Virology 417 (2011) 179–187

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Use of a tandem affinity purification assay to detect interactions between West Nile and dengue viral proteins and proteins of the mosquito vector

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ARTICLE INFO

Article history:
Received 4 April 2011
Accepted 2 June 2011
Available online 23 June 2011

Keywords:
Dengue
West Nile
Mosquito
Arbovirus
Flavivirus
TAP assay

ABSTRACT

West Nile and dengue viruses are (re)emerging mosquito-borne flaviviruses that cause significant morbidity and mortality in man. The identification of mosquito proteins that associate with flaviviruses may provide novel targets to inhibit infection of the vector or block transmission to humans. Here, a tandem affinity purification (TAP) assay was used to identify 18 mosquito proteins that interact with dengue and West Nile capsid, envelope, NS2A or NS2B proteins. We further analyzed the interaction of mosquito cadherin with dengue and West Nile virus envelope protein using co-immunoprecipitation and immunofluorescence. Blocking the function of select mosquito factors, including actin, myosin, PI3-kinase and myosin light chain kinase, reduced both dengue and West Nile virus infection in mosquito cells. We show that the TAP method may be used in insect cells to accurately identify flaviviral–host protein interactions. Our data also provides several targets for interrupting flavivirus infection in mosquito vectors.

Introduction

Dengue and West Nile viruses are members of the flavivirus family, which also includes yellow fever, Japanese encephalitis and tick-borne encephalitis viruses. Flaviviruses are enveloped, single-stranded RNA viruses that cause significant human illness and mortality. Dengue is among the most important human infectious diseases globally. There are an estimated 100 million cases per year, with over 500,000 cases of potentially fatal dengue hemorrhagic fever (Gubler, 2002; site, 2007). West Nile virus can cause serious illness in man, resulting in encephalitis and death, and is soon expected to be endemic in most of the United States and South America (Mackenzie et al., 2004; Rappole et al., 2000). There is no specific treatment for either West Nile or dengue virus, and efforts to create an effective dengue vaccine have been hindered due to safety concerns and potential antibody-dependent enhancement (Gubler, 2002). Arboviruses exist in nature in cycles between an arthropod vector and a vertebrate host. West Nile virus primarily persists in mosquitoes and birds, with humans acting as "dead-end" hosts who do not generally transmit virus (Mackenzie et al., 2004). The cycle of dengue virus is unusual in that humans are the principal vertebrate hosts, therefore the virus can infect mosquitoes as they bite an infected human and the transmission may go on to a new human host (Blair et al., 2000; Mackenzie et al., 2004).

The flavivirus particle contains 3 structural proteins, the capsid (C), envelope (E) and membrane (M) proteins. The structural proteins are the only viral proteins found in viral particles, and the E protein is thought to be the only protein exposed on the surface of the virion, embedded in the lipid bilayer surrounding the capsid, membrane and genetic material, and involved in virus entry into the cell (Kuhn et al., 2002). The virion contains a positive-sense, single stranded RNA genome that is translated into a single polyprotein. This polyprotein is cotranslationally cleaved by host cell and viral proteases to produce the individual viral proteins: the 3 structural proteins, C, E and M, and seven non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Rice, 1996). The E protein is responsible for binding the host cell receptor to allow virus entry into the cell as well as fusion with the host cell membrane to release the viral genetic material (Brinton, 2002; Chen et al., 1996). C is the only viral protein found within the lipid bilayer of the mature virion and is known to complex with the viral RNA to form the nucleocapsid (Mukhopadhyay et al., 2005). It has recently been shown that the flavivirus C protein can trigger cellular innate immune signaling mechanisms during infection as well as in the absence of virus (Chen et al., 2009). The NS proteins perform various functions within mammalian cells during infection. NS1 aids in replication and also has a secreted form that may function in the extracellular fluid. NS5 is the RNA-dependent RNA polymerase and NS3 has protease activity and may also be involved in virus assembly. The NS3 protein forms a complex with NS2B as a cofactor and contains a serine-protease activity domain

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doi:10.1016/j.virol.2011.06.002

at its N-terminus that is responsible for proteolytic processing of the viral polyprotein (Rice, 1996). The functions of NS2A, NS2B, NS4A and NS4B proteins remained largely unknown until recent experiments revealed anti-immune activity, including the inhibition of interferon production, activity and signaling. NS2A inhibits interferon production and signaling and NS4A to bind Jak-STAT and interferes with signaling (Munoz-Jordan et al., 2003; Rice, 1996). A structural analysis of flaviviral NS4B has suggested functions in ER-stress response, altered gene expression and replication efficacy (Welsch et al., 2007). Since viruses contain a very limited number of proteins, it is likely that each viral protein interacts with several cellular proteins to enhance the environment for infection and decrease the ability of the cell to inhibit virus infection and production.

In the past decade, there has been an expansion in research focused on changes in mosquito gene expression due to pathogen infection. Much of this research has focused on the responses of *Anopheles* mosquitoes to malaria infection and recent reports illustrate that several host factors are required, and beneficial, for the malarial life cycle in mosquitoes (Ghosh et al., 2001; Xu et al., 2005). There have also been reports on Aedes gene expression during flaviviral infection with an emphasis on innate immune genes (Sim and Dimopoulos, 2010; Souza-Neto et al., 2009; Xi et al., 2008). Fluorescent differential display has also been used to identify genes in the midgut of *Culex* mosquitoes with altered expression upon infection with West Nile virus (Smartt et al., 2009). Since these studies focus on the levels of mosquito gene transcripts, they may miss host factors that are important for infection but do not have altered transcription levels. In addition, these assays do not provide any information regarding the protein interactions that occur during pathogen gene expression in the mosquito. Uncovering these interactions is crucial to developing a complete picture of the relationship of infectious agents with their transmission vectors.

Several methods commonly used to identify protein interactions include the yeast two-hybrid method, cotranslocation and co-immunoprecipitation (Honda et al., 2007; Hui et al., 2006). Though relatively successful, these methods cannot provide a comprehensive list of interacting proteins in live cells. In addition, none of these methods can be easily adapted for use with mosquito cells due to the lack of readily available arthropod reagents and antibodies. The tandem affinity purification (TAP) assay is an accurate and reliable way to explore protein interactions occurring naturally in live cells. The TAP method enables the purification of interacting protein complexes under near-to-physiological conditions and has been successfully used to identify a number of mammalian cellular proteins that bind viral proteins, including the influenza RNA polymerase, human papillomavirus E1 helicase and the Epstein–Barr virus nuclear antigen 5 (Cote-Martin et al., 2008; Forsman et al., 2008; Jorba et al., 2008). We applied the TAP assay, followed by mass spectrometry, to characterize mosquito components that bind to West Nile and dengue viral proteins. We chose to express NS2A and NS4B nonstructural proteins to search for mosquito proteins that may be involved in the immune response to viral infection, as they are known to interact with components of the mammalian immune system during infection (Liu et al., 2004; Munoz-Jordan et al., 2005). In addition, we chose to look for mosquito binding partners of E and C, as they are the dominant proteins found in the mature flavivirion. We show that the TAP method can be used to identify viral–host protein interactions in insect cells. Identification of insect factors important for arboviral infection may lead to novel ways to break the human–vector transmission cycle.

