

3.4.2 Dengue Infection Does Not Inhibit Type II IFN Signaling

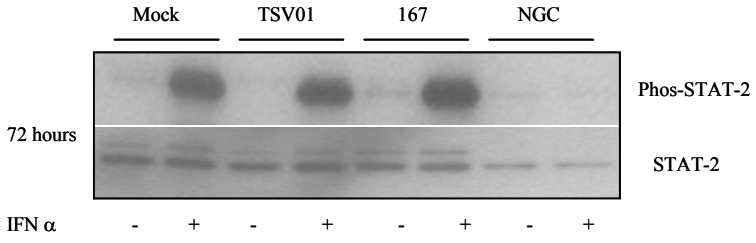
IFN-α/β signaling requires phosphorylation and subsequent hetero-dimerization of both STAT-1 and STAT-2, so we looked at STAT-2 activation in the A549 infection described before. Again, NGC but neither TSV01 nor SG 167 inhibited STAT-2 phosphorylation upon treatment with IFN-α as seen in *figure 25 A*. Interestingly we did not observe a significant reduction in STAT2 protein level in NGC infected cells as compared with TSV01 or SG 167 infected cells at early time points but only after 72 hours of infection (*figure 25 B*). We speculate that STAT-2 degradation reported by (Jones *et al*., 2005) is a relatively later event in viral survival.

STAT-1 activation and homo-dimer formation is common to both type I and type II IFN signaling pathways. As NGC inhibited STAT-1 phosphorylation upon IFN-α/β stimulation, we next asked if NGC would also inhibit STAT-1 activation in response to IFN type II signaling. A549 cells were infected with 5 moi each of TSV01, NGC and SG 167 respectively and twenty four hours post infection, they were treated with IFN-γ (500 ng/ml) for 30 minutes. As seen in *figure 25 C*, activation of STAT-1 was normal in dengue virus (all three strains) infected cells as compared to mock infected cells. Our results clearly demonstrated that dengue virus manipulates the type I but not type II IFN response to infection in a viral strain-dependent manner.

A)



B)



C)

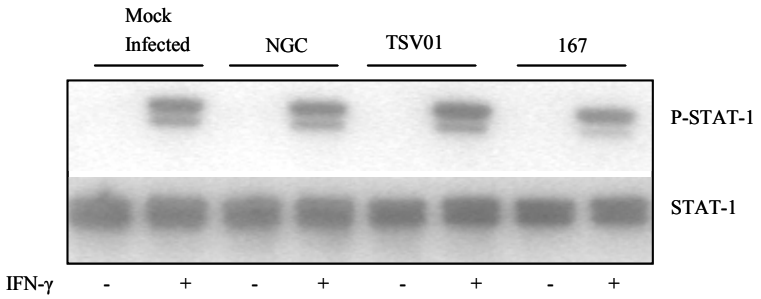


Figure 25: Differential regulation of IFN signaling by dengue viral strains is limited to type I but not type II IFN A) A549 cells were infected with 5 moi each of NGC, TSV01 and SG 167. 48 hours post infection cells (in indicated lanes) were treated with 500 U/ml of IFN-α for 30 minutes. Cell lysates were run on an 8% SDS gel and immunoblotted with anti phospho STAT-2 and STAT-2 antibodies. NGC suppressed activation of STAT-2 in addition to STAT-1. B) Similar treatment 72 hours after infection reveals that STAT-2 protein degradation happens at later stages of infection with NGC but not TSV01 or SG 167. C) A549 cells were infected as before with the three strains and stimulated with IFN-γ (in indicated lanes). NGC did not suppress phosphorylation of STAT-1 in response to IFN-γ confirming that viral suppression of the IFN response is limited to the type I system.

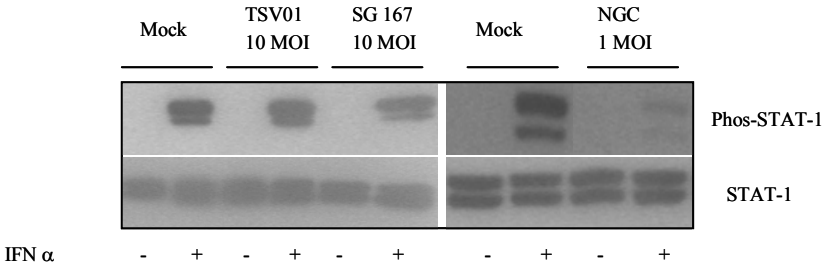
3.4.3 Varying Initial Input of the Virus Does Not Alter the Interferon Signaling Profile of Different Strains

Infection of A549 cells with NGC, TSV01 and SG 167 and showed variations in their respective infectivities. Supernatants from A549 cells infected with TSV01 and SG 167 formed lesser number of plaques in BHK cells as compared to those from NGC (see *figure 24* plaque assays). We therefore wanted to verify that the STAT-1 signaling inhibition is not a factor of this difference in infectivity or viral load.

A549 cells were infected with 1 moi of NGC and 10 moi each of TSV01 and SG 167 and stimulated with IFN-α as described earlier. Culture supernatants which were assessed by plaque assay for infectivity showed that 1 moi of NGC yielded comparable number of plaques as 10 moi of SG 167 and, to a lesser extent, 10 moi of TSV01 (*figure 26 B*). However as seen in *figure 26 A*, infection with 1 moi of NGC inhibited STAT-1 phosphorylation whereas both TSV01 and SG 167 did not inhibit STAT-1 activation even at 10 moi. This suggests that the viral strain-dependent signaling

profiles are independent of the viral load at the point when IFN stimulus is given to the cell. These results indicate that the host interferon response is independent of the viral load and point towards the presence of a virus specific factor that regulates this response.

A)



B)

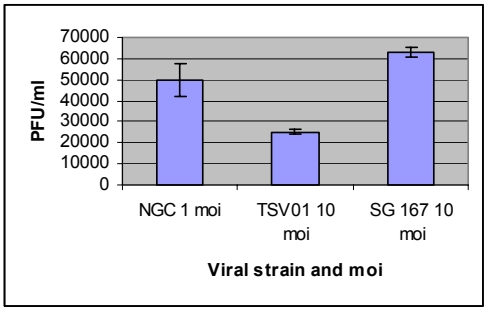


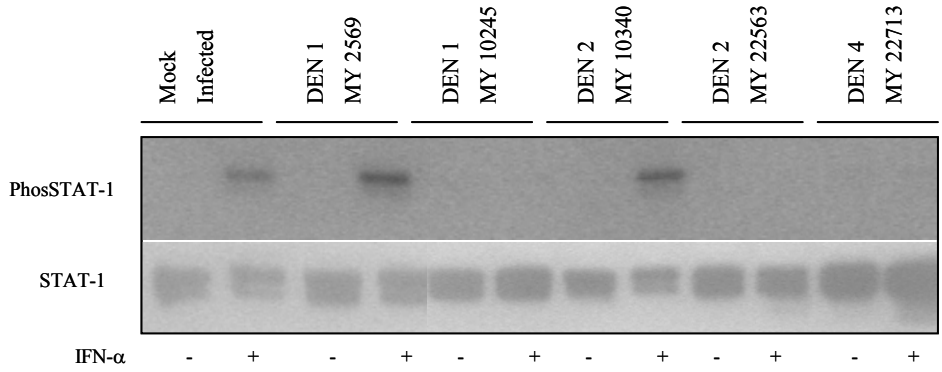
Figure 26: Varying viral load does not alter the interferon signaling profile of different strains. A) A549 cells were infected with 1 moi of NGC and 10 moi each of TSV01 and SG 167. While 1 moi of NGC inhibited STAT-1 phosphorylation, increasing initial input of TSV01 or SG 167 did not inhibit STAT-1 signaling. B) Culture supernatants were assessed for viral load of each viral strain by plaque assay (n=3) and represented in a bar graph. Note that infection of A549 with 10 moi of either SG 167 or TSV01 gave almost as many plaques as that of 1 moi of NGC but failed to inhibit STAT-1 as opposed to NGC.

3.4.4 Differential Response to IFN Signaling in Clinical Isolates of Dengue Points to Its Potential Clinical Relevance

Studies on interferon antagonism of dengue virus were done using either high passage laboratory strains (Munoz-Jordan *et al.*, 2003) or in cells harbouring dengue replicon (Jones *et al.*, 2005). While our data with a low passage clinical isolate SG 167 showed that it did not antagonise the IFN response, we extended our study to a panel of other

low passage clinical isolates. We used clinical isolates MY 2569, MY 10245 (DEN1), MY 10340, MY 22563 (DEN2) and MY 22713 (DEN4) to infect A549 cells at moi 1 for 36 hours and stimulated these cells with IFN-α. As seen in *figure 27 A*, MY 10245, MY 22563 and MY 22713 inhibited STAT-1 phosphorylation in response to IFN treatment and whereas MY 10340 and MY 2569 did not. Note that plaque assay from culture supernatants of these infections validated our earlier observation that strain-dependent IFN antagonism is not directly related to viral load. For example MY 10245 and MY 10340 gave comparable number of plaques but differed in their abilities to inhibit IFN signaling (*figure 27 B*). These results also confirmed that the difference in IFN signaling profiles of dengue viral strains occured across at least two serotypes (DEN-1 and DEN2) signifying that that this phenomenon is independent of serotype. We are currently working on understanding the clinical outcome of infection of these two kinds of strains from the perspective of their ability to induce IFN response.

A)



B)

Strain	pfu/ml
MY 22713	250000
MY 10245	40000
MY 2569	6000
MY 10340	11500
MY 22563	0

Figure 27: Differential response to IFN signaling in other clinical isolates of dengue. A) In order to extend the panel of low passage clinical isolates of dengue we used MY 2569, MY 10245 (DEN1), MY 10340, MY 22563 (DEN2) and MY 22713 (DEN4) from Malaysia. A549 cells were infected with 1 moi of each of these viruses and cells were stimulated with IFN-α in indicated lanes. While MY 2569 and MY 10340 did not inhibit the IFN signaling, the other strains prevented STAT-1 activation upon IFN stimulation. B) Plaque assays (n=3) were done with culture supernatants to control for infection and are

represented as an average pfu/ml of indicated viral strains. Note that MY22563 did not yield any plaques in a standard plaque assay format although it suppressed the IFN response.

3.4.5 Dengue Virus Inhibits the Expression of IFN Stimulated Antiviral Genes in a Strain-Dependent Manner

We showed that some strains of dengue inhibit the IFN-α/β signaling at the STAT-1 phosphorylation level and this could possibly be via inhibition of Tyk-2 phosphorylation as seen in dendritic cells (Jones *et al*., 2005). As discussed earlier, the antiviral effects of IFN are exerted through proteins such as Mx, OAS, ADAR, PKR etc. We therefore looked at the strain-dependent ability of dengue virus to suppress the antiviral genes downstream of JAK/STAT signaling using the same TLDA approach as before but in this case, the infected cells were stimulated with IFN. We also included SG 167 (which did not suppress IFN signaling) and MY 22713 (that suppresses IFN signaling) in our study. A549 cells were infected with 10 moi each of TSV01, SG 167, NGC, and MY 22713 and IFN-α (1000 U/ml) was added to the medium eighteen hours post infection. The samples were collected 24 hours later for RNA extraction and TLDA analysis. Heat inactivated viruses were used as controls in this experiment and four biological repeats were used for analysis and cut offs were employed as described earlier. As shown in *table 8*, interferon stimulated genes such as PKR, OAS, ADAR and Mx which render the anti viral activity are suppressed by NGC infection (negative values) but not by TSV01. SG 167 largely behaved like TSV01 and did not suppress IFN stimulated genes whereas MY 22713 suppressed a few but not all the genes that were suppressed by NGC. These results provide clear evidence that dengue virus counters the antiviral activities of type I IFN at the gene expression level in a strain-dependent manner.

