Further Analysis of the Interaction of the Cellular Protein TIAR with the 3’ Terminal Stem-Loop of the West Nile Virus (WNV) Minus-Strand RNA

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ABSTRACT

Cellular T-cell intracellular antigen-1 related protein (TIAR) binds to the 3' terminal stem-loop of the West Nile virus minus-strand RNA [WNV 3'(-) SL RNA]. TIAR binding sites were previously mapped on loop 1 (L1) and loop 2 (L2) of the 3' (-) SL RNA and mutations of these sites in a WNV infectious clone inhibited virus replication. In the present study, data from in vitro binding assays suggested that multiple TIAR proteins bind to each WNV 3' (-) SL RNA in a positively cooperative manner. The tertiary structure of WNV 3' (-) SL RNA was predicted and it was suggested that L2 forms an exposed loop while L1 forms an embedded loop. We propose that TIAR binds first to L2 and that this interaction facilitates the binding of a second TIAR molecule to L1. Data from in vitro assays also showed that TIAR binds specifically to the WNV 3' (-) SL RNA but not to the complementary WNV 5' (+) SL RNA and that the C-terminal prion domain of TIAR contributes to RNA binding specificity. Immunoprecipitation experiments indicated that TIAR interacts with the WNV 3' (-) SL RNA in cells. Colocalization of TIAR and
viral dsRNA in the perinuclear region of WNV-infected cells was visualized using a proximity ligation assay. In WNV-infected, TIAR-overexpressing cells, increased extracellular virus yields, intracellular viral protein and RNA levels, and an increased ratio of viral plus-strand RNA to minus-strand RNA were observed. These data suggest that TIAR enhances WNV plus-strand RNA synthesis from the minus-strand template.

WNV infections induce small TIAR foci formation in primate cells but not rodent cells. The TIAR foci are located in the perinuclear region and differ in size and location from arsenite-induced stress granules (SGs). However, the small TIAR foci contain many SG components, such as G3BP, PABP, and eIF3A, but not HuR. Arsenite-induced SG formation is still inhibited by WNV infection in these cells. eIF2α phosphorylation was observed in some infected cells that contained WNV-induced TIAR foci but viral NS3 protein accumulation was not inhibited. The data suggest that WNV-induced TIAR foci in primate cells are not canonical SGs.

INDEX WORDS: West Nile virus, Flavivirus, TIAR, TIA-1, Viral RNA replication, Stress granules
FURTHER ANALYSIS OF THE INTERACTION OF THE CELLULAR PROTEIN TIAR
WITH THE 3' TERMINAL STEM-LOOP OF THE WEST NILE VIRUS (WNV)
MINUS-STRAND RNA

by

HSUAN LIU

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December 2013
DEDICATION

I dedicate this dissertation to my family. To my parents, Chih-Yue Liu (劉啟岳) and Hsiu-Chu Liu Yu (余秀菊), who gave me the greatest environment to grow up and fully supported of my education, especially the decision of study in the US. To my husband, Pei-Ju Chin, who will also become a PhD soon. Without his love and unconditional support, this dissertation would not exist.
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LIST OF ABBREVIATIONS