Results

Expression of tagged flaviviral proteins in mosquito cells

A tandem affinity purification (TAP) assay along with mass spectrometry was used to identify mosquito proteins that interact with flaviviral proteins. First, the activity of the nTAP plasmid promoter in mosquito cells and level of expression of the tagged viral proteins were assessed to confirm that the TAP assay would function in these insect cells. The green fluorescent protein (GFP) gene was amplified using the polymerase chain reaction (PCR) and cloned into the nTAP vector. This new plasmid, nTAP–GFP, was transfected into C6/36 mosquito cells and showed a high level of GFP expression by immunoblot and immunofluorescence (Fig. 1). The genes for 2 structural (envelope and capsid) and 2 nonstructural proteins (NS2A, NS4B) from both West Nile and dengue viruses were cloned into the nTAP vector as fusion proteins with the streptavidin-binding protein (SBP) and calmodulin-binding protein (CBP) tags (Fig. 2A). These plasmids were transfected into C6/36 cells and expression levels were examined at 48 h. C6/36 cells are derived from the *Aedes albopictus* mosquito, which is a secondary vector for dengue infection worldwide, and these cells are highly susceptible to both dengue and West Nile virus infection in the laboratory. Immunoblots of the cell lysates, using an antibody against the CBP tag, revealed bands of the appropriate size for all 8 viral proteins (Fig. 2B). Immunofluorescence analysis confirmed that all of the proteins were expressed in transfected mosquito cells. The West Nile and dengue virus NS2A, NS4B and envelope proteins localized to the cytoplasm, and the capsid proteins were detected in the nucleus (Fig. 2C), consistent with reported data regarding flaviviral protein localization (Bulich and Aaskov, 1992; Mackenzie et al., 1998; Miller et al., 2006; Senigl et al., 2004). These data suggest that tagged flaviviral fusion proteins may be used in mosquito cells to identify interacting arthropod proteins.

Identification of mosquito proteins that interact with West Nile and dengue virus NS2A, NS4B, capsid and envelope proteins

The nTAP-viral gene plasmids (Fig. 2A) were transfected into C6/36 cells and the TAP assay performed on cell lysates to isolate tagged viral proteins along with interacting mosquito proteins. The nTAP–GFP plasmid and the empty nTAP plasmid served as controls. The final solution eluted from the calmodulin resin was subjected to LC/MS–MS for peptide sequencing and identification. The tags and viral proteins were correctly detected in the appropriate cell lysates and any proteins

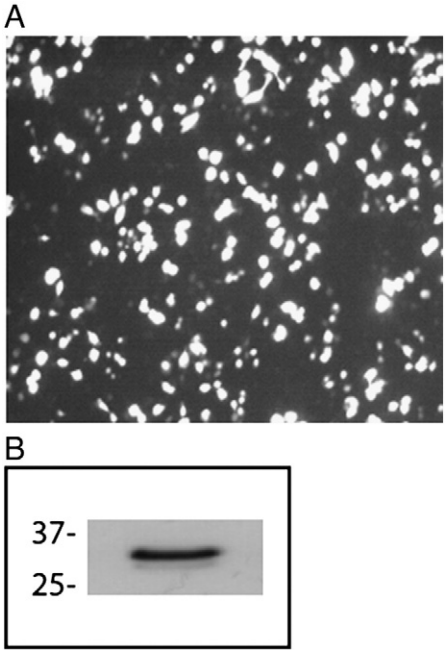

Fig. 1. Expression of TAP-tagged green fluorescent protein (GFP) in mosquito cells. NTAP-tagged GFP was expressed in C6/36 cells. Cells were (A) fixed at 48 h and analyzed by fluorescence microscopy for GFP expression or (B) lysed and analyzed by immunoblot for TAP-tagged GFP.

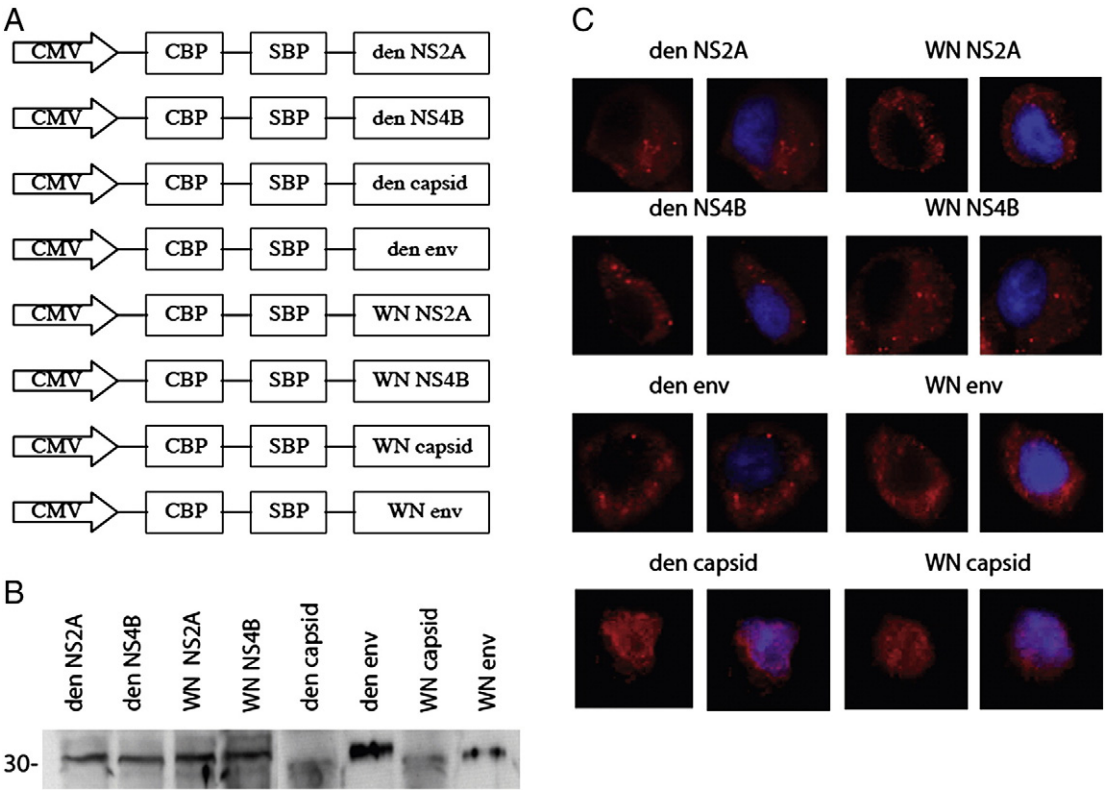

Fig. 2. Expression of TAP-tagged viral proteins. A. Schematic of the TAP plasmids used to express viral proteins fused to streptavidin-binding protein (SBP) and calmodulin binding protein (CBP). Arrow represents the cytomegalovirus promoter (CMV). B. Immunoblot analysis of viral proteins. Tagged proteins were expressed in C6/36 cells, cells were lysed and analyzed by immunoblot with an antibody against CBP. Lanes 1–8: 1. dengue NS2A, 2. dengue NS4B, 3. dengue capsid, 4. dengue envelope, 5. WNV NS2A, 6. WNV NS4B, 7. WNV capsid,and 8. WNV envelope. C. Immunofluorescence analysis. Tagged proteins were expressed in C6/36 cells and analyzed by fluorescence microscopy using an antibody against CBP. Left panel: viral protein expression (red); right panel: location of the nucleus in the cell with DAPI stain (blue).

pulled out using empty vector or nTAP–GFP expression were considered background and eliminated from further study. Mosquito genes were identified using sequence alignment of the identified peptides with the recently published *Aedes aegypti* genome (Nene et al., 2007). A total of 18 mosquito proteins were identified as potential interacting partners of the 8 flaviviral antigens. The mosquito proteins were identified by using the (Table 1). Capsid proteins bound histones, consistent with the localization of capsid to the nucleus (Fig. 2B). The viral envelope proteins bound several cell-surface proteins, including cadherin, laminin alpha-1 and dystroglycan-like protein, suggesting that these proteins could be possible viral receptor molecules. The NS proteins bound multiple translation factors, such as elongation factor 1-alpha and the 40S and 60S ribosomal proteins: consistent with the localization of these proteins to the cytoplasm (Fig. 2B) and reports of flaviviral NS protein involvement in RNA replication and translation (Khromykh et al., 2000; Umareddy et al., 2006). Some of the flaviviral proteins also bound one or more mosquito proteins involved in the cytoskeleton and cellular trafficking, such as actin, myosin and beta-tubulin.