Pathway/function	Gene	NGC	22713	TSV01	167
IFN	IFNB1	144.5	268.1	-	70
IFN Induction	DDX58 (RIG-1)	-3.8	-	-	-
IFN Induction	IFIH1	-	-	2.2	-
IFN Induction	IRF-7	-7.2	-4	-	-
IFN Receptor	IFNAR2	-	-	2.1	-
IFN Receptor	IFNGR1	-	-	3.3	-
IFN Receptor	IFNGR2	-	-	2.4	-
IFN Signaling	JAK2	-	3.8	2.5	-
IFN Signaling	NMI	-3	-	-	-
IFN Signaling	PIAS1	-	-	2.1	-
IFN Signaling	SOCS1	-	-	-	-
IFN Signaling	SOCS3	2.9	2.1	2.2	-
IFN Signaling	STAT1	-4	-2.2	-	-
IFN Signaling	STAT2	-2.3	-2	2.2	-
IFN Signaling	STAT5A	2.8	2.7	2.8	-
IFN Signaling	TNFAIP3	11		8.1	-
IFN induced	EIF2AK2	-12.8	-3.2	-	-
IFN induced	G1P2	-3.9	-	-	-
IFN induced	G1P3	-4.6	-2.2		-
IFN induced	IFI35	-4.7	-2.2	5.6	-
IFN induced	IFI44	-4	-	-	-
IFN induced	IFIT1	-8.4	-	2.5	-
IFN induced	IFITM1	-3.1	-	-	-
IFN induced	IL6	48.1	-	22	-
IFN induced	ISG20	-	-	2.6	-
IFN induced	ISGF3G	-4.5	-	-	-
IFN induced	MX1	-8	-3.4	-	-
IFN induced	OAS1	-5.7	-	-	-
IFN induced	OAS2	-4.3	-	-	-
IFN induced	OAS3	-4.6	-	-	-
IFN induced	OASL	-	5.3	2.4	-
IFN induced	RSAD2	2.8	4.2	2.3	-
IFN induced	ADAR	-3.1	-	-	-
IFN induced	B2M	-	-	-	-
Proteosome	PSMB9	-	-	-	-
Chemokine	CXCL10	25.1	6.8	19.8	-

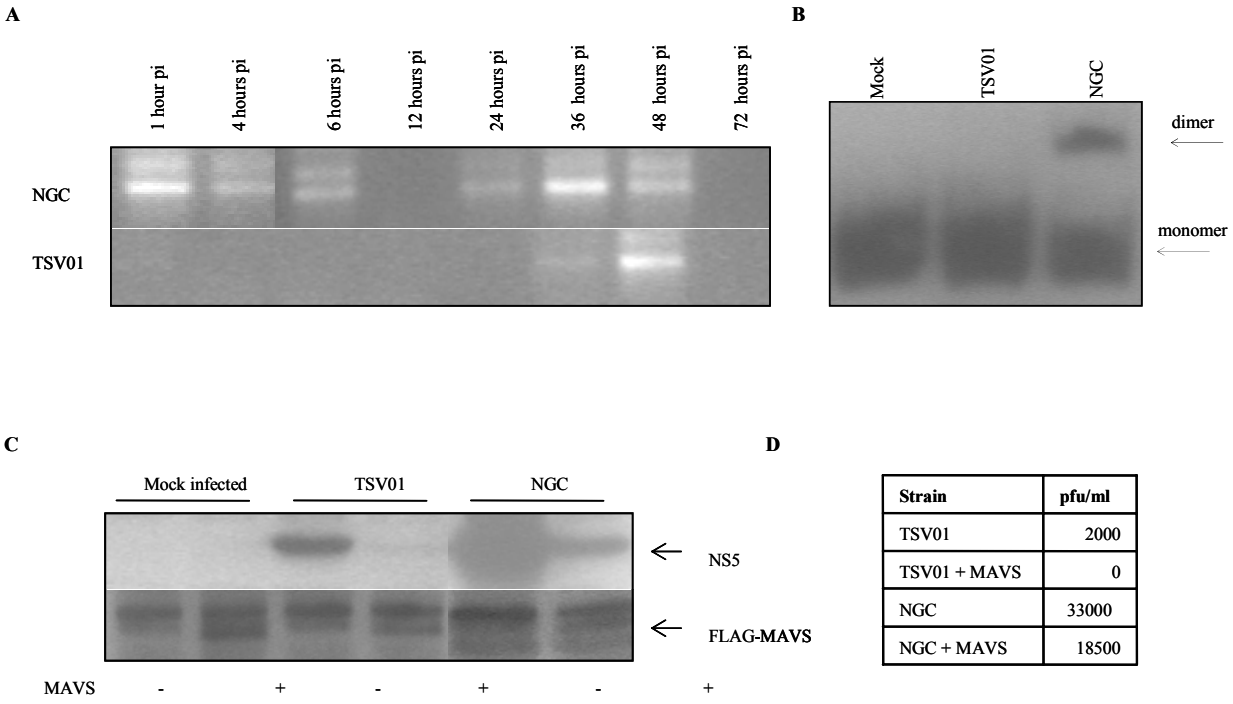
Table 8: Expression of antiviral genes upon stimulation by exogenous IFN is suppressed by NGC but not TSV01. A549 cells were infected with live and heat inactivated TSV01, SG 167, NGC, and MY 22713 strains and 18 hours post infection, treated with IFN-β. RNA was extracted 24 hours post IFN treatment and TLDA was performed. The results are presented in this table as a comparison of live virus versus heat inactivated (fold change) and negative values indicate suppression. As seen here, NGC and to a lesser extent MY 22713 inhibited the antiviral genes such as Mx, OAS (1-3), and ADAR.

3.4.6 Differential Regulation of IFN Induction by Different Strains of Dengue

These results indicated that there are at least two subsets of dengue strains: inhibiting strains that reduce type I IFN signaling exemplified by NGC and MY 22713 and non-inhibiting strains that do not reduce type I IFN signaling exemplified by TSV01 and SG 167. We speculated that the non-inhibiting strains have evolved other ways of antagonising the IFN response. The transcription factor interferon regulatory factor 3 (IRF-3) is a cytoplasmic protein which upon activation, dimerizes and accumulates in nucleus where it participates in production of several cytokines including IFN-α/β. RIG-1 and MDA-5 activate IRF-3 in response to dsRNA via another CARD domain containing anti viral protein MAVS (IPS-1). Recently it has been shown that HCV infection fails to trigger dsRNA signaling pathway in Huh-7 cells but does not inhibit Jak/STAT signaling pathway (Cheng *et al*., 2006). We therefore asked if the non-inhibiting strains of dengue could act on the dsRNA signaling pathway.

To address this, we first checked whether both these types activate IRF-3 in A549 cells to the same extent. A549 cells were infected with 5 moi each of NGC and TSV01 and the cells were collected 1, 4, 6, 12, 24, 36, and 48 hours post infection. RNA was extracted and RT PCR was done with primers specific for IRF-3. As shown in *figure 28 A*, infection with NGC induced IRF-3 almost immediately (1 hour post infection) whereas infection with TSV01 failed to induce IRF-3 almost until 36 hours after infection. NGC mediated IRF3 activation was surprisingly lost at around 12 hours of infection but reappeared at around 24 hours. We speculate that this reflects the kinetics of uncoating of the virus and might be a factor of access of the naked viral RNA to cellular helicases (RIG I) that transmit the signal to IRF3 at that time point of infection but this needs to be verified in a replication kinetics study. We next looked at activation of IRF-3 via its dimerization using a previously published assay for IRF-3 dimerization (Lin *et al*., 2006a). A549 cells were infected for 48 hours with 5 moi each of TSV01 and NGC and the lysates run on native gel. Immunoblotting with IRF-3 antibody showed that TSV01 did not induce dimerization of IRF-3 unlike NGC (*figure 28 B*). This suggests that TSV01 either inhibited or delayed the dsRNA signaling pathway that leads to IFN production. TLDA results also showed that NGC elicited a much higher IFN-β gene expression as compared to TSV01 (see *table 8*).

MAVS is a mitochondrial antiviral protein that is upstream of IRF-3 activation and it's over expression leads to enhanced IFN-α/β production (Seth *et al*., 2006, Seth *et al*., 2005). Since TSV01 did not inhibit IFN signaling, we hypothesized that over expression of MAVS would counter the infection whereas NGC infection might be resistant. A549 cells were transfected with FLAG tagged MAVS plasmid and 24 hours after transfection were infected with 5 moi each of NGC and TSV01. Cell culture supernatants were collected after twenty four hours of infection and cell lysates analyzed by western blot for the presence of MAVS and NS5 protein of the virus (*figure 28 C*). As seen in *figure 28 D*, MAVS could totally counter TSV01 infection (no plaques) whereas NGC infection was resistant to the effects of MAVS over-expression. Note that MAVS could inhibt NGC to a certain extent and this could be because at around 24 hours of infection, NGC did not totally suppress STAT-1 activation (see *figure 24 B*). Additionally, while many studies have shown that IFN can inhibit dengue infection only by treating the cells prior to infecting them, we have used IFN to successfully inhibit TSV01 infection in a post-infection setting (*supplementary figure S2*). Collectively, these results show that while the strains that inhibit STAT-1 induce IFN and are resistant to IFN production, the non-inhibiting strains delay IFN induction and are sensitive to IFN production.



Strain	pfu/ml
TSV01	2000
TSV01 + MAVS	0
NGC	33000
NGC + MAVS	18500

Figure 28: Differential activation of dsRNA signaling pathway for IFN induction by TSV01 and NGC. A) A549 cells were infected with 5 moi of TSV01 or NGC and cells were collected 1, 3, 6, 24, 36 and 48 hours post infection. RNA was extracted and RT PCR was done with IRF-3 primers. While NGC stimulated IRF-3 expression in early stages of infection, TSV01 did not. B) A549 cells were infected with 5 moi of TSV01 or NGC and cells were collected 24 hours post infection, run on a native gel and immunoblotted with IRF-3 antibody to detect its activation via dimerization. C) A549 cells were transfected with FLAG tagged MAVS and 24 hours post transfection were infected with 5 moi of either NGC or TSV01. 24 hours later, cells were harvested and immnoblotted with FLAG and NS5 antibodies to detect transfection and viral infection respectively. D) Cell culture supernatants from the above experiment were titrated for decrease in viral replication by plaque assay in BHK cells. While MAVS abolished replication of TSV01, NGC survived MAVS as seen by the pfu/ml (average of three asaays).

3.5 Discussion

This report demonstrates that dengue viral genome variations determine the type I IFN response to infection and has implications on the clinical outcome of infection. Infection with dengue virus can lead to a range of symptoms from a benign febrile illness to a life threatening DHF/DSS but pathogenesis of severe dengue has been a matter of debate for decades (Vaughn *et al*., 2000). Pre-existing heterotypic immunity is a strong risk factor for the development of DHF (Burke *et al*., 1988, Guzman *et al*., 1991, Halstead, 1970, Sangkawibha *et al*., 1984) and several mechanisms have been proposed to explain how sequential dengue infections might cause severe disease, including abnormal T cell activation and cytokine release or apoptosis (Mongkolsapaya *et al*., 2003, Rothman & Ennis, 1999). The leading hypothesis is that DHF is associated with ADE of dengue virus replication (Halstead, 1979, Kliks *et al*., 1988, Kliks *et al*., 1989). Contemporary opinion is that severe dengue is a consequence of a complex interplay of epidemiological, viral virulence and host response factors (Bravo *et al*., 1987, Kouri *et al*., 1987).

Interestingly, patient studies as early as the 1950's have indicated the presence of viral virulence factors that cause severe dengue (Bravo *et al*., 1987, Gubler *et al*., 1981, Halstead, 1970, Pandey & Igarashi, 2000, Rosen, 1977, Rosen, 1989, Sabin, 1952). Both phylogenetic (Leitmeyer *et al*., 1999, Messer *et al*., 2003, Rico-Hesse *et al*., 1997) and epidemiologic studies of dengue in the Pacific Islands (Fagbami *et al*., 1995, Gubler *et al*., 1978), Indonesia (Gubler *et al*., 1979), and the Indian subcontinent (Messer *et al*., 2002) have pointed to specific viral genotypes as being capable of producing DHF epidemics in a population base of variable immune status (Cologna *et al*., 2005). Several studies demonstrated that DEN-2 strains of the Southeast Asian genotype are more pathogenic when compared to the American genotype of DEN-2, suggesting the presence of important biological differences among viral genotypes (Kochel *et al*., 2002, Rico-Hesse *et al*., 1997, Watts *et al*., 1999).

This study provides the first molecular basis for the contribution of viral virulence factors that modulate the host response and conceivably the clinical outcome of dengue infection. We showed that two strains of DEN2- NGC and TSV01- which are very

similar to each other at the amino acid level elicit very diverse patterns of type I IFN response to infection in cell lines. We also identified low-passage clinical isolates that exhibited such strain-dependent variation in the type I IFN response to infection. Based on our results with the type I IFN signaling and TLDA experiments, there seem to be two groups of dengue virus: one that can inhibit the IFN signaling and antiviral effects (inhibiting strains) and the other that cannot (non-inhibiting strains). This strain-dependent modulation of IFN response seems to be independent of the viral serotype.

Pre-treatment but not treatment post infection of cells with IFN has been shown to inhibit dengue (Diamond *et al*., 2000). However a Cuban report (Limonta, 1984) on the use of recombinant IFN-α to treat dengue patients as well as recent studies in monkeys infected with dengue (Ajariyakhajorn *et al*., 2005) demonstrate the effectiveness of IFN in countering dengue infection. Such inconsistencies in the literature are well explained by the presence of IFN insensitive and sensitive dengue viral strains as evinced by this study. TLDA analysis confirmed that NGC and MY 22713 (that inhibit STAT-1 phosphorylation) elicit more IFN-β production as compared to TSV01 and SG 167 (that do not inhibit STAT-1 phosphorylation). Furthermore, over-expression of MAVS inhibited TSV01 but not NGC infection. These results reveal the presence of divergent sensitivities of dengue strains to type I IFN while our signaling data provides the mechanism of such sensitivity.

TSV01 exhibited a very virulent phenotype *in vivo* upon infection of IFN-α/β receptor double knock-out mice (Dr. Wouter Schul, personal communication) indicating that virulence is correlated with the ability of the virus to effectively counter the IFN signaling. Moreover, a recently published study on West Nile viral strains has correlated disease severity to their IFN signaling profiles (Keller *et al*., 2006). Although in the case of West Nile viral strains, pathogenic lineage has been clearly differentiated from non pathogenic lineage, our results advocate that this phenomenon is pan-flaviviral.

One of the strains used in our study (SG 167) isolated in Singapore from an outbreak in 2005 did not inhibit IFN type I signaling. The 2005 dengue outbreak in Singapore was from predominantly DEN1 and DEN3 serotypes and very few severe dengue cases

(19 out of 12,700 as reported by the Ministry of Health, Singapore) have been reported during that year's outbreak. On the other hand, the DEN2 prototype NGC has been shown to be neurovirulent in mice (Meiklejohn *et al.*, 1952). Therefore we speculate that strains that cause severe dengue antagonize the innate immune response whereas those that cause milder dengue do not. But precise correlation of the IFN response profiles of viral strains with their respective clinical outcomes would require patient-based studies. These studies have been initiated in collaboration with Dr. Duane Gubler and are currently ongoing.