AU-rich element (ARE)
Baby hamster kidney (BHK)
Capsid (C)
Dengue virus (DENV)
Double-strand RNA-binding protein 76 (DRBP76)
Double-stranded RNA (dsRNA)
Endoplasmic reticulum (ER)
Envelope (E)
Eukaryotic elongation factor-1 alpha (eEF-1α)
Eukaryotic translation initiation factor 2 alpha (eIF2α)
Eukaryotic translation initiation factor 3 subunit A (eIF3A)
Fetal bovine serum (FBS)
General control nondepressible 2 (GCN2)
Glutathione S-transferase (GST)
Glyceradehyde-3-phosphate dehydrogenase (GAPDH)
GTPase-activating protein SH3 domain-binding protein (G3BP)
Heat shock protein 70 (Hsp70)
Heme-regulated initiation factor 2α (HRI)
Heterogenous nuclear protein (hnRNP)
Horseradish peroxidase (HRP)
Indirect immunofluorescence assay (IFA)
Isothermal titration calorimetry (ITC)
Japanese encephalitis virus (JEV)
Loop 1 (L1)
Loop 2 (L2)
Lupus autoantigen (La)
Membrane protein (M)
Methyltransferase (MTase)
Mouse embryo fibroblast (MEF)
Multiplicity of infection (MOI)
Nonstructural protein (NS)
Nuclear magnetic resonance (NMR)
Phosphate-buffered saline (PBS)
PKR-like ER-associated eIF2α kinase (PERK)
Poly(A)-binding protein (PABP)
Poly(rC)-binding protein 2 (PCBP2)
Polyacrylamide gel electrophoresis (PAGE)
Polypyrimidine tract-binding protein (PTB)
Pre-membrane protein (prM)
Prion-related domain (PRD)
Processing-body (P-body)
Protein data bank (PDB)
Protein kinase R (PKR)
Proximity ligation assay (PLA)
Reverse transcriptase (RT)
RNA helicase A (RHA)
RNA recognition motif (RRM)
RNA-dependent RNA polymerase (RdRp)
Stem loop (SL)
Stress granule (SG)
T cell intracellular antigen-1 (TIA-1)
T cell intracellular antigen-1 related (TIAR)
Tris-buffered saline (TBS)
Tristetraprolin (TTP)
Untranslated region (UTR)
West Nile virus (WNV)
CHAPTER 1

INTRODUCTION

1.1 Epidemiology and medical importance of West Nile virus

West Nile virus (WNV) is a mosquito-borne virus. The virus is maintained and spread in a bird-mosquito-bird cycle. Humans and horses can be infected as incidental hosts. Most WNV infections in humans result from mosquito bites but infections through blood transfusion, organ transplantation, and transplacental transmission have been reported (Iwamoto et al, 2003; Nguyen et al, 2002; Pealer et al, 2003). The majority of infected people have asymptomatic infections. Twenty to thirty percent of persons infected with WNV have mild illnesses, including fever, headaches, muscle weakness, and disorientation, and less than one percent of infected persons develop severe neuroinvasive diseases, which can sometimes be fatal (Petersen & Marfin, 2002). WNV is endemic in parts of Africa, the Middle East, south and central Asia, and Australia (Hall et al, 2001; Mackenzie et al, 2004). Since the initial outbreak in New York City in 1999, WNV has spread throughout North America, the Caribbean and Central America and into South America (Blitvich, 2008). Recently, more frequent WNV outbreaks have occurred and there has been an increased number of patients with severe disease in Europe and North America (Hayes et al, 2005; Sambri et al, 2013). WNV is regarded as an emerging virus. There is no specific treatment beyond supportive therapy currently available for WNV infections and no WNV vaccines are available for humans (Kramer et al, 2007).
1.2 Virion morphology and genome organization of flaviviruses

WNV is a member of the genus *Flavivirus* (known as flaviviruses) in the family *Flaviviridae*. Other flaviviruses that are well studied include yellow fever virus, dengue virus (DENV), and Japanese encephalitis virus (JEV). Flavivirus virions are small (50 nm in diameter) and spherical and composed of a nucleocapsid core and an outer lipid envelope. The nucleocapsid core contains one viral genomic RNA and multiple viral capsid (C) proteins. The envelope contains viral membrane (M) and envelope (E) proteins embedded in a lipid bilayer (Lindenbach et al, 2013).

Like other flaviviruses, the WNV genome is a positive-sense (+), single-stranded RNA that is approximately 11 kb in length with a type 1 cap structure at the 5' end but lacking a 3' polyadenylated tail (Lindenbach et al, 2013). This viral RNA contains a 96 nt 5' untranslated region (UTR), a single 10 kb open reading frame, and a 631 nt 3' UTR. The secondary structures of the 5' and 3' terminal genomic RNA regions are conserved among flaviviruses (Brinton & Dispoto, 1988; Brinton et al, 1986; Thurner et al, 2004). The 5' terminal stem-loop (SL) (75 nts) is smaller than the 3' terminal SL (96 nts) and the shapes of the two SLs are different (Brinton et al, 1986; Shi et al, 1996). The secondary structure of the 3' terminus of the minus-strand RNA differs from that of the complementary 5' genomic RNA due to G-U base pairs (Dong et al, 2008; Shi et al, 1996). The genome encodes three structural proteins, C, prM, and E, and seven non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5. The N-terminus of NS5 has methyltransferase activity for capping the 5' end of viral genome RNA. The C-terminus of NS5 has RNA-dependent RNA polymerase (RdRp) activity. NS5 and the NS3 protease/RNA helicase constitute the major enzymatic components of the viral RNA synthesis complex (Lindenbach et al, 2013).
1.3  **Flavivirus replication**