Analysis of the interaction between West Nile and dengue virus envelope proteins and a mosquito cadherin homolog

The TAP assay identified 18 mosquito proteins as binding partners of West Nile and dengue virus proteins. We decided to focus on the interaction between mosquito cadherin and the flaviviral envelope proteins for further analysis. Cadherin is a glycoprotein often found on the surface of cells and could be a receptor for dengue or West Nile virus in mosquitoes, as the envelope protein is thought to bind the cellular receptor during virus entry into a host cell. A BLAST search for cadherin against the *A. aegypti* genome (Nene et al., 2007) using the mosquito

Table 1
Mosquito proteins identified as binding partners of select flaviviral proteins.

Flaviviral protein	Mosquito protein	*A. aegypti* protein ID
Dengue capsid	Myosin heavy chain	gi|157118639
	Enolase	gi|157121051
	Histone 2A	gi|157141861
	Histone 4	gi|108880599
	Histone 2B	gi|157138408
	Actin	gi|677901
West Nile capsid	Histone 3	gi|157138412
	Histone 2B	gi|157138408
	Kinectin	gi|157137337
	Actin	gi|677901
Dengue envelope	Myosin heavy chain	gi|157118639
	Myosin light chain	gi|108876629
	Cadherin	gi|108883460
	Dystroglycan-like protein	gi|108870400
	Laminin alpha-1, 2 chain	gi|108883156
West Nile envelope	Myosin heavy chain	gi|157118639
	Myosin light chain	gi|108876629
	Enolase	gi|157121051
	PI3 kinase	gi|157132541
	Beta tubulin	gi|108881720
Dengue NS2A	Elongation factor 1-alpha	gi|56684617
	40S ribosomal protein S6	gi|193806324
	60S ribosomal protein L4	gi|157124831
	Myelinprotein expression factor	gi|157137653
West Nile NS2A	Myosin heavy chain	gi|157118639
	40S ribosomal protein S6	gi|193806324
	Beta tubulin	gi|108881720
Dengue NS4B	Elongation factor 1-alpha	gi|56684617
	Myosin light chain	gi|108876629
	Actin	gi|677901
West Nile NS4B	Elongation factor 1-alpha	gi|56684617
	Actin	gi|677901

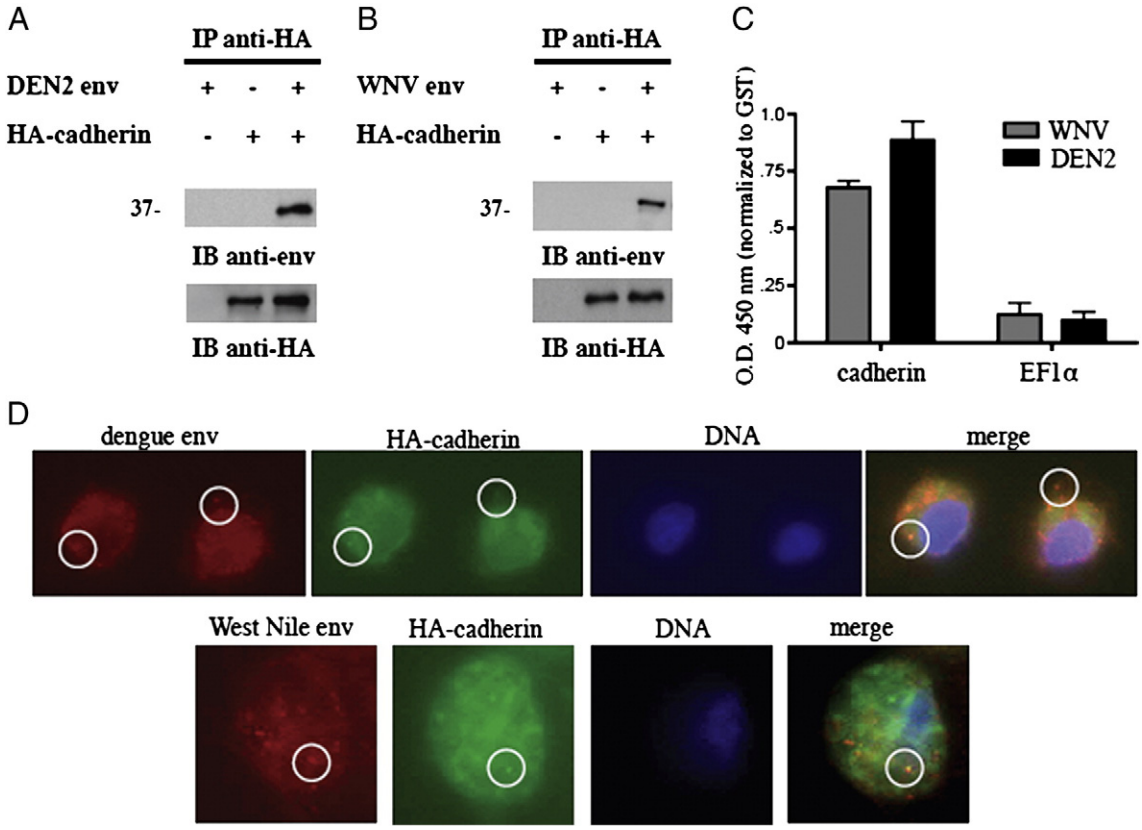

Fig. 3. Mosquito cadherin precipitates and colocalizes with dengue and West Nile virus envelope protein. C6/36 cells were cotransfected with an HA-tagged mosquito cadherin and tagged viral envelope gene expression plasmids. A, B. At 48 h, cells were lysed and cadherin was precipitated using an antibody to the HA-tag. Immunoblot analysis was done using antibodies to virus envelope (top panel) and the HA-tag antibody (bottom panel. IBs are shown for coimmunoprecipitation with HA-tagged mosquito cadherin and tagged flaviviral envelope proteins (A. dengue virus; B. West Nile virus).). IB, immunoblot; −, absence of; +, presence of; env, envelope protein. C. Graph of ELISA measuring levels of dengue or West Nile virus that bound GST-tagged cadherin or EF1α, normalized to levels that bound GST alone. D. C6/36 cells were again cotransfected with an HA-tagged mosquito cadherin and tagged viral envelope gene expression plasmids. At 48 h, cells were fixed and stained with antibodies to virus envelope (top panel—dengue virus, bottom panel—West Nile virus) and the HA-tag antibody. Cells were analyzed by fluorescence microscopy and representative images are shown. DAPI stain was used to visualize the nucleus. White circles highlight areas of colocalization between cadherin and viral envelope proteins.

peptide sequences identified in the TAP assay, revealed a sequence with 30.8% identity and 58.1% similarity to human E-cadherin, a mammalian ligand important for cell adhesion (van Roy and Berx, 2008). The extracellular calcium-binding domains were conserved (Handschuh et al., 2001), suggesting that this is a functional membrane adhesion cadherin protein. To test this, an HA-tagged gene encoding mosquito cadherin was cloned into the pAc5.1/V5-HisA insect expression plasmid. Mosquito cells were transiently transfected with flaviviral envelope and HA-cadherin plasmids. A co-immunoprecipitation assay using an HA-antibody to capture HA-cadherin was done with the cell lysates and revealed West Nile and dengue envelope proteins in the precipitate along with cadherin (Figs. 3A and B). The experiment was repeated using empty TAP vector as a control and we did not see any co-immunoprecipitation between the TAP tags and HA-tagged mosquito cadherin. An ELISA assay was done with inactivated virions and purified GST-tagged mosquito cadherin to confirm functional interaction between cadherin and viral envelope proteins. Purified GST alone and GST-tagged EF1α were used as controls. As shown in Fig. 3C, both dengue and West Nile viruses bound to mosquito cadherin but not to purified EF1α protein. To visualize where the proteins interacted during expression in the cell, HA-cadherin and the tagged viral proteins were again co-transfected into mosquito cells. The cells were fixed and stained with an antibody against HA and antibodies against viral envelope proteins, and analyzed by microscopy. Both envelope and cadherin proteins were found in the cytoplasm and were colocalized in distinct foci near the plasma membrane (Fig. 3C). Some cadherin was also found near or along the plasma membrane and nuclear membrane, representative of the many functions of the protein in the cell (Yap et al., 2007). To illustrate that the interaction between the envelope and mosquito cadherin protein was specific, a separate immunofluorescence experiment was done using HA-tagged elongation factor 1-alpha (EF1α). This mosquito protein was found to bind nonstructural proteins NS2A and NS4B in the TAP assay but was not found to interact with the envelope protein of either dengue or West Nile virus. The tagged viral proteins were again transfected into mosquito cells along with HA-EF1α and cells were fixed and stained as before. Protein expression was visualized using fluorescent microscopy and no colocalization was seen between either envelope protein and mosquito EF1α (Fig. S1).