Earlier studies that compared Southeast Asian DEN2 strains with those from South America have demonstrated mutations in the viral 3'UTR as well as prM, E, NS4B and NS5 that correlate to the differences in viral survival and replication fitness (Alvarez *et al.*, 2005, Edgil *et al.*, 2003). In our studies in A549 cells, NGC and TSV01 do not exhibit similar infectivities but when the viral load of TSV01 was increased to 10 moi and compared to 1 moi of NGC, the IFN signaling profiles of these strains remain unaltered. Moreover, MY 10340 and MY 10245 which show similar levels of infectivities differ in their ability to inhibit IFN signaling. Our results with viral load titrations clearly demonstrate that the differences in viral load alone cannot explain the differences in the IFN inhibition profiles of viral strains. This leads us to believe that a viral virulence factor might account for differential response of dengue strains to type I IFN.

NS4B of dengue is thought be the IFN antagonist but NS4B cloned from TSV01 did not antagonise the IFN response. The publication (Munoz-Jordan *et al.*, 2003) which showed that NS4B suppresses IFN signaling used NS4B of a DEN2 infectious clone pD2/IC-30P-A which is 100% identical to that of NGC and differs from TSV01 by 4 amino acids (F14L, A19T, I48V, L112F as seen in *figure 29*). It is interesting to note that three of these changes in the NS4B sequence seem to be in the region (aa 1-125) that is important for IFN antagonism (Munoz-Jordan *et al.*, 2003). While it is tempting to think that NS4B is the critical IFN antagonist, membrane topology studies of NS4B (Miller *et al.*, 2006) present a logistic challenge to this hypothesis because this region has been shown to reside in the ER lumen. Moreover, while it has been shown that the 2K fragment is essential for IFN antagonism, the existence of uncleaved 2K-NS4B in