Flavivirus replication occurs in the cytoplasm (Figure 1.1). Virions enter cells via receptor-mediated endocytosis. The low pH of late endosomes induces fusion between the viral membrane and the endosomal membrane that releases the viral genome into the cytoplasm. The genomic RNA is first translated into one long polypeptide, which is co- and post-translationally cleaved into the three structural proteins and seven non-structural proteins by the viral NS3 serine protease domain and its NS2B cofactor, or by cellular proteases. The non-structural proteins are not carried in the virion and only function in the infected cell in viral replication and virion assembly (Lindenbach et al, 2013). The viral RdRp NS5 complexed with the other viral NS proteins, four of which are membrane associated, starts to *de novo* synthesize minus-strand RNA from the genome RNA (Choi & Rossmann, 2009) and then uses the complementary minus-strand RNA produced as a template for copying genomic RNA. At early times after infection, the amounts of nascent genomic and minus-strand viral RNAs are equal whereas at later times viral proteins have induced cell remodeling and genomic RNA synthesis is more than 10-100 times more efficient than minus-strand RNA synthesis (Chu & Westaway, 1985; Cleaves et al, 1981). Replication complexes can be visualized in the perinuclear region of an infected cell with antibody to double stranded RNA (dsRNA) or antibody to a viral non-structural protein (Westaway et al, 1999; Westaway et al, 1997). Recent electron tomography followed by three-dimensional reconstruction produced a model of the architecture of the endoplasmic reticulum (ER) invaginations that contain the viral replication complexes (Figure 1.2) (Welsch et al, 2009). Replication complexes line the inside wall of the virus-induced ER vesicles (Mackenzie et al, 1996; Welsch et al, 2009). Nascent genomic RNAs function as templates not only for minus-
strand RNA synthesis but also for viral protein translation. At later times after infection, some of the genomic RNA is assembled into nascent virions on the ER membrane that bud into the ER lumen. Immature virions are transported by the exocytic pathway through the Golgi and then to the cell surface in vesicles. Mature virions are formed in these vesicles by cleavage of prM to M by the cell protease furin. The virus containing vesicles fuse with the plasma membrane releasing the progeny virions (Mackenzie & Westaway, 2001; Welsch et al, 2009).

Among flaviviruses, sequences located in the 5' UTR as well as in the adjacent capsid coding region and in the 3' UTR of the viral genome form conserved secondary RNA structures (Thurner et al, 2004). Deletions or mutations in the 5' terminal SL, named SLA, in a WNV or DENV infectious clone or in the terminal SL of the 3' UTR in a WNV infectious clone inhibited virus replication (Cahour et al, 1995), but did not affect viral translation (Davis et al, 2007; Emara et al, 2008; Filomatori et al, 2006; Lodeiro et al, 2009). Hybridization of the 5'-3' cyclization sequences and the adjacent sequences of flavivirus genomes brings the 5' SL which can bind to NS5 in close proximity to the 3' end of the genome facilitating minus-strand initiation (Dong et al, 2008; Friebe & Harris, 2010; Friebe et al, 2011; Zhang et al, 2008a).