Expression of identified mosquito genes is altered during West Nile and dengue virus infection

In order to further analyze the role the identified mosquito proteins may play in West Nile and dengue infection, the levels of gene expression were determined after viral infection of mosquito cells. At 24 post-infection, cells were lysed, RNA was isolated and cDNA was made. Expression was quantified using QPCR and normalized to mosquito actin. All of the genes had increased expression upon West Nile infection, with the exception of H2A, which was slightly decreased (Fig. 4). Dengue infection resulted in increased gene expression for all proteins except H3 and laminin. Myosin light and heavy chains, ribosomal proteins 40S S6 and 60S L4 and cadherin had over 3-fold increased expression during infection with both viruses. West Nile

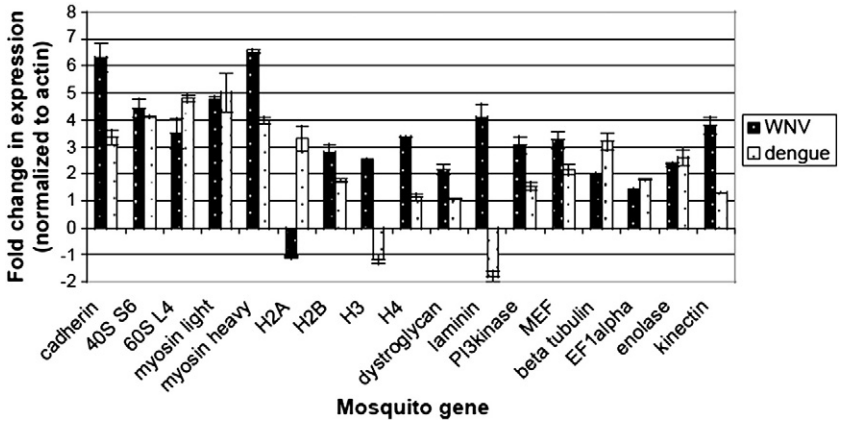

Fig. 4. Expression of interacting mosquito proteins is altered during dengue and West Nile infection. C6/36 cells were infected with dengue or West Nile virus (MOI 0.1). At 48 h post-infection, RNA was extracted from the cells and cDNA was made. Quantitative real-time PCR (qRTPCR) was done on the cDNA for the mosquito genes identified in the TAP assay. Values were normalized to mosquito actin expression and experiments were done in triplicate. Mean values are shown +/− standard deviation. $p<0.005$ for all values when compared to gene expression levels in uninfected cells.

infection had the greatest effect on cadherin and myosin heavy chain expression in the mosquito cell, with over 6-fold increase in expression.

A beneficial role for the interacting mosquito proteins in West Nile and dengue virus infection: inhibition of identified proteins inhibits virus infection

The viral proteins may have bound the mosquito proteins because they are required for infection. If so, then blocking the mosquito proteins should reduce dengue and/or West Nile virus infection. To confirm that the mosquito proteins were necessary for virus infection, chemical inhibition was used to reduce the influence of selected mosquito proteins, where reagents were available. Five chemical inhibitors were used, cytochalasin D (which blocks actin polymerization), latrunculin A (which reduces the amount of F-actin), 2,3-butandione-2-monoxime (2,3-BDM: which inhibits myosin), LY294 (which inhibits PI3-kinase) and ML7 (which inhibits myosin light chain kinase). The drug concentrations were chosen based on amounts previously used in the literature and, at the concentrations used, none of the inhibitors had any significant effect on cell viability or cell number before virus was added. Using the trypan blue exclusion test, we found that over 90% of cells were viable at the highest concentration of drug used for all five chemical inhibitors, which was comparable to cells grown in media alone. All five inhibitors reduced both dengue and West Nile virus infection in a dose-dependent manner (Fig. 5). ML7 had a much greater impact on dengue virus than West Nile virus. In contrast, both cytochalasin D and latrunculin A reduced West Nile virus infection to a larger degree than dengue virus. We saw the following IC50s for West Nile and dengue virus infection, respectively: cytochalasin D — 6 and 2 μg/mL, ML7 — >50 and 47 μM, LY294 — both >15 over 50 μM, 2,3-BDM — both >50 mM and latrunculin A — 4.8 and 5.2 nM.

Discussion

There has been a recent increase in research focused on the effects of flaviviral infection on mosquito protein expression. Sessions et al. showed that a number of genes have altered expression upon dengue infection of *Drosophila* cells, including several that encode proteins involved in endocytosis, RNA-binding and the unfolded protein response. They further demonstrated that many of these proteins

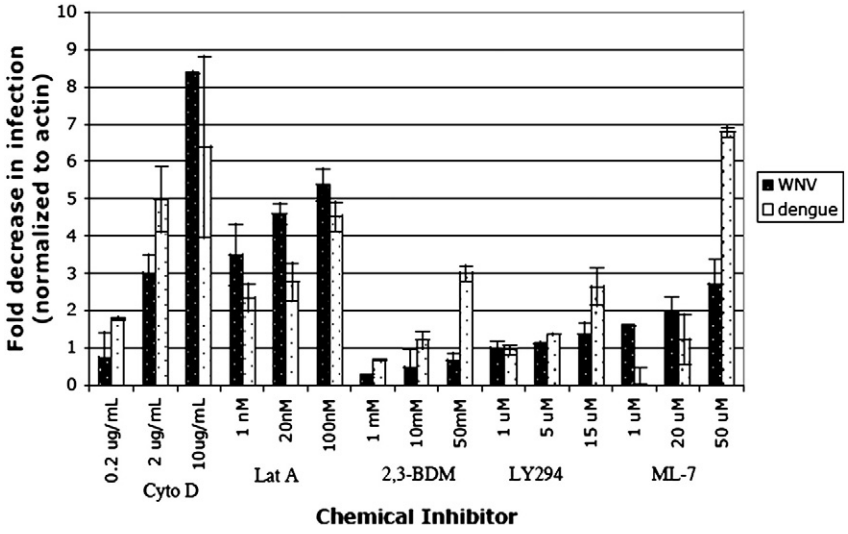

Fig. 5. Inhibition of viral infection with chemical knockdown of mosquito proteins. Chemical inhibitors were added to 24-well plates of C6/36 cells at the concentrations indicated. Cells were incubated at room temperature for 1 h and then infected with dengue or West Nile virus (MOI 0.1). At 24 h post-infection, RNA was extracted from the cells and cDNA was made. QPCR was done for viral envelope protein on the cDNA and values were normalized to mosquito actin expression in each well. Each inhibitor experiment was done in triplicate. Mean values are shown +/− standard deviation. Cyto D = cytochalasin D, LatA—latrunculinA. Cell viability was >85% using trypan blue exclusion test at the maximum concentration of each chemical inhibitor used. p Value<0.005 for each inhibitor at the IC50 concentration versus media alone using one-way ANOVA and Bonferroni post-test.

were important for infection of *Aedes* mosquitoes as well as mammalian cells (Sessions et al., 2009). Another study investigating the protein profile of dengue-infected *Aedes* mosquitoes found several proteins in infected mosquitoes that were not present in uninfected mosquitoes, though the proteins were identified by size only (Rohani et al., 2005). Though these assays provide valuable information about the alteration of mosquito transcripts in response to flavivirus infection, they do not provide information about protein interactions at the cellular level during infection. This study examined the interactions between mosquito cell proteins and the envelope, capsid, NS2A and NS4B viral proteins of West Nile and dengue viruses using the TAP assay. This is the first reported use of the TAP assay to identify interacting host cell partners of flaviviral proteins as well as the first time this method has been used in insect cells. Many pathogens use insects as transmission vectors and this assay presents an efficient way to identify insect binding partners of pathogen antigens under native cellular conditions.