the viral life cycle is debatable. Full genome sequencing and alignment studies are on the way with the clinical isolates. Preliminary results of such alignments imply that the secondary structure of the 3' UTR region correlates with IFN antagonism. Genetic complementation studies would of course be needed to validate these viral factors which could be used as markers for severe dengue.

```
            1                                                    50
TSV01_NS4B  NEMGFLEKTK KDFGLGSIAT QQPESNILDI DLRPASAWTL YAVATTFITP
  NGC_NS4B  NEMGFLEKTK KDLGLGSITT QQPESNILDI DLRPASAWTL YAVATTFVTP
            51                                                   100
TSV01_NS4B  MLRHSIENSS VNVSLTAIAN QATVLMGLGK GWPLSKMDIG VPLLAIGCYS
  NGC_NS4B  MLRHSIENSS VNVSLTAIAN QATVLMGLGK GWPLSKMDIG VPLLAIGCYS
            101                                                  150
TSV01_NS4B  QVNPITLTAA LLLLVAHYAI IGPGLQAKAT REAQKRAAAG IMKNPTVDGI
  NGC_NS4B  QVNPITLTAA LFLLVAHYAI IGPGLQAKAT REAQKRAAAG IMKNPTVDGI
            151                                                  200
TSV01_NS4B  TVIDLDPIPY DPKFEKQLGQ VMLLVLCVTQ VLMMRTTWAL CEALTLATGP
  NGC_NS4B  TVIDLDPIPY DPKFEKQLGQ VMLLVLCVTQ VLMMRTTWAL CEALTLATGP
            201                                                248
TSV01_NS4B  ISTLWEGNPG RFWNTTIAVS MANIFRGSYL AGAGLLFSIM KNTTNTRR
  NGC_NS4B  ISTLWEGNPG RFWNTTIAVS MANIFRGSYL AGAGLLFSIM KNTTNTRR
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Figure 29: Alignment of NS4B region of NGC versus TSV01. Note that these differ only in the four amino acids highlighted in orange.

Viral genomic analysis in parallel with patient-based studies will lead to a better understanding of the relationship between viral genomic variations and clinical out come and ultimately, the identification of a disease severity marker. Given the global emergence of dengue, the importance of being able to handle impeding dengue epidemics cannot be overemphasised. Currently, treatment for dengue is symptomatic and relies largely on patient monitoring. This requires increased health resources and cannot be used to manage disease in case of a large outbreak. There is obviously an urgent need for reliable early biomarkers that can be used as markers of disease severity. Our IFN induction and signaling studies provide an excellent handle in terms of a viral severity factor that can predict whether one particular strain can cause severe dengue or not. Simultaneously, these studies also open avenues for IFN therapy to rapidly stop the spread of IFN sensitive strains in case of out breaks. Finally, understanding of both host and viral components of the IFN regulation in infection

may provide important new strategies for detection and control of infection and eventually, creation of novel antiviral compounds.

CHAPTER 4

Role of UPR in Dengue infection

4 ROLE OF UPR IN DENGUE INFECTION

4.1 Introduction

4.1.1 Endoplasmic Reticular Stress and the Unfolded Protein Response

Eukaryotic endoplasmic reticulum (ER) consists of an extensive membranous network that provides an oxidative environment for post-translational modification, folding and oligomerization of newly synthesized secretory and transmembrane proteins. It also serves as a Ca2+ store and as the site for synthesis of sterols and lipids (Schroder & Kaufman, 2006). Several endogenous imbalances in cells, such as massive protein production, loss of calcium homeostasis, inhibition of N-linked glycosylation, and accumulation of unfolded proteins, often contribute to malfunction of the ER termed as the ER stress. It can also be induced using drugs such as tunicamycin, which inhibits N-linked glycosylation, thapsigargin, an intracellular Ca2+ pump inhibitor and dithiothreitol (DTT), a strong reducing agent which prevents the formation of disulfide bonds. The unfolded protein response (UPR) is a cellular adaptive response to alleviate ER stress (Kaufman, 1999, Zinszner *et al*., 1998). The UPR regulates adaptive responses to ER stress by sustaining a balance between folding demand and capacity of the ER. These include (1) enhancement of the folding capacity via induction of ER resident molecular chaperones and protein foldases, (2) reduction in the folding demand by up regulation of ER associated degradation (ERAD), (3) attenuation of general translation and (4) stimulation of ER synthesis to dilute the unfolded protein load.

4.1.1.1 Components of the Unfolded Protein Response

In mammalian cells, the UPR is mediated by three proximal sensors: protein kinase (PKR)-like ER resident kinase (PERK), activating transcription factor 6 (ATF6) and the ER transmembrane protein kinase/endoribonuclease (IRE1) which are regulated by the ER chaperone immunoglobulin heavy chain binding protein-BiP (*figure 30*). In non-stressed cells, the UPR sensors are bound by BiP in an inactive state whereas in cells undergoing ER stress, accumulation of misfolded proteins dissociate BiP from the UPR

sensors (Bertolotti *et al*., 2000, Shen *et al*., 2002). PERK or IRE1 homodimerize through their respective luminal domains upon dissociation from BiP of each protein. This induces auto phosphorylation and subsequent activation of these two UPR sensors. In parallel, dissociation of ATF6 from BiP leads to its translocation to the Golgi where it is cleaved to release an active transcription factor responsible for induction of many genes involved in the UPR (reviewed in (Harding *et al*., 2002, He, 2006, Schroder & Kaufman, 2006)).

1) IRE1 causes cleavage of the 28S rRNA, resulting in translational repression. IRE1 also mediates unconventional splicing of X-box binding protein 1 (XBP1) for its translation into an active transcription factor. Spliced XBP-1 activates ERAD genes such as ER degradation-enhancing α-mannosidase-like protein (EDEM) that recognize misfolded proteins for degradation. XBP-1 also activates UPR genes with an ER stress element (ERSE) (Calfon *et al*., 2002, Iwawaki *et al*., 2001, Sidrauski & Walter, 1997, Tirasophon *et al*., 1998).

2) PERK is specifically activated by ER stress and phosphorylates the α subunit of the eukaryotic translation initiation factor 2 (eIF2α). Three other kinases that are similar in the kinase domains but differ in the regulatory domains, respond to distinct stress stimuli to phosphorylate eIF2α at the identical residue serine 51: PKR, heme-regulated inhibitor of translation, and GCnonderepressing 2 (GCN2). Phosphorylation of eIF2α causes global inhibition of protein synthesis but also induction of activating transcription factor 4 (ATF4) that transactivates C/EBP homologous protein (CHOP) and Growth arrest and DNA Damage-inducible protein 34 (GADD34). CHOP facilitates cell death through apoptosis under ER stress whereas GADD34 relieves ER stress induced translation inhibition (Harding *et al*., 2000, Harding *et al*., 1999, He, 2006, Novoa *et al*., 2003, Okada *et al*., 2002).

3) ATF6 has a cytosolic N-terminal domain and an ER luminal C-terminal domain. As a result of its activation, the N-terminal domain of ATF6 is released by proteolysis, translocates to the nucleus and induces the expression of genes coding for chaperones or folding enzymes harboring an ER-stress element (ERSE) in their promoters. ATF6

also upregulates the expression of XBP1 mRNA, a substrate of IRE1 (Benjamin, 2006, Okada *et al*., 2002).

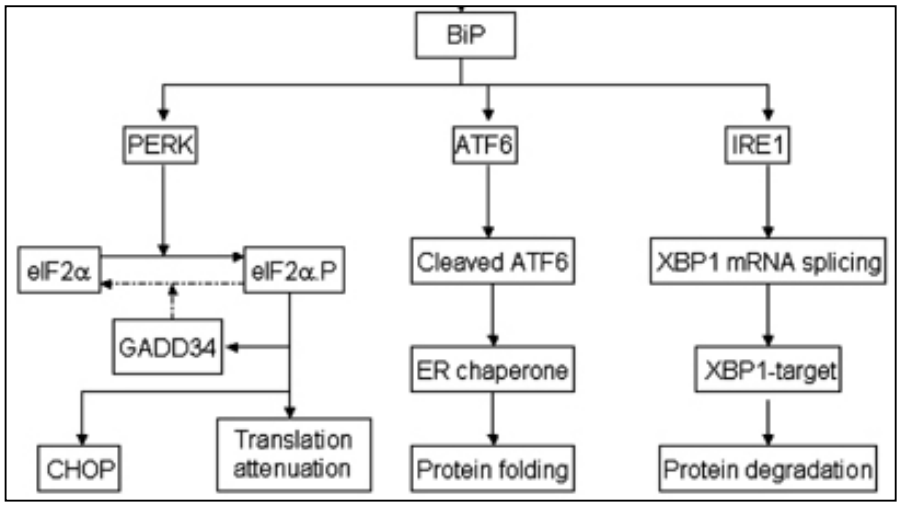


Figure 30: Mediators of UPR. Under ER stress conditions, unfolded proteins bind to the master control protein BiP, which thereby releases ER stress transducers, including PERK, ATF6, and IRE1. Thus, PERK undergoes dimerization, autophosphorylation, and subsequent activation. Activated PERK phosphorylates eIF2α, which results in attenuation of general translation and induction of GADD34 and CHOP. Release of ATF6 from BiP leads to the translocation of ATF6 to the Golgi apparatus, where ATF6 is cleaved to yield a truncated form that is capable of stimulating the expression of chaperone genes in the nucleus. Release of IRE1 from BiP permits its dimerization and activation. Activated IRE1 facilitates the splicing of XBP1 mRNA, which encodes a transcription factor leading to the expression of the UPR genes that target proteins to degradation (Adapted from (He, 2006)).

4.1.2 Modulation of the UPR by Viruses

Viruses rely on the utilization of cellular machinery and resources to complete their life cycle. Flaviviruses, for example, replicate in the cytoplasm and bud from the membranes of the ER and Golgi apparatus to release mature virion via intrinsic secretory pathways (Lindenbach & Rice, 2003). As seen in *figure 31*, flaviviruses induce convoluted membranes (CM), paracrystalline arrays (PC) and spherical smooth membrane structures (SMS) in infected cells and this proliferation and hypertrophy of the ER membranes is one of the major morphological changes in flavivirus-infected cells (Chu & Westaway, 1992, Leary & Blair, 1980, Westaway *et al*., 2003).

Moreover, the large amount of viral proteins synthesized in infected cells overwhelm the ER folding capacity and subsequently activate the UPR which in turn modulates various signaling pathways leading to cell survival or cell death.

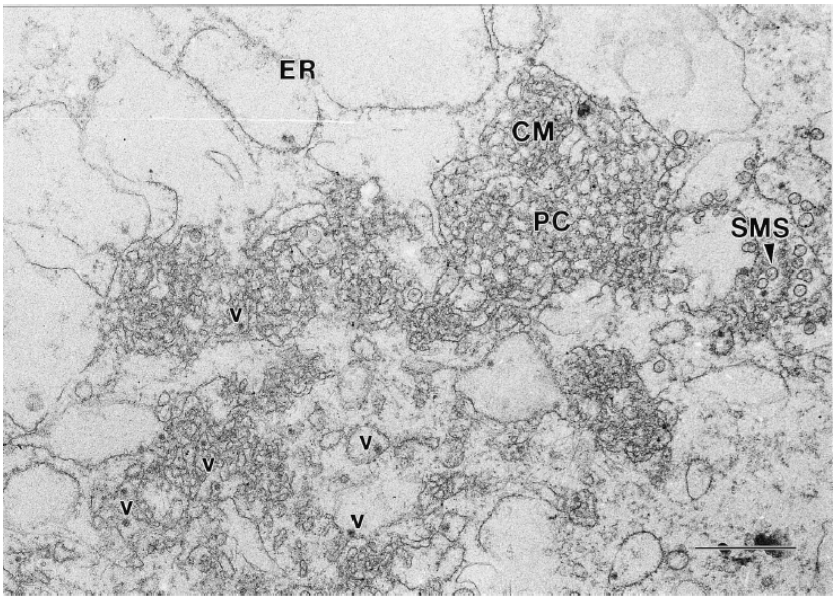


Figure 31: Vero cells infected with Kunjin virus for 24 hours show virus-induced structures including masses of CM, PC, and spherical SMS about 100 nm in diameter. Virus particles (v) and proliferating ER are in close proximity to the induced membranes. Taken from (Westaway *et al*., 1997b).

Several studies unveiled the induction of ER stress components in virus infection. For instance the master regulator of UPR- Bip is induced in cells infected with paramoxyviruses (Bitko & Barik, 2001), hanta viruses (Li *et al*., 2005b), hepaciviruses (Liberman *et al*., 1999) as well as flaviviruses such as cytopathic strains of BVDV (Jordan *et al*., 2002) and JEV (Su *et al*., 2002). Activation of PERK has been reported in infection with herpes simplex virus (Cheng *et al*., 2005), cytomegalovirus (Isler *et al*., 2005) and BVDV (Jordan *et al*., 2002). IRE1-XBP1 pathway has been recently shown to be activated in cells infected with JEV and dengue (Yu *et al*., 2006a) whereas the ATF6 pathway has been reported to be activated by HCV (Tardif *et al*., 2002). HCV infection also triggers ER stress through a mechanism involving ER calcium depletion (Benali-Furet *et al*., 2005). In addition, some viruses are hypothesized to exert their pathogenic effects by triggering CHOP mediated apoptosis (Jordan *et al*., 2002, Li *et al*., 2005b, Su *et al*., 2002).

While it is apparent that viruses activate the ER stress components, it is also becoming increasingly evident that many viruses have evolved mechanisms to cope with UPR responses (that limit viral replication) by inhibiting one or more steps in these signaling pathways. The herpes simplex encodes a GADD 34 homolog - $\gamma_1$34.5 protein which dephospholyates eIF2α and overcomes the PERK response (Cheng *et al.*, 2005, He *et al.*, 1997). African swine fever virus overcomes activation of CHOP by thapsigargin (Netherton *et al.*, 2004) whereas cytomegalovirus overcomes translation inhibition despite activation of eIF2α phosphorylation (Isler *et al.*, 2005) but the mechanisms of viral activities that achieve these are not known. XBP1 trans-activating activity, such as EDEM induction, is repressed in cells expressing HCV replicon (Tardif *et al.*, 2004). Further examples of such viral modulation of the UPR have been reviewed in (He, 2006, Schroder & Kaufman, 2006) and are summarized in *table 9*.

Activation of UPR is essential for the cell to survive the inevitable ER stress caused during viral infection but it is detrimental to viral replication but some viruses exert their pathogenic effects by UPR mediated apoptosis. It is therefore thought that a balance between viral stimulation and inhibition would determine the pathogenesis or replication of viral infection (He, 2006). It may be advantageous for viruses to modulate the UPR, inhibiting the effects that would be detrimental to the infection, while maintaining those that may be beneficial. For example, replication of hepatitis C virus has been shown to stimulate the ATF6 pathway (Tardif *et al.*, 2002), but attenuate the IRE1–XBP1 pathway (Tardif *et al.*, 2004).

Virus	Family	Genome	UPR component
African swine fever virus	*Asfarviridae*	dsDNA	BiP and PERK
Cytomegalovirus	*Herpesviridae*	dsDNA	XBP-1, ATF4, ATF6, and PKR
Herpes simples virus	*Herpesviridae*	dsDNA	PERK and PKR
Hepatitis B virus	*Hepadnaviridae*	dsDNA	BiP
Papillomavirus	*Papillomaviridae*	dsDNA	GADD34 and PKR
Vaccinia virus	*Poxiviridae*	dsDNA	PERK and PKR
Tula virus	*Bunyaviridae*	ssRNA	BiP
Bovine viral diarrhea virus	*Flaviviridae*	ssRNA	BiP
Hepatitis C virus	*Flaviviridae*	ssRNA	BiP, PERK, XBP-1, and PKR
Japanese encephalitis virus	*Flaviviridae*	ssRNA	BiP and CHOP
Influenza A virus	*Orthomyxoviridae*	ssRNA	BiP, $P58^{IPK}$, and PKR
Respiratory syncytial virus	*Paramyxoviridae*	ssRNA	BiP
Simian virus 5	*Paramyxoviridae*	ssRNA	BiP
Mouse retrovirus	*Retroviridae*	ssRNA	Bip
Vesicular stomatitis virus	*Rhabdoviridae*	ssRNA	BiP, PERK and PKR

Table 9: Viruses that activate UPR and the components that are activated are reveiwed in (He, 2006, Schroder & Kaufman, 2006) and are summarized in this table.

4.2 Aim of the Study