Viral RdRps can copy primed RNA templates in vitro with very little template specificity. Cellular proteins binding to the 3' terminal region of a viral RNA are thought to facilitate recruitment of the RdRp so that it specifically targets and initiates the 3' ends of viral RNA templates in infected cells (Brinton, 2001). The 3' terminal sequences/structures of viral genomic and minus-strand RNAs act like promoters for viral RNA synthesis (Lai, 1998). A number of experimental strategies have been used to identify host proteins that interact with viral RNAs, including viral RNA affinity columns or co-immunoprecipitation with RdRps followed by mass spectrometry (Brinton, 2002). Recently, RNAi screens, yeast single-gene deletion
(YKO) and essential gene (yTHC) libraries have been used as systematic genome-wide approaches with candidates confirmed by further experiments (Cherry et al, 2005; Nagy & Pogany, 2006). Many identified host proteins interacting with viral RNAs are RNA-binding proteins. The functions of RNA-binding proteins in cells include RNA splicing, RNA transport, RNA stabilization, RNA chaperoning and RNA unwinding, posttranslational modification, translational repression, and RNA processing and decay (Li & Nagy, 2011). Interestingly, some eukaryotic translation elongation factors were reported to facilitate virus replication (Li et al, 2013). Cell proteins binding to viral RNAs have been reported to facilitate viral translation, to regulate the switch from viral translation to replication, to recruit the RdRp to genome RNA, to facilitate RC assembly, to be a component of the RdRp holoenzyme, to facilitate minus-strand or genome RNA synthesis, or to stabilize viral RNA (Lai, 1998; Li & Nagy, 2011; Nagy & Pogany, 2012; Nagy & Richardson, 2012). The viral replication related functions of cell proteins that interact with the genomes of two plus-strand RNA viruses, poliovirus and tomato bushy stunt virus (TBSV), have been well studied (Li & Nagy, 2011; Nagy & Pogany, 2012).

Poliovirus is a member of the genus Enterovirus in the family Picornaviridae. The poliovirus replication complexes are assembled on the trans-Golgi network membrane. The viral RdRp precursor, 3CD, and cell poly(rC)-binding protein 2 (PCBP2) bind to different sites on the 5' cloverleaf-like structure of the genome RNA and cell poly(A)-binding protein (PABP) binds to the 3' end poly(A) tail. Genome circularization occurs by interaction between PCBP2 and PABP. This leads to cleavage of 3CD, the interaction of the viral 3D<sup>pol</sup> RdRp with the 3' end of the genome and initiation of minus-strand RNA synthesis using the viral di-uridylated VpG protein (VpG-UU) as a primer (Barton et al, 2001; Herold & Andino, 2001). Cell heterogenous nuclear protein C (hnRNP C), which binds to the 5' and 3' termini of the viral minus-strand RNA and
viral 3CD, was proposed to partially unwind the newly synthesized dsRNA replicative form (RF), which consists of hybridized plus-strand RNA and minus-strand RNA, and to facilitate initiation of viral plus-strand RNA synthesis (Brunner et al., 2005; Ertel et al., 2010; Roehl & Semler, 1995).

TBSV is a plant virus in the genus *Tombusvirus* of the family *Tombusviridae*. The replication complexes of TBSV consist of the viral RdRp, p92, and RNA chaperone, p33 and the cell proteins, eukaryotic elongation factor 1α (eEF1α) and heat shock protein 70 (Hsp70) and are assembled on the peroxisome membrane. eEF1α binds to the 3′ UTR of the TBSV genome RNA and facilitates the proper positioning of the RdRp on the template genome RNA for efficient initiation of minus-strand RNA synthesis (Li et al., 2010). Cell glyceradehyde-3-phosphate dehydrogenase (GAPDH) recruits the RdRp to the viral minus-strand RNA facilitating plus-strand RNA synthesis and promoting asymmetric RNA production (Wang & Nagy, 2008).