We identified 18 mosquito proteins as binding partners of West Nile and dengue proteins. The normal cellular functions of the identified host proteins varied greatly within the set, yet the class of host proteins that each individual flaviviral protein bound was remarkably constant. The nonstructural proteins of both West Nile and dengue viruses bound multiple host cell translation factors, such as elongation factor 1-alpha (EF1-alpha) and 40S and 60S ribosomal proteins, which corresponds with reports in the literature of flaviviral nonstructural protein involvement in viral RNA replication and translation (Khromykh et al., 2000). Three flaviviral proteins, dengue NS2A and dengue and West Nile NS4B, bound to mosquito EF1-alpha. Recent research has shown that EF1-alpha binds flaviviral RNA, presumably to aid in viral replication (Blackwell and Brinton, 1997). This is the first report of EF1-alpha having a direct interaction with a flaviviral protein and the reasons for the binding could be to aid in viral replication or translation or to impair normal cellular processes. The NS2A proteins of both dengue and West Nile viruses were found to bind 40S and 60S ribosomal proteins and this interaction is likely linked to flaviviral RNA translation. There have been several reports of viral RNA interactions with ribosomal proteins, including Epstein–Barr virus noncoding RNA EBER1, which recruits ribosomal L22 during infection, presumably to aid in viral replication (Fok et al., 2006). In addition, the hepatitis C (HCV) IRES region associates with ribosomal S5 and it was suggested that this interaction functions to position HCV RNA on the 40S ribosomal subunit during translation (Fukushi et al., 2001). This is the first report of a flaviviral protein binding either 40S or 60S ribosomal protein. If these translation factors are required during the processing of the viral genome or for viral replication, the flaviviral proteins may have evolved mechanisms to bind and localize the proteins in an appropriate area of the cell.

Both dengue and West Nile virus envelope proteins bound several mosquito cell-surface proteins, which may be putative virus receptors. We found that laminin bound to the dengue envelope protein, and several laminin binding proteins and receptors have already been suggested to act as receptors or cofactors for both dengue and West Nile virus entry in mosquito and mammalian cells (Bogachek et al., 2008; Sakoonwatanyoo et al., 2006). This indicates that the TAP method can accurately identify host proteins known to bind components of the virion surface by using the envelope protein. As our assay analyzed protein interactions within the cell, not on the cell surface, the viral envelope could be binding receptor proteins to prevent localization to the plasma membrane. Viruses have been shown to bind their receptors intracellularly to prevent protein from reaching the cell surface, thus inhibiting further virus entry. A good example of this is the down-regulation of CD4, the HIV-1 receptor, during infection of a T cell with HIV-1 (Clapham and McKnight, 2002). In addition to laminin, the dengue envelope bound a mosquito homolog of cadherin as well as a dystroglycan-like protein, which are both glycoproteins thought to be expressed on the cell-surface. Alpha-dystroglycan is known to be the receptor for the arenaviruses lymphocytic choriomeningitis virus and Lassa virus in mammalian cells (Cao et al., 1998). Cadherin is known to be expressed on the surface of mammalian cells (van Roy and Berx, 2008) and the mosquito homolog could be a putative receptor or co-factor for both dengue and West Nile viruses. In endothelial cells, vascular endothelial cadherin is downregulated in association with increased cellular permeability due to dengue virus infection (Dewi et al., 2008). We chose to focus on the interaction between the flaviviral envelope protein and the mosquito cadherin for further analysis. The interaction between the two proteins was confirmed using immunoblot analysis. The cadherin binding was specific and significant for both dengue and West Nile virus envelope proteins. In addition, immuno-fluorescence analysis provided evidence that the two proteins colocalize when co-expressed in the mosquito cell. It remains to be seen whether the mosquito cadherin protein can act as a flaviviral receptor during infection, yet both the specific binding of the envelope protein to cadherin and the colocalization of the proteins within the cell suggest this possibility. The West Nile virus envelope protein was also found to bind mosquito PI3-kinase in our assay and the PI3-kinase signaling pathway is known to be activated during flavivirus infection to inhibit apoptosis. Blocking PI3-kinase using the chemical inhibitor LY294 reduced both West Nile and dengue infection in mosquito cells in a dose-dependent manner, suggesting that the protein may play a role in the viral life cycle.

Most of the flaviviral proteins investigated here also bound one or more proteins involved in the cytoskeleton and with cellular trafficking, such as actin, myosin and beta tubulin. Viral proteins may be expected to bind these proteins, as viruses must rely on host cellular factors for the trafficking of their own proteins and components in the cell. Both structural and non-structural proteins bound myosin light and heavy chains, suggesting an important role for myosin in the flaviviral life cycle. Additionally, blocking myosin and myosin light chain kinase with two chemical inhibitors, 2,3-BDM and ML-7, significantly reduced both dengue and West Nile virus infection in mosquito cells. Mammalian myosin Vc protein has been shown to be involved in the release of dengue from liver cells (Xu et al., 2009), and the mosquito myosin is likely playing a role in viral trafficking, either during entry or assembly and release of virus. West Nile virus NS2A and envelope protein both bound beta-tubulin, which are building blocks of microtubules and an important component of the cytoskeleton. Japanese encephalitis virus NS3 protein has been shown to associate with tubulin and microtubules during virus replication (Chiou et al., 2003). Another group reported that a tubulin-like protein in C6/36 cells binds dengue 2 virus, presumably through the envelope protein on the virus surface (Chee and AbuBakar, 2004), suggesting that tubulin could be acting as a receptor or cofactor for entry. In addition, tubulin is known to bind enolase, another protein found to bind West Nile virus envelope and dengue capsid proteins in our assay. Enolase has been shown to be required for Sendai virus transcription via its interaction with tubulin (Ogino et al., 2001) and there may be a similar requirement for the two proteins in flavivirus infection. HCV replication complexes (RCs) are known to associate with microtubules and HCV NS3 and NS5A interact with tubulin and actin during replication. It is thought that HCV RCs move along microtubules to travel within the cell (Lai et al., 2008) and this method may be utilized by dengue and West Nile flaviviruses. Mosquito actin bound both structural and nonstructural proteins from West Nile and dengue viruses. Actin has been indicated in various roles in the life cycles of several viruses, including replication of influenza A (Arcangeletti et al., 2008), the assembly of HIV-1 (Jolly et al., 2007) and the release of vaccinia (Arakawa et al., 2007). The inhibition of actin polymerization in mosquito cells using latrunculin-A and cytochalasin D lowered the infection of the cells with both dengue and West Nile viruses, though it had a greater impact on West Nile infection. Actin is likely involved in several aspects of the viral life cycle and it is clear that both dengue and West Nile have at least a partial requirement for actin polymerization during infection. Since blocking the function of these mosquito proteins decreased infection with both viruses, they are likely

beneficial and/or necessary for infection. Future studies may include an investigation into these interactions using RNAi in cells derived from the *A. aegypti* mosquito, as C6/36 cells are known to be dysfunctional in the RNAi response, (Brackney et al., 2010).

Conclusions

This study identified mosquito binding partners of flaviviral proteins that may play important roles as host factors during viral infection of mosquito cells. This list provides a useful starting point for the field of flaviviral pathogenesis in the mosquito and present targets for further investigation of the mosquito–flaviviral relationship. We identified and confirmed binding between viral proteins and mosquito cadherin. We also confirmed the importance of actin, myosin, PI3-kinase and myosin light chain kinase in West Nile and dengue infection of mosquito cells. Exploration of the interactions between West Nile and dengue viral proteins with the proteins of the mosquito transmission vector is necessary, in hopes of identifying proteins vital to the viral life cycle and perhaps novel candidates that will help break the human–vector infection cycle.

Materials and methods

Cell lines and cultivation

C6/36 *Aedes albopictus* cells (ATCC, VA) were used for all transfection and infection studies. The cells were grown at 29 °C in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gemini, CA), 1% penicillin–streptomycin and 1% tryptose phosphate broth (Sigma, MO).