In recent years it is becoming clear that the ability of viruses to regulate the cellular responses to infection determine the consequence of infection. An understanding of such responses would also therefore yield critical information to control dengue infection. While the viral dsRNA intermediates trigger the IFN response to infection, unfolded or misfolded proteins in productive infection trigger the UPR response. Both arms of the host response are interconnected and are thought to act in a coordinated manner to control infection. It is also hypothesized that UPR mediated apoptosis might play a crucial role in dengue pathogenesis. UPR responses to flaviviral infections have not been extensively reported before. The aim of this study was to characterize the UPR response to dengue infection and to identify potential ways of modulating these responses such that they can affect the outcome of dengue infection.

4.3 Materials and Methods

Viruses and Cell Lines: DEN-2 (TSV01, NGC) and DEN 1 (MY 10245) were used in this study. The propagation of virus was carried out in C6/36 cells utilizing RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Gibco). Virus titers (plaque forming unit per ml, PFU/ml) were determined by a plaque-forming assay on BHK-21 cells as previously described. Viral infections for ER stress experiments were done on the A549 cell line propagated in F12 medium (Gibco).

ER Stress Treatment, Preparation of Cell Lysates and Immunoblot: Cells were grown to ~80% confluence. Thapsigargin (1-2 μM) was added for one hour or cells infected with dengue virus for the indicated period of time. Cells were then washed once in phosphate-buffered saline and lysed on ice in 150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P-40, 0.25% Na deoxycholate, 1 mM Na_3VO_4, 50 mM NaF, and Complete protease inhibitors (Roche). Protein concentration was measured using the Bradford method; normalised and equal amounts of proteins were loaded on SDS-PAGE and analyzed by immunoblot with specific antibodies.

RNA Extraction and RT PCR Analyses: Total RNA was isolated using Qiashedder/Rneasy RNA purification columns (Qiagen). Reverse transcription was done using oligodT primer (1st Base, Singapore) and PCR was done with primers indicated below. Commercially available β-actin primers were ordered from 1st Base, Singapore. PCR products were separated by electrophoresis on a 3% agarose gel and visualized by ethidium bromide staining.

XBP1 F AAACAGAGTAGCAGCTCAGACTGC
XBP1 R TCCTTCTGGGTAGACCTCTGGGAG
Gadd34 F GTGGAAGCAGTAAAAGGAGCAG
Gadd34 R CAGCAACTCCCTCTTCCTCG

Reagents: Salubrinal and Thapsigargin were from Calbiochem. Anti-phospho EIF2α and EIF2α antibodies were from Cell signaling. Anti-E monoclonal (4G2) antibody was generated in house, secondary antibody for ELISA (anti-mouse HRP) was

purchased from Santacruz and secondary antibody for immunoflorescence (anti-mouse texas red) was purchased from Jackson immunoresearch. siRNAs against RISC and IRE1 were purchased from Dharmacon whereas siRNA against RHAU (Tran *et al*., 2004) was donated by Dr. Yoshi Nagamine, Friedrich Miescher Institute, Switzerland. The GFP-ATF6 plasmid was a kind gift from Dr. Eric Chevet of Mc. Gill University, Canada.

Enzyme Linked Immunosorbent Assay (ELISA): An ELISA based technique to assess flaviviral replication was developed in house by Dr. Wouter Schul. Breifly, cells are seeded on the day before infection such that they are about 80% confluent. They are then infected with dengue virus for 48 hours, washed in PBS, and fixed for 4 minutes in cold methanol. The fixed cells are incubated with anti-E (4G2) antibody (1:100) for 1hour and anti-mouse-HRP for another one hour. After washes, Tetra methyl benzidine substrate (Sigma) is added and absorbance readings at 450 nm are used to measure virus infection.

4.4 Results

4.4.1 Dengue Infection Induces Phosphorylation of EIF2α

Phosphorylation of the EIF2α factor by stress-sensing kinases such as PKR and PERK initiates a global translational shutoff. EIF2α phosphorylation also induces ATF4 that stimulates the expression of CHOP and GADD34. We first asked if dengue infection induces phosphorylation of EIF2α. A549 cells were infected for 6, 24, 48 and 72 hours with DEN2 (TSV01) and DEN1 (MY 10245) viruses. Cells were harvested at indicated time points in m-RIPA lysis buffer and analsyed by immunoblotting with antibodies against phospho-EIF2α and EIF2α. As seen in *figure 32*, dengue infection phoshorylated EIF2α at around 24 hours post infection.

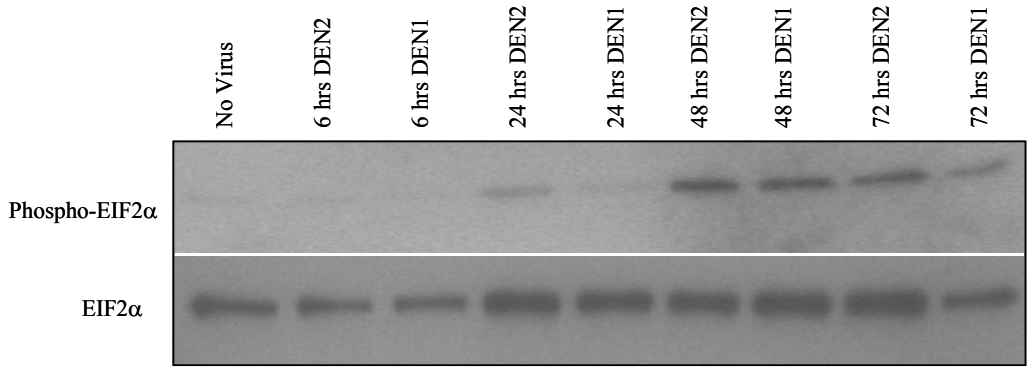


Figure 32: Dengue infection induces phosphorylation of EIF2α. A549 cells were infected with DEN2 or DEN1 viruses and lysed at indicated time points in m-RIPA buffer. Immuno-blotting with antibody that recognizes phosphorylation of EIF2α at serine 51 revealed that dengue phosphorylated EIF2α at around 24 hours post infection. The lower panel shows immuno-blotting with EIF2α antibody.

Translation is not attenuated by dengue infection (Edgil *et al*., 2006) although EIF2α is phosphorylated. This implies that a viral mediated function may act downstream of EIF2α phosphorylation. We therefore next asked if the compensatory factor GADD34 (regulatory sub unit of protein phosphatase 1) that dephospholyates EIF2α is induced

specifically by dengue infection. A549 cells were infected as before and RNA extracted for RT PCR analysis to check for expression of GADD34 mRNA (370 bp) as seen in *figure 33*. Thapsigargin, a well-recognized inducer of ER stress, served as a positive control in these tests. Dengue infection also induced the expression of GADD34 at around 24 hours post infection most likely to compensate for the induction of EIF2α phosphorylation. Interestingly, DEN1 induced more GADD34 expression and less phospholyation of EIF2α at around 24 hour time point as compared to DEN2 as seen in *figures 32* and *33*.

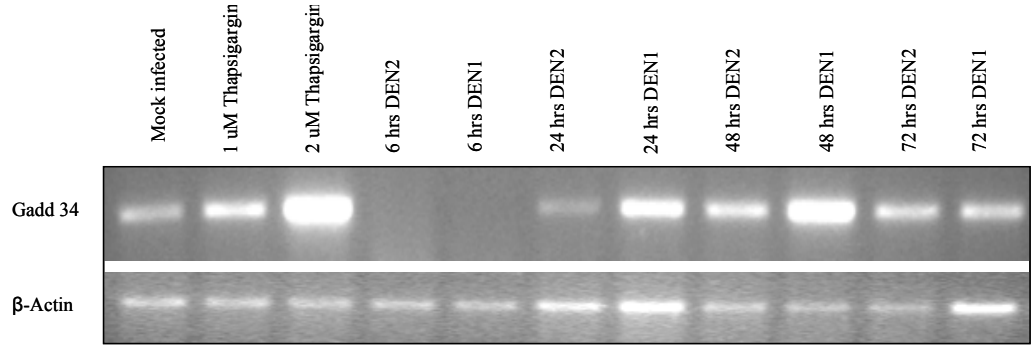


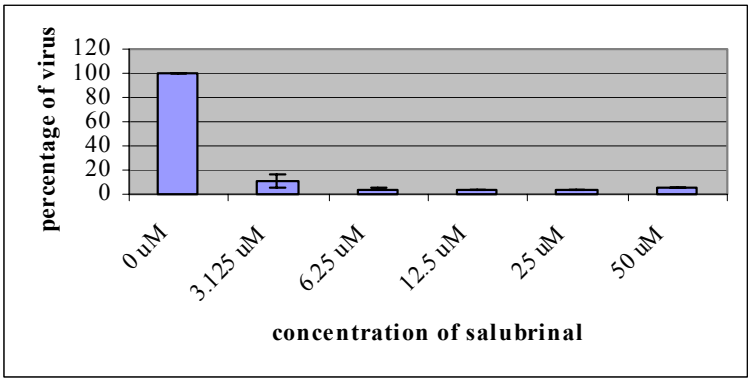
Figure 33: Dengue infection induces GADD34 mRNA expression. A549 cells were infected with DEN2 or DEN1 viruses, RNA collected at indicated time points and RT PCR was done with GADD34 specific primers. Both DEN2 and DEN1 induced GADD34 expression at around the same time as EIF2α phosphorylation. The lower panel shows RT PCR with β-actin primers as a loading control.

4.4.2 Salubrinal Inhibits Dengue Infection

Recently, salubrinal has been shown to be a non-toxic, selective inhibitor of the protein complex (containing the protein phosphatase 1 and its cofactor GADD34) that dephosphorylates eIF2α. Moreover, it has been shown to inhibit replication of herpes simplex virus (HSV) by blocking virus mediated eIF2α dephosphorylation (Boyce *et al*., 2005). Since dengue virus also seems to activate the eIF2α dephosphorylation event by inducing GADD34 expression, we hypothesized that salubrinal might inhibit dengue infection.

A549 cells were pre-treated with salubrinal for one hour and infected with medium containing salubrinal and DEN2 virus (10 moi) for 48 hours. Plaque assays were used to quantify viral replication. The number of plaque forming units (pfu) in cells not treated with virus was assumed as 100% and those in cells treated with indicated amounts of salubrinal are represented as percentage of the former in *figure 34 A*. As expected, salubrinal inhibited dengue virus even at very low concentrations indicating that modulation of the UPR impacts infection. We verified these results in a different assay format. For this, A549 cells were infected with 10 moi of DEN2 and the indicated concentrations of salubrinal were added one hour post infection to the cells. Two days later, the cells were fixed in cold methanol and an ELISA with anti-E antibody was done to quantify viral replication. The results are represented in a bar graph (*figure 34 B*) as percentage of virus in cells treated with indicated concentrations of salubrinal as compared to those treated with no compound (100%). Interestingly, post infection treatment of A549 cells with salubrinal exhibited a dose-dependent inhibition pattern as seen in *figure 34 B*. Since salubrinal is not toxic to cells at the concentrations used in our assay (Boyce *et al.*, 2005), we speculate that pre-treatment of salubrinal can prime the cells to counter infection more effectively than treatment post-infection. But in both cases, it is clear that salubrinal countered dengue infection. Further characterization of salubrinal mediated inhibition of dengue replication in terms of EC50, moi, viral strains and cell lines is ongoing.

A)



B)

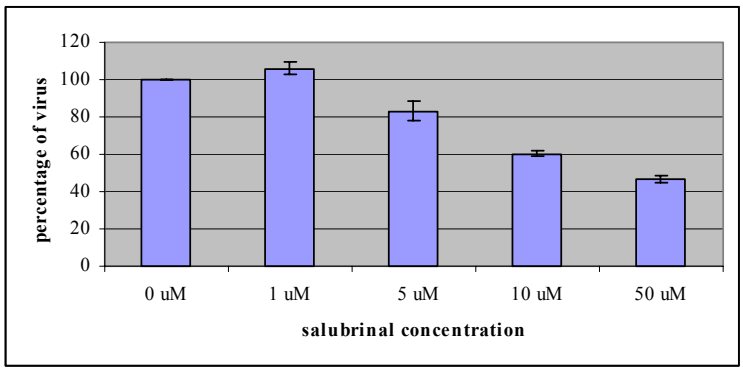


Figure 34: Salubrinal inhibits dengue replication. A) A549 cells were pre-treated for one hour with indicated concentrations of salubrinal and infected with DEN2. Supernatants were collected 48 hours post infection and pfu/ml determined by plaque assays and viral replication in the presence of salubrinal is represented as a percentage of that in cells without salubrinal. B) A549 cells were infected with DEN2 and treated one hour post infection with indicated concentrations of salubrinal for 48 hours. Cells were fixed in cold methanol and an ELISA was performed with anti E-antibody culture to score for viral replication. The results are plotted in a bar graph as percentage of virus in cells treated with indicated concentrations of salubrinal as compared to untreated cells (100%). Note that pre-treatment of cells with salubrinal almost completely abolished dengue replication but treatment after infection showed a dose-dependent inhibitory action of salubrinal.

4.4.3 Dengue Infection Activates the XBP1 Pathway

During ER stress IRE1 processes XBP1 mRNA (473 bp) by an unconventional splicing that results in the removal of a 26-nucleotide intron and a translational frame shift. The spliced form of XBP1 is translated into a transcription factor and activates proteins of the degradation pathway (EDEM) that target misfolded or unfolded proteins whereas the unspliced form results in an inactive protein. To assess whether dengue infection triggers the IRE1-XBP1 pathway, we analyzed XBP1 mRNA splicing in DEN2 and DEN1 infected A549 cells in which an RNA virus has been shown to induce ER stress (Bitko & Barik, 2001). The cDNA of XBP1 was amplified by RT-PCR and the products analyzed on a 3% agarose gel as previously described (Iwakoshi *et al.*, 2003, Shang & Lehrman, 2004). Infection with DEN2 and DEN1, but not mock infection, caused XBP1 splicing which peaked around 48 hours post infection as seen in *figure 35*. Thapsigargin was used as a positive control in these tests. It was also

noticed that cells treated infected with DEN2 seemed to express higher levels of XBP1 than did mock-infected cells or DEN1 infected cells.

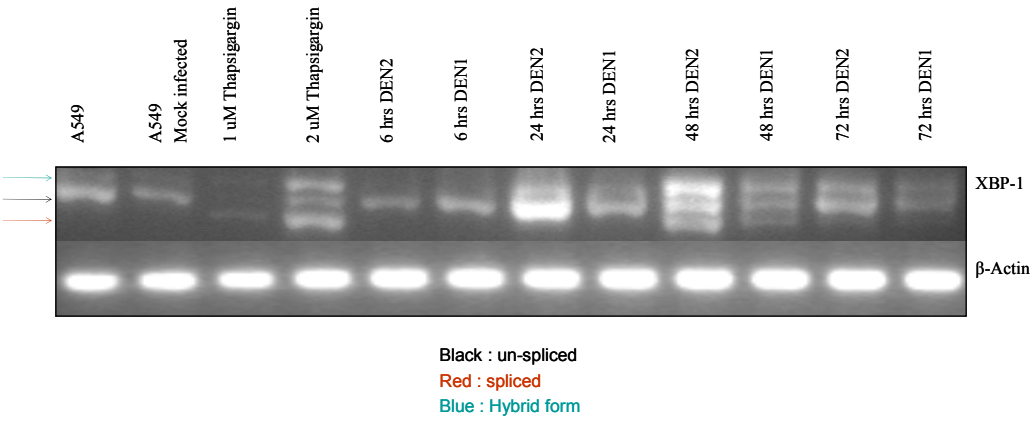


Figure 35: XBP1 is spliced in dengue infected A549 cells. A549 cells were infected with DEN2 and DEN1 and RNA was extracted at indicated time points for RT PCR analysis with XBP1 primers. The PCR products were run on a 3% agarose gel to separate the spliced (red arrow), unspliced (black arrow) and the hybrid (blue arrow) forms. Thapsigargin was used as a positive control for induction of XBP1 splicing. Unspliced form of XBP1 was present in mock infected cells as well as at early (6 hours) time point of infection. After 24 hours of infection, the spliced form as well a hybrid of the spliced and unspliced mRNAs as described in (Shang & Lehrman, 2004) appeared and the peak of XBP-1 splicing seemed to be at around 48 hours of infection. Note that DEN2 seemed to increase the level of mRNA as compared to mock infection as well as infection with DEN1. The panel below shows RT PCR products of β-actin as loading control.

XBP1 splicing is a result of oligomerisation and activation of IRE1 and it is clear from our studies that XBP1 splicing is induced by dengue replication. We hypothezied that manipulating this event might modulate the outcome of infection. A549 cells were transfected with siRNA directed against IRE1 and 6 hours later were infected with DEN2 (1 moi). siRNA directed against a human RNA helicase RHAU (Tran *et al.*, 2004) and the RISC complex were used as controls. Twenty four hours after infection, the amount of virus was quantified by ELISA based assay described earlier. As seen in *figure 36*, there was an increase in the amount of virus produced in cells treated with siRNA against IRE1 (approximately 45% more virus) as opposed to untreated cells (taken as 100% virus). This suggests that the IRE1-XBP1 pathway of the UPR plays a role in control of viral replication. However, these experiments will need further

verification of the specificity of the siRNA to IRE1 and detailed characterisation is ongoing.

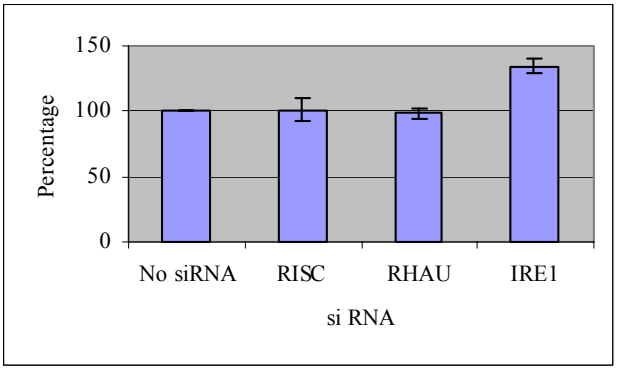


Figure 36: Enhanced dengue replication in cells treated with siRNA against IRE1. A549 cells were transfected with siRNA against IRE1 and infected with DEN2. si RNA againt human RHAU and RISC proteins were used as controls. Dengue replication was scored by an ELISA with anti-E antibody and represented as a percentage of viral replication in cells with no siRNA transfection compared to those with indicated siRNAs.

4.4.3 Dengue Infection Activates the ATF6 Pathway