Two sets of cellular proteins that bind to the 3′ terminal stem-loops of the genomic and minus-strand RNAs of WNV were previously identified (Blackwell & Brinton, 1995; Shi et al., 1996). One of the three proteins that binds to the genome 3′ SL was identified as eukaryotic elongation factor-1 alpha (eEF-1α) (Blackwell & Brinton, 1997). The interaction between the genome 3′ SL RNA and eEF-1α positively regulates WNV minus-strand RNA synthesis (Davis et al., 2007), and it was proposed that the interaction of eEF-1α with the genome 3′ SL RNA opens the bottom part of the main stem of this structure (Davis et al., 2013). Four proteins were detected binding to the minus-strand 3′ SL RNA and one was identified as T-cell intracellular antigen-1 (TIA-1) related protein (TIAR) (Li et al., 2002). The amino acid sequence of TIAR has 80% similarity to that of TIA-1 (Beck et al., 1996). Hence, an interaction between TIA-1 and the minus-strand 3′ SL RNA in infected cells could not be excluded. Binding studies showed that the
binding activity of TIA-1 to the minus-strand 3' SL RNA was 10 times weaker than to TIAR (Li et al, 2002). The binding sites for TIAR were mapped to the two AU-rich loops of the minus-strand 3' SL RNA and mutations of the mapped binding sites in a WNV infectious clone inhibited virus replication (Emara et al, 2008). WNV progeny particle production was decreased in TIAR knock-out cells compared to wild type cells (Li et al, 2002). These data suggested that TIAR/TIA-1 may facilitate WNV genome RNA synthesis in infected cells. Additional studies on TIAR/TIA-1 are described in this dissertation.

1.4 T-cell intracellular antigen -1 (TIA-1) and its related protein, TIAR

T cell intracellular antigen-1 (TIA-1) and its related protein, TIAR, are RNA binding proteins. These proteins bind to the AU-rich element (ARE) RNA sequences at the 3' UTR of some cellular mRNAs to mediate translational silencing (Gueydan et al, 1999; Kedersha et al, 2000) and bind to AU-rich sites in pre-mRNAs to regulate alternative mRNA splicing (Izquierdo & Valcarcel, 2007b; Le Guiner et al, 2001). TIAR and/or TIA-1 are also involved in global translational arrest by sequestering mRNAs (without AREs) in stress granules (SGs) under stress conditions and TIA-1 regulates Fas-mediated apoptosis (Kedersha et al, 1999; Tian et al, 1995).

TIAR/TIA-1 contain three RNA recognition motifs (RRMs), RRM1, RRM2, and RRM3, at the N-terminus and a glutamine-rich auxiliary domain at the C-terminus. Each RRM domain is about 100 amino acids long. The C-terminal auxiliary domain is about 90 amino acids in length and contains a prion-related domain (PRD). The PRD domain functions in protein aggregation during SG formation (Gilks et al, 2004). The RRM2 is known to be responsible for specific binding to U-rich RNAs (Dember et al, 1996). However, it has been shown that the RRM1, RRM3, and PRD domains increase RNA binding activity and that RRM3 contributes to RNA
8

binding specificity (Dember et al, 1996; Kim et al, 2007; Kim et al, 2011). TIAR also binds to C-rich RNA and T-rich ssDNA with lower binding activity than to U-rich or AU-rich RNA (Kim et al, 2013; Kim et al, 2007; Suswam et al, 2005). TIAR and TIA-1 each have two isoforms, TIAR a/b and TIA-1 a/b, due to alternative splicing (Beck et al, 1996). The major isoform of TIAR in cells is TIARb. Expression levels of TIA-1a and TIA-1b are equal in most types of cells (Beck et al, 1996). In TIAR knockout cells, the expression of TIA-1b is upregulated to a greater extent than TIA-1a, suggesting that TIAR regulates the relative levels of TIA-1a and TIA-1b in cells (Izquierdo & Valcarcel, 2007b).