TAP expression plasmid constructs

All plasmids were prepared using Qiagen miniprep kits (Valencia, CA) after standard transformation into DH5α competent bacterial cells. The tagged virus protein nTAP expression plasmids were made by cloning the coding regions for each viral protein into the N-terminal TAP plasmid (Stratagene, CA). Oligos can be found in Table S1.

Transfection of plasmids

All plasmids were transfected into C6/36 mosquito cells using Effectene (Qiagen, CA) according to manufacturer's instructions. Briefly, for a 10-cm^2 plate, 10 μg of DNA was mixed with 500 μL buffer EC and 32 μL enhancer was added. This was allowed to incubate for 5 min at RT. Then 30 μL Effectene reagent was added and the solution vortexed briefly. After a 10 min incubation, the solution was added to the cells. Expression was observed after 24 h and peaked at 48 h, as measured by levels of GFP expression.

TAP assay

The TAP assay was used to identify mosquito cell proteins that interacted with flaviviral proteins after expression in C6/36 cells. Briefly, at 48 h post-transfection, cells were washed with PBS(-) and lysed using 1X lysis buffer containing protease inhibitors. All steps were done at 4 °C to maintain the protein interactions. The cell lysates were applied to streptavidin resin, incubated at 4 °C for 2 h, washed, and bound proteins eluted off. A second purification step was done with calmodulin resin and the proteins were boiled off into PBS(-). The eluted proteins were analyzed at the Yale University W.M. Keck Foundation core facility. The eluate was subjected to trypsin digestion followed by LC/MS–MS (liquid chromatography and mass spectometry) for peptide sequencing and identification using the recently completed *A. aegypti* mosquito genome (Nene et al., 2007). Putative mosquito proteins were identified via amino acid sequence identity to both known mosquito proteins and their mammalian counterparts using the BLAST software on the NCBI website. Mosquito proteins found to bind the tags alone as well as proteins found to bind tagged green fluorescent protein were eliminated as putative interacting partners.

Expression of mosquito proteins

Mosquito genes were identified using sequence alignment of identified peptides with the recently published *A. aegypti* genome (Nene et al., 2007). RNA was isolated from mosquito cells using RNeasy (Qiagen, CA) according to manufacturer's instructions and cDNA made by reverse transcription with a Superscript kit (Invitrogen, CA) according to the manufacturer's instructions. For expression in insect cells, mosquito genes were amplified from the cDNA, fused to GFP and cloned into an insect expression vector pAc5.1/V5-HisA (Invitrogen, CA).

Protein production

The protein coding sequences were cloned into the pGEX-6P-2 plasmid and transformed into BL-21-Gold (DE3) competent cells (Stratagene, CA) using a standard transformation protocol. The bacteria was grown in LB media overnight at 37 °C, diluted in fresh media and grown to mid-log phase (A600 = 0.7). The expression of GST-tagged protein was induced by adding isopropyl-beta D-thiogalactoside (IPTG) to 0.1 mM final concentration and allowed to grow for 4 h at 37 °C. The cells were lysed, Triton X-100 was added to a final concentration of 1% and cells were shaken at 25 °C for 30 min to solubilize proteins. The crude extract was centrifuged at 10,000 × *g* for 5 min at 4 °C and the supernatant was incubated with glutathione sepharose (GS) 4B (GE, NJ) for 30 min at 25 °C. The GST-tagged proteins were eluted by adding elution buffer (50 mM Tris–HCl, 10 mM reduced glutathione, pH8.0), incubating at 25 °C for 10 min and centrifugation at 500 × *g* for 5 min.

ELISA analysis

5 μg of GST or GST-tagged mosquito protein was coated onto a 96-well ELISA plate (Thermo Fisher Sci, MA) and incubated overnight at 4 °C. The plate was blocked with 1% BSA in PBS(-) and incubated with inactivated virus for an hour at RT. The proteins were washed off, antibodies were added for 30 min at RT, washed off and secondary-HRP was added for 30 min at RT, washed off and TMB substrate was added for 20 min at RT. Stop solution was added and the O.D. of the wells was read at 450 nm.

Flavivirus infection

C6/36 cells at 75% confluence were infected with dengue 2 New Guinea C or West Nile NY99 virus (generous gifts of Dr. John Anderson, Connecticut Agricultural Experiment Station, CT) at an MOI of 0.1 or 1.0, as indicated in the figure legends. Virus was added directly to media, washed off after 4 h when new media was added and cells were left for 24 h, at which point the various assays were conducted. Viral stocks were propagated in C6/36 cells for 7 days before supernatant was centrifuged, virus collected and stored at −80 °C.

Use of chemical inhibitors

All chemicals were purchased from Sigma (MO). Cytochalasin D was provided as a 5 mg/mL stock in DMSO, latrunculin A was made as a 25 mg/mL stock in DMSO, 2,3-butanedione monoxime (2,3-BDM) was made as a 100 mg/mL stock in methanol, LY294 was made as a 5 mg/mL stock in DMSO and ML-7 was dissolved in ethanol:water (1:1). All were diluted in DMEM immediately before use. C6/36 cells were incubated with each inhibitor for 1 h at room temperature and

then dengue or West Nile virus was added at an MOI of 0.1, making sure to keep the same final concentration of drug in the media throughout the infection process. At 24 h, cells were lysed, RNA was isolated using the RNEasy kit (Ambion, TX) and cDNA was made using the Superscript kit (Invitrogen, CA). This cDNA was used in a quantitative RT-PCR reaction to determine copy number of viral envelope gene using the following oligos and a standard plasmid with known gene copy number:

Dengue 5′-caggctgaggatggacaaactac-3′ & 5′-caaaagggatcttacatggagaac-3′
West Nile 5′-ctactgtggagtcgcacgg-3′ & 5′-cctctccatattctccaagc-3′

Cell viability assay

The cells were incubated in chemical inhibitors as described above and then assayed for viability using 0.4% trypan blue solution (Sigma, MO) in a 1:1 ratio with a cell solution in PBS(-). The cells were analyzed by microscopy and percentage of viable cells was calculated.

qRTPCR

Expression levels of mosquito genes as well as viral envelope proteins were quantified via a qRT-PCR reaction using mosquito cell cDNA. The qRT-PCR reaction was done with SYBR green mix (Biorad, CA) in an iCycler machine (Biorad, CA). All assays were done in triplicate using cDNA made from one RNA isolation. The reaction conditions were as follows: 95 °C for 5 min. followed by 50 cycles of 20 s at 95 °C, 20 s at 56 °C, 8 s at 72 °C, and a final extension step of 5 min at 72 °C. Gene expression changes were calculated based on changing Ct value normalized to actin expression. Oligos can be found in Table S2.

Western blots

The plasmids containing the gene for desired protein expression were transfected into C6/36 cells and at 48 h the cells were lysed. The lysate was boiled in SDS–PAGE buffer with 2% beta-mercaptoethanol, run on a 12% SDS–PAGE gel and the proteins were then transferred to nitrocellulose. The nitrocellulose was blocked with 5% milk in 1% TBST for 1 h at RT and then incubated with the appropriate primary antibody overnight at 4 °C. The nitrocellulose was washed and then incubated with a horseradish peroxidase secondary antibody for 1 h at RT and then washed again. The protein blots were incubated with ECL substrates (Amersham, NJ) for 5 min at RT and then detected on Kodak film. Antibodies used were against calmodulin binding protein (CBP) (Abcam, MA), dengue virus (Millipore, MA), West Nile envelope protein (L^2, CT) and the HA tag (Santa Cruz Biotechnology, CA).