It is known that XBP1 is produced after ATF6 activation (Lee *et al.*, 2002, Yoshida *et al.*, 2001). We therefore checked if the ATF6 pathway might be also activated by dengue infection. In response to ER stress, ATF6 migrates from ER to the Golgi, where it is processed to its active form that translocates to the nucleus. We assessed the presence of this nuclear form of ATF6 as a marker of its activation during infection with dengue. GFP tagged ATF6 was transfected into A549 cells and infected with 10 moi of DEN2 (NGC) 24 hours after transfection. One day poat infection, the cells were fixed in cold methanol and immuno-florescence was done with anti-E protein antibodies. As seen in *figure 37*, ATF6 was cytoplasmic in mock infected cells whereas a large part of it translocated to the nucleus in DEN2 infected cells suggesting that dengue virus activated the ATF6 pathway of the UPR. Studies have been initiated to understand the effects of viral strain, moi and cell types on the ATF6 pathway and modulation of this pathway using mutant forms of ATF6 is also underway.

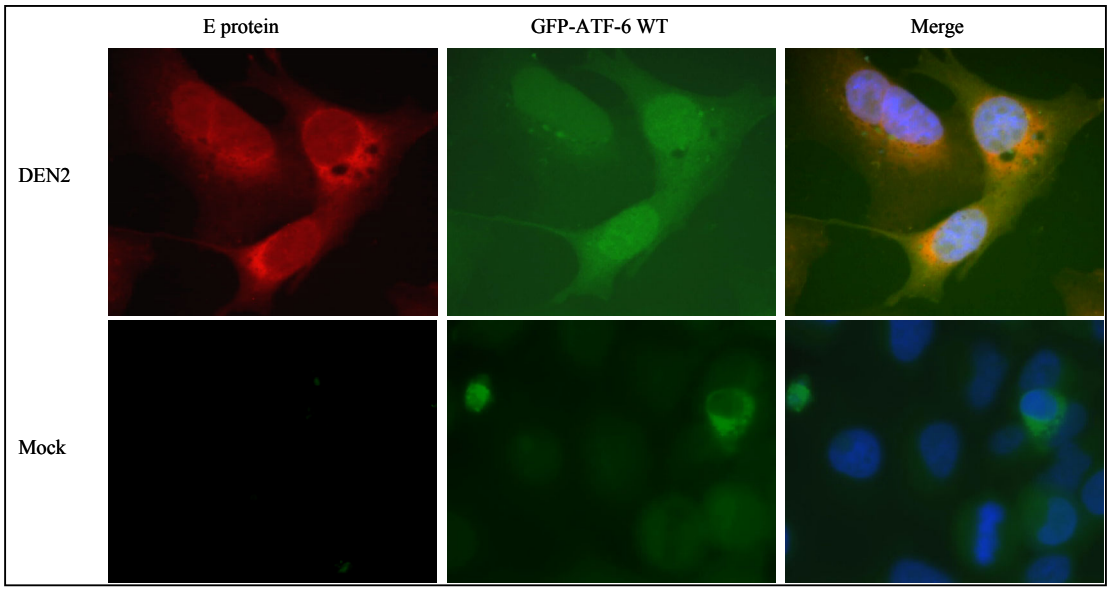


Figure 37: Dengue infection activates the ATF6 pathway. A549 cells were transfected with GFP tagged ATF6 plasmid and infected with DEN2 virus. Twenty four hours post infection, mock infected and DEN2 infected cells were fixed in cold methanol and stained for virus infection using an antibody directed against E protein. ATF6 was largely cytoplasmic in mock infected A549 cells (lower panel) whereas it translocated to the nucleus in cells infected with DEN2 (top panel) indicating that dengue infection activated the ATF6 pathway.

4.5 Discussion

Many positive-strand RNA viruses need to modify intracellular membranes of their host cells in order to create a compartment suitable for virus replication (Carette *et al.*, 2000, Schlegel *et al.*, 1996, van der Meer *et al.*, 1999). Although this phenomenon has been well documented, little is known about how viruses induce intracellular membrane proliferation but it is increasingly becoming clear that viruses like other ER stress signals induce membrane proliferation and other UPR components. Replication and maturation of the flaviviruses occur in close association with the host endoplasmic reticulum and flaviviruses have also been shown to induce membrane rearrangements in course of productive infection (Chambers *et al.*, 1990a, Westaway *et al.*, 1997b). Moreover, it has been shown that JEV (Su *et al.*, 2002), BVDV (Jordan *et al.*, 2002) and HCV infections (Tardif *et al.*, 2002) induce ER stress and consequent UPR. We initiated this study to characterize the UPR response to dengue infection. The activation of PERK, as revealed by a mobility shift assay for PERK phosphorylation, has been used as an early marker for ER stress (Harding *et al.*, 1999). Employing a similar approach, we tried to determine the activation status of PERK in dengue-infected A549 cells by using an anti-PERK antibody (Cell Signaling). Nonetheless, even for positive control using the ER stress inducers thapsigargin and DTT, we failed to see any PERK signals and this could be because the endogenous level of PERK in A549 cells was too low to be detected. Thus, the potential role of PERK activation in the dengue induced UPR is unclear from our present study. However, in our micro array analyses described earlier, different strains of DEN2 induced the expression of PERK and PKR to a different extent. Moreover, we could detect phospholyation of PKR by DEN2 (*supplementary figure S1*) and dengue virus induced the phosphorylation of EIF2α in A549 cells. It is therefore possible that both PKR and PERK kinases might separately phosphorylate EIF2α in response to dengue infection.

Despite this phosphorylation event, translation is not attenuated in dengue virus infected cells. We consequently suspected that dengue virus might activate a compensatory factor. The $\gamma_1$34.5 protein of herpes simplex virus is highly homologous to GADD34 and has been shown to alleviate translational arrest in cells treated with thapsigargin and DTT (Cheng *et al.*, 2005). RT PCR analyses of GADD34 levels

showed that dengue induces the expression of GADD34 at around the same time as the EIF2α phosphorylation event. This leads us to believe that dengue virus compensates the EIF2α phosphorylation event by enhancing the expression of the protein phosphatase 1 (whose regulatory subunit is GADD34) that dephosphorylates EIF2α and overcomes the block in translation. To corroborate our hypothesis, we analyzed the antiviral effect of salubrinal on dengue. Salubrinal was recently discovered as a small molecule inhibitor of the protein complex (containing the protein phosphatase 1 and its cofactor GADD34) that dephosphorylates EIF2α and it has been shown to inhibit the replication of HSV (Boyce *et al*., 2005). Salubrinal dramatically reduced dengue infection both in pre- and post-infection settings and in different assay formats. We therefore conclude that up regulation of GADD34 by the dengue virus is critical for its replication and speculate that this event might compensate for the virus-induced phosphorylation of EIF2α. Ongoing studies aim to identify the viral factor responsible for the up regulation of GADD34 as well as the mechanism of action. Studies on the mode of action of salubrinal and its efficacy on various viral strains are also on the way.

Dengue infection also triggered the IRE1-XBP1 pathway of the UPR by activation of the XBP-1 splicing event. During the course of this study, another group published similar results with JEV and DEN2 (Yu *et al*., 2006a). Furthermore, they also found that the XBP1 downstream genes such as EDEM1 and p58 (IPK) were induced in dengue infected cells. Interestingly, transactivation of XBP1 target genes are suppressed in HCV (Tardif *et al*., 2004). The discrepancy of XBP1 induction between these viruses might reflect the differences in infection patterns of these viruses; while HCV usually causes chronic infection, JEV and dengue cause acute infection. It is noteworthy that knock down of XBP1 had no effect on viral production suggesting that XBP1 splicing is beneficial but not essential for virus production. But increased cytopathic effects were noticed in XBP1 knock out cells in response to dengue infection indicating that XBP1 alleviates ER stress induced by dengue infection (Yu *et al*., 2006a). Additionally, knock down of IRE1 increased the replication of dengue virus in this study. These knock down studies point out that a fine balance between activation and inhibition of the UPR response is crucial for productive infection and it is tempting to speculate that dengue virus modulates the IRE1 arm of the UPR. The ATF6 pathway also seems to be

activated by dengue virus infection. In our studies XBP1 mRNA is elevated in DEN2 infection as opposed to that of DEN1 and ATF6 has been shown to up regulate the expression of XBP1(Yoshida *et al*., 2001). It would therefore be exciting to study the mechanism of activation of ATF6 especially in terms of viral serotypes and strains and its effect on virus infection.

Our study is one of the first to report activation of UPR by dengue virus and has focused mostly on understanding the initial events in dengue infection mediated UPR owing to time constraints. One of the critical questions that remain to be addressed is how does dengue infection activate ER stress and UPR. In dengue infected cells, three viral proteins are glycosylated and accumulated in the ER lumen, namely, the precursor of membrane protein (prM), the envelope protein (E), and the nonstructural protein NS1 and accumulation of these in the ER may contribute to UPR induction. These and several nonstructural proteins of dengue (NS2A, NS2B, 2K-NS4B and NS2B-NS3) have been shown to induce XBP-1 splicing but none of them to the extent that whole virus is capable of (Yu *et al*., 2006a). Some of the flaviviral non structural proteins are hypothesized to be viroporins (Chang *et al*., 1999) and may cause homeostasis imbalance of calcium and other ions in the ER, thereby triggering the UPR. Moreover, during virus maturation, virions budding out from the ER appear to consume the constituents of phospholipid and sterol of the ER membrane, which may not only activate the UPR but also induce ER proliferation (Su *et al*., 2002).

Initiation of the UPR is critical for cell survival and consequently for viral replication. However prolonged/excessive UPR can lead to cell death. Therefore differential regulation of ER stress by viruses would dictate viral pathogenesis versus replication. Although the pathogenesis of dengue related disease remains poorly understood, virus-induced cell death may be a crucial pathogenic event. It has been suggested that apoptosis is an innate defence mechanism, which allows the organism to control virus infection by elimination of infected cells through phagocytosis (Despres *et al*., 1996). However, several viruses have been shown to induce apoptosis, which can be detrimental to the host (Carrasco *et al*., 1996, Geisbert *et al*., 2000, Koga *et al*., 1994, Lewis *et al*., 1996). Apoptotic cell death has been implicated as a cytopathological mechanism in response to dengue infection both *in vitro* and *in vivo* (Carrasco *et al*.,

1996, Despres *et al*., 1996, Despres *et al*., 1998, Matsuda *et al*., 2005). These observations suggest that virus-induced apoptosis may contribute to the pathogenesis of dengue.

While the molecular pathways by which viruses induce apoptosis are not well understood, it is thought that apoptosis may be initiated in response to viral proteins or cellular signals and regulated by cellular proteins such as bcl-2, p53, myc, and c-fos. Several viruses also induce apoptosis mediated by ER stress. Infection of JEV exhibits severe cytopathic effects caused by CHOP and P38 MAPK mediated apoptosis. Tula virus infection activates the JNK pathway while BVDV activates caspase 12 to initiate apoptosis (Jordan *et al*., 2002, Li *et al*., 2005b, Su *et al*., 2002). It is therefore conceivable that UPR responses to dengue infection might play an important role in dengue pathogenesis. Further patient-based studies with various strains of dengue would be needed to confirm the role of virus mediated UPR in dengue pathogenesis. Our study provides a good starting point for evaluation of the UPR responses to infection and should pave way for a better understanding of the viral regulation of these processes. Finally, modulators of UPR such as salubrinal that inhibit dengue replication open up an avenue toward cell-protective agents that target the endoplasmic reticulum for anti-viral therapy.

CHAPTER 5
Conclusions and Outlook

5 CONCLUSIONS AND OUTLOOK

The aim of this work was to understand viral and host components that modulate the replication process of dengue virus with a view that such comprehension is crucial for elucidation of potential avenues of antiviral therapy. The small hydrophobic non structural protein of NS4B is one of the least known of all the non structural proteins of the *Flaviviridae* family and the hydrophobicity profiles and membrane topologies of this protein are conserved across the family. Emerging biological functions of NS4B, such as its role in cytopathogenicity (Qu *et al*., 2001) and its interactions with other replicase components (Khromykh *et al*., 2000, Koch, 1999, Lin *et al*., 1997, Piccininni *et al*., 2002) indicate that NS4B may be an important scaffold for macromolecular assemblies that affect cell biology as well as viral RNA replication. Moreover, NS4B has domains on both the cytoplasmic and luminal faces of the ER membrane suggesting that it could be an ideal candidate for mediating and coordinating interactions within and between these two compartments. Mutational studies on dengue NS4B have indicated its role in maintaining the balance between efficient replication in the mosquito vector and the human host (Hanley *et al*., 2003). Recent findings of Munoz *et al* have proposed that flaviviral NS4B plays an important role in antagonising the IFN response to infection (Munoz-Jordan *et al*., 2005). HCV NS4B has been shown to induce the UPR response (Zheng *et al*., 2005) and also to target cellular proteins such as P21/Waf1, RNase L and p53 to degradation (Florese *et al*., 2002, Kato *et al*., 2002). These findings prompted us to define the function of NS4B in dengue life cycle. We set out to also map the previously uncharacterized type I IFN response as well as the UPR response to dengue infection in the same context.

In *Chapter 2* of this thesis, we characterized an interaction between NS4B and the C-terminal part of NS3. NS4B decreased the ssRNA binding activity of the viral helicase and mutations in NS4B that affect this ability lead to altered replication efficiency of the virus. As NS4B did not affect the ATPase function of NS3, it is unlikely that it enhances the helicase activity by altering the rate of progression of the helicase on dsRNA. Our results point towards a scenario wherein NS4B interacts with NS3 and dissociates it from ssRNA rendering the helicase molecule susceptible to rebind dsRNA, thereby increasing its unwinding activity. It is also possible that NS4B renders

directionality to the helicase. Additionally, given that NS4B is an integral membrane protein with several transmembrane domains, it might also interact with and sequester the viral replicase complex to the intracellular membranes by its interaction with the NS3 protein. Interestingly, no interaction was observed between NS4BN, NS4BC or NS4BM with NS3 pointing to the critical contribution of protein conformation in the interactions within the replication complex. This thesis provides one of the first lines of evidence of the direct role of NS4B in flaviviral replication. Further analyses would be required to understand the relevance of these findings *in vivo* but our study provides an insight into the workings of the coordinated processes that are important for viral replication. Moreover, it is hoped that knowledge of activity of modulators of viral enzymatic activities will help accelerate ongoing research programs aimed at developing compounds with specific antiviral activity.