Immunofluorescence assays

For immunofluorescence assays, the C6/36 cells were fixed in 4% paraformaldehyde for 15 min at RT and then permeablilized with 0.1% Triton-X 100 for 1 min at RT before incubation with appropriate antibody. Antibodies used were against calmodulin binding protein (CBP) (Abcam, MA), dengue virus (Millipore, MA), West Nile envelope protein (L^2, CT) and the HA tag (Santa Cruz Biotechnology, CA). Cells were blocked in 1% BSA (Fisher Scientific, PA) in PBS (-) for 20 min at room temperature and were then incubated with primary antibody diluted in 1% BSA 1/250 for 20 min at RT. Cells were washed, incubated with labeled secondary antibody diluted in 1% BSA 1/500 for 20 min at RT and analyzed by fluorescent microscopy. A DAPI stain (Sigma-Aldrich, MO) was also incubated with the cells for 1 min at RT to illustrate the location of nucleus in the mosquito cell.

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2011.06.002.

Acknowledgments

This work was generously supported by NIH grants UO1 AI070343 and T32 AI07404. E.F. is an investigator with the Howard Hughes Medical Institute. We thank the Yale University Keck Facility proteomics and mass spectrometry resource for their assistance. T.C. and E.F. designed the experiments and wrote the manuscript. T.C., J.C., A.N., F.F. and M.N.K. assisted with QPCR and cloning viral genes.

References

Arakawa, Y., Cordeiro, J.V., Schleich, S., Newsome, T.P., Way, M., 2007. The release of vaccinia virus from infected cells requires RhoA-mDia modulation of cortical actin. Cell Host Microbe 1 (3), 227–240.
Arcangeletti, M.C., De Conto, F., Ferraglia, F., Pinardi, F., Gatti, R., Orlandini, G., Covan, S., Motta, F., Rodighiero, I., Dettori, G., Chezzi, C., 2008. Host-cell-dependent role of actin cytoskeleton during the replication of a human strain of influenza A virus. Arch. Virol. 153 (7), 1209–1221.
Blackwell, J.L., Brinton, M.A., 1997. Translation elongation factor-1 alpha interacts with the 3′ stem-loop region of West Nile virus genomic RNA. J. Virol. 71 (9), 6433–6444.
Blair, C.D., Adelman, Z.N., Olson, K.E., 2000. Molecular strategies for interrupting arthropod-borne virus transmission by mosquitoes. Clin. Microbiol. Rev. 13 (4), 651–661.
Bogachek, M.V., Protopopova, E.V., Loktev, V.B., Zaitsev, B.N., Favre, M., Sekatskii, S.K., Dietler, G., 2008. Immunochemical and single molecule force spectroscopy studies of specific interaction between the laminin binding protein and the West Nile virus surface glycoprotein E domain II. J. Mol. Recognit. 21 (1), 55–62.
Brackney, D.E., Scott, J.C., Sagawa, F., Woodward, J.E., Miller, N.A., Schilkey, F.D., Mudge, J., Wilusz, J., Olson, K.E., Blair, C.D., Ebel, G.D., 2010. C6/36 *Aedes albopictus* cells have a dysfunctional antiviral RNA interference response. PLoS Negl. Trop. Dis. 4 (10), 1–10.
Brinton, M.A., 2002. The molecular biology of West Nile Virus: a new invader of the western hemisphere. Annu. Rev. Microbiol. 56, 371–402.
Bulich, R., Aaskov, J.G., 1992. Nuclear localization of dengue 2 virus core protein detected with monoclonal antibodies. J. Gen. Virol. 73 (Pt 11), 2999–3003.
Cao, W., Henry, M.D., Borrow, P., Yamada, H., Elder, J.H., Ravkov, E.V., Nichol, S.T., Compans, R.W., Campbell, K.P., Oldstone, M.B., 1998. Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. Science 282 (5396), 2079–2081.
Chee, H.Y., AbuBakar, S., 2004. Identification of a 48 kDa tubulin or tubulin-like C6/36 mosquito cells protein that binds dengue virus 2 using mass spectrometry. Biochem. Biophys. Res. Commun. 320 (1), 11–17.
Chen, S.O., Fang, S.H., Shih, D.Y., Chang, T.J., Liu, J.J., 2009. Recombinant core proteins of Japanese encephalitis virus as activators of the innate immune response. Virus Genes 38 (1), 10–18.
Chen, Y., Maguire, T., Marks, R.M., 1996. Demonstration of binding of dengue virus envelope protein to target cells. J. Virol. 70 (12), 8765–8772.
Chiou, C.T., Hu, C.C., Chen, P.H., Liao, C.L., Lin, Y.L., Wang, J.J., 2003. Association of Japanese encephalitis virus NS3 protein with microtubules and tumour susceptibility gene 101 (TSG101) protein. J. Gen. Virol. 84 (Pt 10), 2795–2805.
Clapham, P.R., McKnight, A., 2002. Cell surface receptors, virus entry and tropism of primate lentiviruses. J. Gen. Virol. 83 (Pt 8), 1809–1829.
Cote-Martin, A., Moody, C., Fradet-Turcotte, A., D'Abramo, C.M., Lehoux, M., Joubert, S., Poirier, G.G., Coulombe, B., Laimins, L.A., Archambault, J., 2008. Human papillomavirus E1 helicase interacts with the WD repeat protein p80 to promote maintenance of the viral genome in keratinocytes. J. Virol. 82 (3), 1271–1283.
Dewi, B.E., Takasaki, T., Kurane, I., 2008. Peripheral blood mononuclear cells increase the permeability of dengue virus-infected endothelial cells in association with downregulation of vascular endothelial cadherin. J. Gen. Virol. 89 (Pt 3), 642–652.
Fok, V., Mitton-Fry, R.M., Grech, A., Steitz, J.A., 2006. Multiple domains of EBER 1, an Epstein–Barr virus noncoding RNA, recruit human ribosomal protein L22. RNA 12 (5), 872–882.
Forsman, A., Ruetschi, U., Ekholm, J., Rymo, L., 2008. Identification of intracellular proteins associated with the EBV-encoded nuclear antigen 5 using an efficient TAP procedure and FT-ICR mass spectrometry. J. Proteome Res. 7 (6), 2309–2319.
Fukushi, S., Okada, M., Stahl, J., Kageyama, T., Hoshino, F.B., Katayama, K., 2001. Ribosomal protein S5 interacts with the internal ribosomal entry site of hepatitis C virus. J. Biol. Chem. 276 (24), 20824–20826.
Ghosh, A.K., Ribolla, P.E., Jacobs-Lorena, M., 2001. Targeting Plasmodium ligands on mosquito salivary glands and midgut with a phage display peptide library. Proc. Natl. Acad. Sci. U. S. A. 98 (23), 13278–13281.
Gubler, D.J., 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol. 10 (2), 100–103.
Handschuh, G., Luber, B., Hutzler, P., Hofler, H., Becker, K.F., 2001. Single amino acid substitutions in conserved extracellular domains of E-cadherin differ in their functional consequences. J. Mol. Biol. 314 (3), 445–454.
Honda, A., Okamoto, T., Ishihama, A., 2007. Host factor Ebp1: selective inhibitor of influenza virus transcriptase. Genes Cells 12 (2), 133–142.
Hui, E.K., Barman, S., Tang, D.H., France, B., Nayak, D.P., 2006. YRKL sequence of influenza virus M1 functions as the L domain motif and interacts with VPS28 and Cdc42. J. Virol. 80 (5), 2291–2308.
Jolly, C., Mitar, I., Sattentau, Q.J., 2007. Requirement for an intact T-cell actin and tubulin cytoskeleton for efficient assembly and spread of human immunodeficiency virus type 1. J. Virol. 81 (11), 5547–5560.