We also tried to validate Jordan *et al*'s observation that flaviviral NS4B antagonises the type I IFN response to infection (Munoz-Jordan *et al*., 2005, Munoz-Jordan *et al*., 2003) but NS4B cloned from a different strain of the same serotype used in their report (DEN2) failed to antagonise the IFN response. A serendipitous discovery of strain dependent variation of type I IFN response to dengue virus opened up the possibility of existence of potential viral "virulence factors" that modulate the host response and conceivably the clinical outcome of dengue infection. Severity in dengue has been a hotly debated issue and several factors (viral, host, epidemiological) have been shown to be associated with the onset of haemorrhagic complications in course of infection (Bravo *et al*., 1987, Kouri *et al*., 1987). Although there is epidemiological evidence that certain strains of viruses cause more severe forms of infection than others, not much ground has been covered with regards to the mechanisms involved. *Chapter 3* of this thesis provides the first molecular basis for the existence of viral virulence factors that modulate the host response and conceivably the clinical outcome of dengue infection. We provide evidence for the presence of two lineages of dengue virus (1) a putative "pathogenic" lineage typified by the strain NGC. These viruses can inhibit the IFN signaling, it's consequent antiviral effects and are insensitive to IFN treatment, (2) a putative "non-pathogenic" lineage typified by TSV01 that is incapable of countering the IFN signaling, it's consequent antiviral effects and is sensitive to exogenous IFN. While this work was ongoing another group has shown that pathogenesis of different

strains of the West Nile virus is a function of the ability of that strain to counter IFN response (Keller *et al*., 2006).

Comprehensive genome sequencing coupled with accurate clinical information would be required to validate our hypothesis and to initiate the search for dengue viral virulence factors. NGC and TSV01 differ in their NS4B sequences by four amino acids and our studies show that TSV01's NS4B did not antagonize the IFN response. It is therefore tempting to assume that NS4B might be the viral virulence factor that we are after. However, membrane topology studies of NS4B (Miller *et al*., 2006) present a logistic challenge to the idea that it can directly antagonise the IFN response. The region of NS4B (aa 54-102) responsible for IFN antagonism resides in the lumen of the ER making a direct interaction between NS4B and cellular cytoplasmic components involved in IFN signaling unlikely. Likewise, while it has been shown that the 2K fragment is essential for IFN antagonism, the existence of uncleaved 2K-NS4B in the viral life cycle is debatable. Moreover, in preliminary sequence comparisons, we could not identify any amino acid that was different between NS4B of TSV01 (IFN sensitive) and NGC (IFN insensitive) and similar between that SG167 and TSV01 (both IFN sensitive). Interestingly, the secondary structures of the 3' UTR regions of these viral strains correlate with their IFN sensitivities. These results of course, have to be corroborated with sequence comparisons of other strains and validated by genetic complementation studies. Our work will prove to be a useful handle for identification of viral factors which could be used as early biomarkers for severe dengue. Simultaneously, these studies also open avenues for IFN therapy to rapidly stop the spread of IFN sensitive strains in case of out breaks.

While flaviviral NS4B has been shown to antagonise the IFN response (Munoz-Jordan *et al*., 2005), HCV NS4B has been shown to induce ER stress (Zheng *et al*., 2005). Viral dsRNA intermediates stimulate the IFN response whereas unfolded or misfolded proteins during infection impose endoplasmic reticular stress and consequent unfolded protein response. Both these reponses are cordinated and do converge at various levels. Infact, the two major mediators of the IFN-induced arm of the innate immune response are evolutionarily related to IRE1 and PERK of the UPR pathways. The kinase/endoribonuclease domain of IRE1 is homologous to RNaseL, and the protein

kinase domain of PERK is related to the PKR. All of these components are required to limit viral protein synthesis and pathogenesis. While the IFN response to viral infection has been extensively studied, little is known about the role of UPR in viral infections. The recent discovery of a small molecule inhibitor of ER stress (salubrinal) that showed antiviral activity has caused much excitement in the field of antiviral research (Boyce *et al*., 2005). Recently, ER stress has been shown to be induced by the three glycoproteins (prM, E, NS1) as well as several non structural proteins of dengue (NS2A, NS2B, 2K-NS4B and NS2B-NS3) but none of the individual factors were capable of initiating the UPR to the the extent that whole virus is capable of (Yu *et al*., 2006a). Some of the flaviviral non structural proteins are hypothesized to cause homeostasis imbalance of calcium ions in the ER (Chang *et al*., 1999) and virions budding out from the ER consume phospholipid and sterol of the ER membrane (Su *et al*., 2002).These results suggests that NS4B might not be the sole factor that induces and interacts with UPR. But its precise role in the process could not be defined in this thesis owing to lack of time.

Instead, in *Chapter 4* of this thesis, we sought to first identify the UPR pathways that are activated by dengue infection with a view to modulate the outcome of infection by interfering with one or more of the UPR components. Dengue infection induced phosphorylation of EIF2α but also up regulated the expression of GADD34 to overcome the block in translation. Moreover an inhibitor of dephosphorylation of EIF2α, salubrinal, inhibited dengue infection. Ongoing studies aim to identify the viral factor responsible for the up regulation of GADD34 as well as the mechanism of action. Studies on the mode of action of salubrinal and its efficacy on various viral strains are also underway. Dengue infection also triggered the ATF6 and the XBP1 pathway of the UPR and knocking down the components of the IRE1-XBP1 pathway resulted in enhanced cytopathic effects and virus production (this study and (Yu *et al*., 2006a)). Our study provides a good starting point for evaluation of the UPR responses to dengue infection and presents a proof of concept on the use of UPR modulators (salubrinal) as potential inhibitors of flaviviral infections. Future work should focus on identification of viral factors that interact with UPR pathways and exploration of other possibilities to manipulate them and influence the outcome of infection. It will also be interesting to understand the role of UPR induced apoptosis on dengue pathogenesis.

In conclusion, the data generated in this thesis have (a) articulated a role for NS4B in dengue replication (b) provided evidence and mechanism for the contribution of viral factors in pathogenesis of dengue and (c) defined the UPR responses that can be manipulated to manage the outcome of dengue infection. Given the global re-emergence of dengue, it is hoped that knowledge gained in this thesis will expedite the quest for an anti-dengue drug.

Supplementary Material

SUPPLEMENTARY MATERIAL

1) Micro Array Analyses of IFN Regulated Genes in Dengue Infection

One of the first questions raised by the IFN antagonist studies by Munoz Jordon *et al* (Munoz-Jordan et al., 2003) is- what IFN specific responses are suppressed by dengue infection? To address this question, we designed a micro array experiment to check for IFN related responses that are suppressed by dengue infection. We hypothesized that a comparison of A549 cells treated with IFN for a specific time point (24 hours) with that of dengue infected A549 cells treated for the same time with IFN would give us a list of IFN induced genes that are suppressed specifically by the virus. We included three time points of infection in this experiment to also obtain a picture of the kinetics of suppression. As we were unaware at that point of the strain-dependent variation in IFN responses, we performed this experiment with the TSV01 strain of dengue virus (which later has been shown to not suppress IFN responses). A549 cells were infected for 30, 48 and 72 hours with TSV01. In all cases, the infected cells were treated with IFN-α (1000U/ml) such that the IFN treatment lasted for 24 hours. For example, IFN was added 6 hours post infection and samples harvested 24 hours later so that the infection proceeded for 30 hours in this sample. Human arrays of 19,800 60mer oligonucleotide probes (representing 18861genes), designed by compugen and manufactured by Sigma-Genosys were used according to the standard protocol described for cDNA microarray (Eisen & Brown, 1999). For every sample at all time points dye swap was performed and a rigorous quality check was done before an array was used for down stream analysis and three biological replicates were used. Following Lowess normalization, differentially expressed genes were selected using (Statistical Analysis of Microarray) SAM. These experiments were done in collaboration with Dr. Martin Hibberd, GIS, Singapore and the analyses were done with help from Dr. Samiuel Hassan, NITD.

Mock infected samples were used as controls and comparison of gene expression levels of A549 cells treated with IFN for 24 hours with those of TSV01 infected A549 cells (also treated with IFN for 24 hours) are presented in *supplementary table ST1*. Some of the IFN responsive genes are highlighted in red to show that TSV01 did not induce

their suppression. This experiment proved to be not useful to answer the question of specific IFN related host responses that are suppressed by dengue infection but it led us to the serendipitous discovery of the role of genomic variations in infectivity. These results were later verified by TLDA analysis described in *Chapter 3*. Note that there was another set of analyses done with heat inactivated viruses as controls but the data is not shown here as the statistical significance was not adequate.

Gene ID	A549+ IFN	A549+ IFN		
		30 hrs infection	48 hrs infection	72 hrs infection
Interferon-induced protein 44 (IFI44)	32.367		27.56692	56.37097
Bone marrow stromal cell antigen 2 (BST2)	28.58958		29.66145	44.02365
Tripartite motif-containing 22 (TRIM22)	23.17956		51.43656	160.0803
B-factor, properdin (BF)	22.62248	4.959204	37.56104	34.91047
N-myc (and STAT) interactor (NMI)	18.32128		5.609375	4.625665
Interferon-induced protein 35 (IFI35)	14.08816		7.713341	7.764795
HLA complex P5 (HCP5)	13.97708		17.52011	26.3914
Melanoma differentiation associated protein-5 (MDA5)	13.44643		23.2992	21.6894
Major histocompatibility complex, class I, A (HLA-A)	12.98645		3.436448	4.607595
Ubiquitin-conjugating enzyme E2L 6 (UBE2L6)	12.55013		8.138683	10.49368
Complement component 1, r subcomponent (C1R)	12.22434		16.87713	9.04107
2'-5'-oligoadenylate synthetase-like (OASL)	11.64117	13.38038	98.9789	87.35375
Ubiquitin-activating enzyme E1-like (UBE1L)	10.93212		17.38148	47.20448
Interferon regulatory factor 7 (IRF7)	10.61341		7.337693	7.263215
Serum amyloid A2 (SAA2)	9.765306		4.517958	166.9187
Dual specificity phosphatase 16 (DUSP16)	9.664557		4.078268	2.851386
Solute carrier family 15, member 3 (SLC15A3)	9.002373		11.90939	52.47368
Interferon stimulated gene 20kDa (ISG20)	8.67352		14.48466	10.95874
2'-5'-oligoadenylate synthetase 3, 100kDa (OAS3)	8.455976		4.44467	6.386691
TNF receptor shedding aminopeptidase regulator (ARTS-1)	8.360417			2.313993
Solute carrier family 25, member 12 (SLC25A12)	8.126972		3.574156	3.939435
Major histocompatibility complex, class I, F (HLA-F)	7.802043		10.38614	15.72263
Beta-carotene 15,15'-monooxygenase 1 (BCMO1)	7.784196		2.132092	2.532351
Guanylate binding protein 1, interferon-inducible, 67kDa (GBP1)	7.60426		45.9	17.98833
G protein-coupled receptor 3 (GPR3)	7.461679		4.648551	4.476378
Myxovirus resistance 2 (mouse) (MX2)	6.905095		66.17082	212.036
Cyclin-E binding protein 1 (CEB1)	6.805046		13.47624	14.26348
Normal mucosa of esophagus specific 1 (NMES1)	6.80283		33.40705	92.86172
Chemokine (C-X3-C motif) ligand 1 (CX3CL1)	6.59			18.1
Vitronectin (VTN)	6.487793			2.416653
Apolipoprotein L, 1 (APOL1)	6.131074		9.41428	11.8096
Solute carrier family 1, member 3 (SLC1A3)	6.076172			10.23762
Lectin, galactoside-binding(LGALS3BP)	5.957362		4.312179	6.877048
Caspase 4, apoptosis-related cysteine protease (CASP4),	5.76511		3.377369	3.439828
Inositol polyphosphate-4-phosphatase, type I, (INPP4A)	5.757692		5.191358	
Pleckstrin homology domain, family A member 4 (PLEKHA4)	5.720149		113.0146	50.75634
Interferon induced transmembrane protein 3 (IFITM3)	5.680995		5.594312	8.735653
Viperin (cig5)	5.677749		12.54804	36.94169

Proteasome subunit, beta type, 8 (PSMB8).	5.446127		5.533729	5.32822
2'-5'-oligoadenylate synthetase	5.243915		13.15304	23.42607
Periaxin (PRX)	5.1875			0.112613
Baculoviral IAP repeat-containing 3 (BIRC3)	5.097124		10.53898	9.601965
Transporter 1, ATP-binding cassette, sub-family B (TAP1)	5.081857	2.658112	7.618455	8.929933
Serine proteinase inhibitor, clade A, member 3 (SERPINA3)	5.00974		2.538395	
Interleukin 18 receptor 1 (IL18R1)	4.96743		5	