Jorba, N., Area, E., Ortin, J., 2008. Oligomerization of the influenza virus polymerase complex in vivo. J. Gen. Virol. 89 (Pt 2), 520–524.
Khromykh, A.A., Sedlak, P.L., Westaway, E.G., 2000. cis- and trans-acting elements in flavivirus RNA replication. J. Virol. 74 (7), 3253–3263.
Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E., Jones, C.T., Mukhopadhyay, S., Chipman, P.R., Strauss, E.G., Baker, T.S., Strauss, J.H., 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell 108 (5), 717–725.
Lai, C.K., Jeng, K.S., Machida, K., Lai, M.M., 2008. Association of hepatitis C virus replication complexes with microtubules and actin filaments is dependent on the interaction of NS3 and NS5A. J. Virol. 82 (17), 8838–8848.
Liu, W.J., Chen, H.B., Wang, X.J., Huang, H., Khromykh, A.A., 2004. Analysis of adaptive mutations in Kunjin virus replicon RNA reveals a novel role for the flavivirus nonstructural protein NS2A in inhibition of beta interferon promoter-driven transcription. J. Virol. 78 (22), 12225–12235.
Mackenzie, J.M., Khromykh, A.A., Jones, M.K., Westaway, E.G., 1998. Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. Virology 245 (2), 203–215.
Mackenzie, J.S., Gubler, D.J., Petersen, L.R., 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat. Med. 10 (12 Suppl.), S98–S109.
Miller, S., Sparacio, S., Bartenschlager, R., 2006. Subcellular localization and membrane topology of the Dengue virus type 2 Non-structural protein 4B. J. Biol. Chem. 281 (13), 8854–8863.
Mukhopadhyay, S., Kuhn, R.J., Rossmann, M.G., 2005. A structural perspective of the flavivirus life cycle. Nat. Rev. Microbiol. 3 (1), 13–22.
Munoz-Jordan, J.L., Laurent-Rolle, M., Ashour, J., Martinez-Sobrido, L., Ashok, M., Lipkin, W.I., Garcia-Sastre, A., 2005. Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. J. Virol. 79 (13), 8004–8013.
Munoz-Jordan, J.L., Sanchez-Burgos, G.G., Laurent-Rolle, M., Garcia-Sastre, A., 2003. Inhibition of interferon signaling by dengue virus. Proc. Natl. Acad. Sci. U. S. A. 100 (24), 14333–14338.
Nene, V., Wortman, J.R., Lawson, D., Haas, B., Kodira, C., Tu, Z.J., Loftus, B., Xi, Z., Megy, K., Grabherr, M., Ren, Q., Zdobnov, E.M., Lobo, N.F., Campbell, K.S., Brown, S.E., Bonaldo, M.F., Zhu, J., Sinkins, S.P., Hogenkamp, D.G., Amedeo, P., Arensburger, P., Atkinson, P.W., Bidwell, S., Biedler, J., Birney, E., Bruggner, R.V., Costas, J., Coy, M.R., Crabtree, J., Crawford, M., Debruyn, B., Decaprio, D., Eiglmeier, K., Eisenstadt, E., El-Dorry, H., Gelbart, W.M., Gomes, S.L., Hammond, M., Hannick, L.I., Hogan, J.R., Holmes, M.H., Jaffe, D., Johnston, J.S., Kennedy, R.C., Koo, H., Kravitz, S., Kriventseva, E.V., Kulp, D., Labutti, K., Lee, E., Li, S., Lovin, D.D., Mao, C., Mauceli, E., Menck, C.F., Miller, J.R., Montgomery, P., Mori, A., Nascimento, A.L., Naveira, H.F., Nusbaum, C., O'Leary, S., Orvis, J., Pertea, M., Quesneville, H., Reidenbach, K.R., Rogers, Y.H., Roth, C.W., Schneider, J.R., Schatz, M., Shumway, M., Stanke, M., Stinson, E.O., Tubio, J.M., Vanzee, J.P., Verjovski-Almeida, S., Werner, D., White, O., Wyder, S., Zeng, Q., Zhao, Q., Zhao, Y., Hill, C.A., Raikhel, A.S., Soares, M.B., Knudson, D.L., Lee, N.H., Galagan, J., Salzberg, S.L., Paulsen, I.T., Dimopoulos, G., Collins, F.H., Birren, B., Fraser-Liggett, C.M., Severson, D.W., 2007. Genome sequence of *Aedes aegypti*, a major arbovirus vector. Science 316 (5832), 1718–1723.
Ogino, T., Yamadera, T., Nonaka, T., Imajoh-Ohmi, S., Mizumoto, K., 2001. Enolase, a cellular glycolytic enzyme, is required for efficient transcription of Sendai virus genome. Biochem. Biophys. Res. Commun. 285 (2), 447–455.
Rappole, J.H., Derrickson, S.R., Hubalek, Z., 2000. Migratory birds and spread of West Nile virus in the Western Hemisphere. Emerg. Infect. Dis. 6 (4), 319–328.
Rice, C.M., 1996. Flaviviradae: the viruses and their replication. In: Fields BN, K.D., Howley, P.M. (Eds.), Field's Virology. Lippincott-Raven, Philadelphia, pp. 931–959 (Vol. 3rd edition).
Rohani, A., Yunus, W., Zamree, I., Lee, H.L., 2005. Protein synthesized by dengue infected *Aedes aegypti* and *Aedes albopictus*. Trop. Biomed. 22 (2), 233–242.
Sakoonwatanyoo, P., Boonsanay, V., Smith, D.R., 2006. Growth and production of the dengue virus in C6/36 cells and identification of a laminin-binding protein as a candidate serotype 3 and 4 receptor protein. Intervirology 49 (3), 161–172.
Senigl, F., Kopecky, J., Grubhoffer, L., 2004. Distribution of E and NS1 proteins of TBE virus in mammalian and tick cells. Folia Microbiol. (Praha) 49 (2), 213–216.
Sessions, O.M., Barrows, N.J., Souza-Neto, J.A., Robinson, T.J., Hershey, C.L., Rodgers, M.A., Ramirez, J.L., Dimopoulos, G., Yang, P.L., Pearson, J.L., Garcia-Blanco, M.A., 2009. Discovery of insect and human dengue virus host factors. Nature 458 (7241), 1047–1050.
Sim, S., Dimopoulos, G., 2010. Dengue virus inhibits immune responses in *Aedes aegypti* cells. PLoS One 5 (5), e10678.
site, P. A. H. O. w., 2007. Number of reported cases of dengue and dengue hemorrhagic fever (DHF) in the Americas, by country: figures for 2007, Vol. 2008.
Smartt, C.T., Richards, S.L., Anderson, S.L., Erickson, J.S., 2009. West Nile virus infection alters midgut gene expression in Culex pipiens quinquefasciatus Say (Diptera: Culicidae). Am. J. Trop. Med. Hyg. 81 (2), 258–263.
Souza-Neto, J.A., Sim, S., Dimopoulos, G., 2009. An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. Proc. Natl. Acad. Sci. U. S. A. 106 (42), 17841–17846.
Umareddy, I., Chao, A., Sampath, A., Gu, F., Vasudevan, S.G., 2006. Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA. J. Gen. Virol. 87 (Pt 9), 2605–2614.
van Roy, F., Berx, G., 2008. The cell–cell adhesion molecule E-cadherin. Cell. Mol. Life Sci. 65 (23), 3756–3788.
Welsch, C., Albrecht, M., Maydt, J., Herrmann, E., Welker, M.W., Sarrazin, C., Scheidig, A., Lengauer, T., Zeuzem, S., 2007. Structural and functional comparison of the non-structural protein 4B in flaviviridae. J. Mol. Graph. Model. 26 (2), 546–557.
Xi, Z., Ramirez, J.L., Dimopoulos, G., 2008. The *Aedes aegypti* toll pathway controls dengue virus infection. PLoS Pathog. 4 (7), e1000098.
Xu, X., Dong, Y., Abraham, E.G., Kocan, A., Srinivasan, P., Ghosh, A.K., Sinden, R.E., Ribeiro, J.M., Jacobs-Lorena, M., Kafatos, F.C., Dimopoulos, G., 2005. Transcriptome analysis of *Anopheles stephensi–Plasmodium berghei* interactions. Mol. Biochem. Parasitol. 142 (1), 76–87.
Xu, X.F., Chen, Z.T., Gao, N., Zhang, J.L., An, J., 2009. Myosin Vc, a member of the actin motor family associated with Rab8, is involved in the release of DV2 from HepG2 cells. Intervirology 52 (5), 258–265.
Yap, A.S., Crampton, M.S., Hardin, J., 2007. Making and breaking contacts: the cellular biology of cadherin regulation. Curr. Opin. Cell Biol. 19 (5), 508–514.