Similar to Taxol resistant associated protein 3 (TRAG-3)	4.62			5.694737
CD38 antigen (p45) (CD38)	4.45141		6.810615	6.264421
ortholog of mouse D11lgp2 (LGP2)	4.369619		47.17931	68.61268
Proteasome subunit, beta type, 9 (PSMB9)	4.310735		9.095462	13.3341
Similar to ATP-binding cassette transporter ABCA3	4.291825		10.49057	
Cathepsin S (CTSS)	4.123377		7.215741	6.332386
Myosin, light polypeptide kinase (MYLK)	4.085			0.19906
Signal transducer and activator of transcription 1b, (STAT1b)	4.078105		2.911302	3.272565
Cholecystokinin (CCK)	3.987805			13.95082
Claudin 15 (CLDN15)	3.962316		4.80625	
Ceruloplasmin (ferroxidase) (CP)	3.915162		6.08	
Lipoma HMGIC fusion partner (LHFP)	3.909977		2.223317	
EH-domain containing 3 (EHD3)	3.723861		2.769231	
Spinocerebellar ataxia 1 (SCA1)	3.683761			0.283164
FXYD domain containing ion transport regulator 4 (FXYD4)	3.6375			3.644068
Midkine (neurite growth-promoting factor 2) (MDK)	3.537049		5.487685	5.247688
Guanylate binding protein 4 (GBP4)	3.353902		32.66553	34.76603
Interferon, gamma-inducible protein 16 (IFI16)	3.343145		12.48837	17.53715
2',5'-oligoadenylate synthetase 1, (OAS1) E18	3.292936		8.074548	12.74829
p8 protein (candidate of metastasis 1) (P8)	3.11747			0.114173
Serum amyloid A1 (SAA1)	3.075503		19.79012	599.2966
Similar to Proteasome activator complex subunit 2 (REG-beta)	2.93597		2.130026	2.601868
Mps One Binder kinase activator-like 2C (MOBKL2C)	2.899625		2.851195	2.341675
Zinc finger CCCH type domain containing 1 (ZC3HDC1)	2.879841		4.983006	7.624932
Beta-2-microglobulin (B2M)	2.789957		2.954607	3.267308
Leucine aminopeptidase 3 (LAP3)	2.673776		3.763862	5.48851
Major histocompatibility complex, class I, C (HLA-C)	2.530394		3.344535	5.342176
Signal transducer and activator of transcription 2, (STAT2)	2.371089		4.78405	4.920911
TAP binding protein related (TAPBP-R)	2.365968		4.669228	5.248919
Mitogen-activated protein kinase kinase kinase 8 (MAP3K8)	2.309016		4.729913	4.558082
Decay accelerating factor for complement (DAF)	2.293075		3.13043	3.075775
TAP binding protein (TAPBP)	2.27191		2.263718	2.152466
Betacellulin (BTC)	2.265653		5	5.762825
SP110 nuclear body protein (SP110)	2.152703		4.917881	6.82428
Serine proteinase inhibitor, clade B, member 8 (SERPINB8)	2.134282		3.802843	3.866445
Complement component 1, s subcomponent (C1S)	2.118801		3.490009	4.806921
Zinc finger protein 288 (ZNF288)	2.038013			2.128842
Tripartite motif-containing 38 (TRIM38)	1.972118		3.456036	4.428769
Tudor repeat associator with PCTAIRE 2 (PCTAIRE2BP)	1.935618		3.478775	4.631448
Complement component 3 (C3)	1.920258		4.756546	8.838114
Peroxisomal proliferator-activated receptor A (PRIC285)	1.904333		8.131592	11.01938
Fibrinogen, B beta polypeptide (FGB)	1.738322			0.241893
Endothelial cell growth factor 1 (ECGF1)	1.68942		3.89891	5.150656
Amphiregulin (AREG)	1.650922		2.171867	
Signal transducer and activator of transcription 1 (STAT1)	1.550502		2.150939	2.124762

PHD finger protein 11 (PHF11)	1.40919		2.450301	3.169474
Sulfide quinone reductase-like (SQRDL)	0.852036		2.313972	2.403518
Nuclear factor of polypeptideenhancer in B-cells (RELB)	0.826732		3.878263	3.523491
Solute carrier family 11 member 2 (SLC11A2)	0.758297			2.239956
Carbonyl reductase 3 (CBR3)	0.75353			2.253757
Carrier family 25, member 28 (SLC25A28)	0.720498			2.213826
Tissue inhibitor of metalloproteinase 4 (TIMP4)	0.709377			0.265552
Phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1)	0.69078		7.275114	4.139661
Tumor necrosis factor related protein 1 (C1QTNF1)	0.677199		3.704447	8.529382
Interferon regulatory factor 1 (IRF1)	0.647949		9.872116	6.711095
Four jointed box 1 (FJX1)	0.640785			2.565646
Intercellular adhesion molecule 5, telencephalin (ICAM5)	0.639946		2.145169	
SERTA domain containing 1 (SERTAD1)	0.632758		2.243452	3.602799
Tumor necrosis factor, alpha-induced protein 2 (TNFAIP2)	0.62732		2.232275	3.021138
Heterogeneous nuclear ribonucleoprotein D-like (HNRPDL)	0.623908		2.407529	
AXIN1 up-regulated 1 (AXUD1)	0.621794		2.626556	
Chromogranin A (parathyroid secretory protein 1) (CHGA)	0.61561		2.558928	2.080425
Collagen, type V, alpha 3 (COL5A3)	0.612529		2.015962	
Internexin neuronal intermediate filament protein, alpha (INA)	0.606638			2.165876
Adrenergic, beta-2-, receptor, surface (ADRB2)	0.604182			2.509434
Protein phosphatase 1, subunit 15A (PPP1R15A)	0.581588		9.857963	3.283209
EH-domain containing 1 (EHD1)	0.573628			2.409249
zinc finger protein 342 (ZNF342)	0.570889			2.53671
nuclear factor of gene enhancer in B-cells (NFKB1)	0.569672		2.039097	
Claudin 1 (CLDN1)	0.567353		2.303239	
DNA-damage-inducible transcript 3 (DDIT3)	0.567065	2.491809	4.147693	
Ribosomal protein S6 kinase (RPS6KC1)	0.55611			2.022211
Glycogenin 2 (GYG2)	0.551709			0.274894
Protease inhibitor 3, skin-derived (PI3)	0.548362		3.464824	12.35261
Solute carrier family 29 member 4 (SLC29A4)	0.539597			0.29291
FBJ murine osteosarcoma viral oncogene homolog B (FOSB)	0.524457		5.596356	3.408666
Angiopoietin-like 4 (ANGPTL4)	0.511181		3.236642	2.889234
v-jun sarcoma virus 17 oncogene homolog (JUN)	0.509877		5.398213	3.721532
Solute carrier organic anion transporter (SLCO2B1)	0.496751			3.617371
Opioid growth factor receptor (OGFR)	0.492931		2.097799	3.260111
Distal-less homeo box 2 (DLX2)	0.491722			3.621762
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4)	0.481577		2.069577	
Mitochondrial solute carrier protein (MSCP)	0.479215			2.165196
Serine/threonine kinase 19 (STK19)	0.470027		2.540541	4.730808
Ras homolog enriched in brain like 1 (RHEBL1)	0.444854		3.357975	3.194012
Septin 1 (SEPT1)	0.439324			0.266403
Zinc finger protein 92 (ZNF92)	0.414232			4.35
Dedicator of cytokinesis 2 (DOCK2)	0.386402			0.291638
PR domain containing 1, with ZNF domain (PRDM1)	0.365758		6.478109	
Annexin A9 (ANXA9)	0.357544		0.13538	0.103197
Zinc finger CCCH type, antiviral 1 (ZC3HAV1)	0.349161		2.284749	3.790421
Cartilage oligomeric matrix protein (COMP)	0.342663			5.197183
Sarcolipin (SLN)	0.334752		0.264873	0.210916
Amiloride binding protein 1 (ABP1)	0.327354		0.171129	
Ring finger protein 128 (RNF128)	0.319386			0.146694
Calcyphosine (CAPS)	0.285247			0.294719
Interleukin 29 (IL29)	0.275689		181.1785	211.0125

Chemokine (C-X-C motif) ligand 10 (CXCL10)	0.216774		32.0467	34.61739
Interferon, alpha-inducible protein 27 (IFI27)	483.4844		33.93065	50.36268
Interferon induced transmembrane protein 1 (9-27) (IFITM1)	102.0304		58.65844	87.53438
Lysosomal-associated membrane protein 3 (LAMP3)	66.12188		78.21212	129.6784
Interferon, alpha-inducible protein (G1P3)	49.10828		4.781011	5.172082
Interferon, alpha-inducible protein (G1P2)	48.46421		11.32955	13.65111
Major histocompatibility complex, class I, B (HLA-B)	39.3198		24.20198	71.81928
Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1)	38.048		34.10837	34.8625
B aggressive lymphoma gene (BAL)	28.19907		7.377417	5.940895
Interferon induced transmembrane protein 2 (IFITM2)	26.99598		5.564	4.037925

Table ST1: Gene expression analysis of TSV01 infected cells treated with IFN compared to uninfected cells treated with IFN. Some of the IFN response genes are highlighted in red to show that TSV01 did not induce suppression of the IFN pathway genes. See text for experimental details.

2) Dengue Infection Induces Activation of PKR

Phosphorylation of the EIF2α factor by stress-sensing kinases such as PKR and PERK initiates a global translational shutoff. EIF2α phosphorylation also induces ATF4 that stimulates the expression of CHOP and GADD34. As dengue virus phosphorylated EIF2α, we looked at activation of PKR. A549 cells were infected for 1, 3, 6 and 24 hours with DEN2 (TSV01) and harvested at indicated time points in m-RIPA lysis buffer and analyzed by immunoblotting with antibodies against Phospho- PKR and PKR. As seen in *figure S1*, dengue infection phosphorylated PKR at around 24 hours post infection.

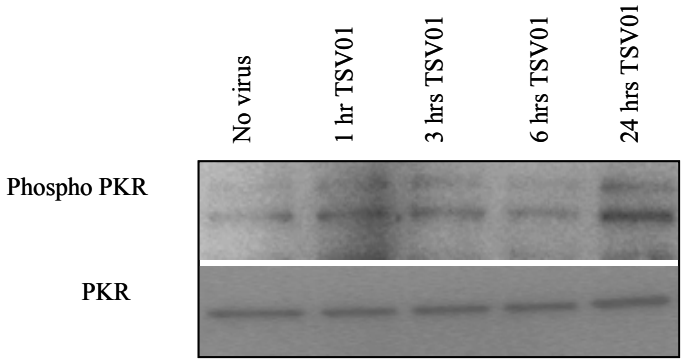


Figure S1: Dengue infection induces phosphorylation of PKR. A549 cells were infected with DEN2 (TSV01) viruses and lysed at indicated time points in m-RIPA buffer. Immuno-blotting with antibody that recognizes phosphorylation of PKR reveals that dengue phosphorylated PKR at around 24 hours post infection. The lower panel shows immuno-blotting with PKR.

3) IFN Inhibts TSV01 in A Post Infection Setting

Type I IFN has been used in invitro studies to counter dengue infection and most studies have reported that pre-treatment but not treatment post infection of cells with IFN can inhibit dengue. We tested this with TSV01 infection. A549 cells were treated either pre- or post- infection with 1000 U/ml of IFNβ as indicated in *figure S2* and infected with 1 moi of TSV01. ELISA was performed one day later and the results are plotted in *figure S2*. Suprisingly, infection with TSV01 could be inhibited by adding IFN-β to A549 cells upto 24 hours after infection suggesting that TSV01 is sensitive to IFN.

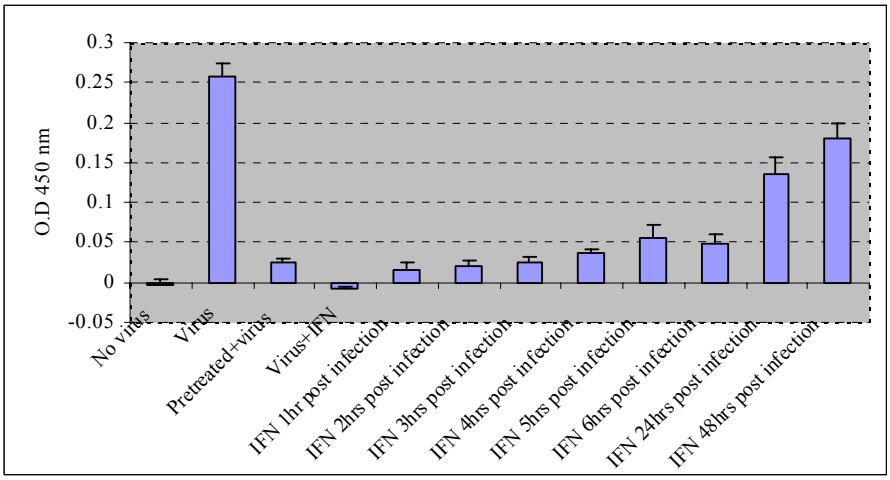


Figure S2: TSV01 infection can be inhibted by IFN-β. A549 cells were treated with IFN-β either pre- or post infection with 1 moi of TSV01. 24 hours later, ELISA was done on these cells as described elsewhere to score for viral replication and the raw results are presented here. Note that TSV01 infection can be countered by addition of exogenous IFN even in later stages of infection.

4) Standard Nomenclature of Dengue Viral Strains

To avoid prevalent confusion in nomenclature of dengue viral stains, a new system has been proposed (Dr. Mark Schrieber, manuscript submitted). The standard nomenclature of the viral stains used in this thesis is represented in *table ST2*.

Strain Name	Standard Nomenclature
NGC	D2/PG/NGC_NITD/1944
TSV01	D2/AU/TSV01_NITD/1993
MY 10340	D2/MY/10340/1997
MY 22563	D2/MY/22563/2000
MY 10245	D1/MY/10245/1997
MY 2569	D1/MY/2569/1995
MY 22713	D4/MY/22713/2001
SG 167	D1/SG/D1SG05ED167/2005

Table ST2: Standard nomenclature of the dengue viral strains used in this study

ABBREVIATIONS

AAF	IFN-α-activated factor
AD	activation domain
ADAR	adenosine deaminase, rna-specific
Ade	adenine
ADE	antibody dependent enhancement
ATF	activating transcription factor 6
ATP	adenosine triphosphate
ATTC	American type culture collection
B2M	beta 2 microglobin
BVDV	bovine viral diarrhea virus
C	capsid
CHOP	C/EBP homologous protein
CM	convoluted membranes
CMV	cytomegalovirus
CXCL10	chemokine, cxc motif, ligand 10
DC-SIGN	dendritic cell-specific ICAM- grabbing non-integrin
DNA-BD	DNA-binding domain
dsRNA	double stranded RNA
DTT	dithiothreitol DENV dengue virus
E	envelope
ECL	enhanced chemiluminiscence
EDEM	ER degradation-enhancing α-mannosidase-like protein
EDTA	ethylenediamine tetraacetic acid
EIF2α	eukaryotic initiation factor 2α
EIF2AK2	EIF2α kinase 2
ELISA	Enzyme linked immunosorbent assay
ER	endoplasmic reticular
ERAD	ER associated degradation
ERSE	ER stress element
FBS	foetal bovine serum
G1P	G protein coupled receptor
GADD34	Growth arrest and DNA damage-inducible protein 34
GAS	IFN-γ-activated site
GCN2	GCnonderepressing 2
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
GST	glutathione S-transferase
HCV	hepatitis C virus
His	histidine
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRP	horse radish peroxidase
HSV	herpes simplex virus
IP-10	interferon-gamma-inducible protein 10
IFIH	interferon induced with helicase c domain protein
IFIT	interferon-induced protein with tetratricopeptide repeats
IFITM	IFN-inducible transmembrane protein
IFN	interferon
IFNAR	interferon, alpha, beta, and omega, receptor
IFNGR	interferon, gamma, receptor
IFNB1	interferon beta 1
IKK	inhibitor of kappa light polypeptide gene enhancer in b cells
IL	interleukin
IMP	inosine monophosphate
IPTG	isopropyl-β-D-thiogalactopyranoside
IRE 1	inositol-requiring 1
IRES	internal ribosome entry site

IRF	interferon regulatory factor
ISG	interferon stimulated gene
ISGF3	IFN-stimulated gene factor 3
Jak	Janus protein tyrosine kinase
JEV	Japanese encephalitis virus
kb	kilobases
kDa	kilodaltons
KUN	kunjin virus
Leu	leucine
LPS	lipo polysaccharide
M	membrane
MAVS	mitochondrial antiviral signaling protein
MMP	Matrix metallo proteases
moi	multiplicity of infection
NFkB	nuclear factor of kappa light chain gene enhancer in b cells
NLS	nuclear localization sequences
NMI	nmyc interactor
NP-40	nonidet P 40
NTP	nucleotide triphosphate
NTPase	nucleotide triphosphatase
OAS	2'5'-oligoadenylate synthetase
OMP	orotidyleate monophosphate
PAGE	polyacrylaamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PERK	PKR-like ER resident kinase
PFU	plaque forming unit
PIAS	protein inhibitor of activated stat1
PKR	protein kinase R
polyIC	poly (inosinic acid) - poly (cytidylic acid)
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9
pTMDs	putative transmembrane domains
RdRp	RNA dependent RNA polymerase
RF	replicative form
RI	replicative intermediate
RIG-1	Retinoid-inducible gene 1
RIPA	radioimmunoprecipitation buffer
RISC	RNA induced silencing complex
RSAD2	radical S-adenosyl methionine domain containing 2
RT	reverse transcriptase
RTPase	RNA triphosphatase
SAM S	adenosyl-methionine
SDS	sodium dodecyl sulphate
ssRNA	single standed
SMS	smooth membrane structures
SOCS	suppressors of cytokine signaling
STAT	signal transducer and activator of transcription
TBK1	tank-binding kinase 1
TLDA	taqman low lensity array
TLR	toll-like receptor
TNF	tumour necrosis factor
TNFAIP3	tumor necrosis factor-alpha-induced protein 3
Trp	tryptophan
Tyk	tyrosine kinase
UAR	upstream AUG region
UPR	unfolded protein response
UTR	untranslated regions
VP	vesicle packets
XBP1	X-box binding protein 1

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