1.5 Characteristics of RRM domain-containing proteins

In eukaryotes, the RRM domain is one of the most abundant protein domains. The function of an RRM is to bind to RNA in a specific manner. The TIAR and TIA-1 proteins previously reported to bind to the 3′ SL of the flavivirus minus-strand RNA both contain three RRM domains. In humans, 2% of the gene products contain at least one RRM. In addition, the RRMs are often present in multiple copies (two to six RRMs) within a single protein (44%). At least 49 structures of RRMs in complex with RNA or DNA have been determined either by nuclear magnetic resonance (NMR) or X-ray crystallography (Daubner et al, 2013). Alignment of different RRM domains shows that two consensus amino acid sequences, called RNP1 and RNP2, are individually located on β3 and β1 beta sheets of the RRM domain. Moreover, the secondary structure of each RRM folds into an αβ sandwich structure with β1α1β2β3α2β4 topology. RNP1 and RNP2 form a surface for RNA binding. One RRM domain binds to 2-8 nts of RNA or ssDNA. The nucleic acid contacts of RRMs in different proteins differ. Even though different RRM domains contain the consensus sequences and have a similar topology, the
structures of RRM domains in different proteins are not identical. The target RNA secondary structure, the combination of two or more RRM domains involved in the interaction with RNA, and the unique N- or C-terminal extensions of an RRM domain can vary the interaction with RNA (Maris et al, 2005). For example, the co-crystal structure of the RRM domain of the U1A spliceosomal protein with a particular RNA hairpin shows that the AUUGCAC loop sequence interacts extensively with RNP1 and RNP2 and also with the C-terminal extension of the RRM domain (Oubridge et al, 1994). The β-sheets of RRM1 and RRM2 of sex-lethal protein face to each other to form a cleft for RNA binding, in contrast, the β-sheets of RRM1 and RRM2 of PABP protein lay side-by-side to create a large binding platform for RNA binding (Maris et al, 2005).

The similarity of the amino acid sequences of RRM1, RRM2, and RRM3 between TIAR and TIA-1 are 79%, 92%, and 91 %, respectively. The consensus RNP1 and RNP2 sequences of each RRM domain for TIAR and TIA-1 are identical, but the consensus sequences of RNP1 and RNP2 of each of the three RRM domains are unique (Kim et al, 2013). The structures of the RRM domains of TIAR and TIA-1 have been reported. The identification codes (ID) of the nuclear magnetic resonance (NMR) structures of TIAR RRM1, RRM2, RRM3, and TIA-1 RRM2 are 2CQI, 2DH7, 1X4G, and 2RNE, respectively in the Protein Data Bank (PDB; www.rcsb.org) (Kim et al, 2013; Kuwasako et al, 2008). The only crystal structure that has been solved is TIA-1 RRM2 (PDB ID: 3BS9) (Kumar et al, 2008). The TIAR/TIA-1 RRMs contain β₁α₁β₂β₃α₂β₄ topology, similar to RRM domains in other cell proteins. Even though no structure of an TIAR RRM domain complexed with RNA has been reported, a chemical shift assay of the binding of TIAR RRM2 and a 6-nt AU-rich RNA (5'-UUUUUU-3') showed that not only the beta-sheet surface of TIAR RRM2 but also the C-terminal extension of RRM2 contributes to RNA binding (Kim et al, 2013). A small x-ray scattering study suggested a two-site binding
interaction of TIAR RRM123 to a 20-nt poly(U) RNA (Bauer et al, 2012). However, the structure of full-length TIAR/TIA-1 complexed to RNA has not been reported.

1.6 Other cell proteins previously reported to bind to the 3' RNAs of flaviviruses

Although several other cell proteins were reported to interact with the 3' terminal sequences of the genomic RNAs of flaviviruses other than WNV, none of these proteins has yet been shown to be directly involved in regulating viral RNA synthesis. NF90, also named double-strand RNA-binding protein 76 (DRBP76), polypyrimidine tract-binding protein (PTB), DEAD-box RNA helicase DDX6, Y box-binding protein-1 (YB-1), and a p100 protein with transcription and RNA transport functions were reported to bind to the 3' UTR of DENV RNA (Anwar et al, 2009; Gomila et al, 2011; Lei et al, 2011; Paranjape & Harris, 2007; Ward et al, 2011). Lupus autoantigen (La) protein was reported to bind to the 3' UTR of JEV RNA (Vashist et al, 2009) and far upstream element binding protein 1 (FBP1) was reported to bind to both the JEV 5' and 3' UTRs (Chien et al, 2011). Using protein knock-down experiments, it was concluded that NF90, PTB, DDX6, and p100 proteins enhance DENV replication and La protein enhances JEV replication (Anwar et al, 2009; Gomila et al, 2011; Lei et al, 2011; Vashist et al, 2009; Ward et al, 2011). In contrast, YB-1 and FBP1 mediate anti-viral effects (Chien et al, 2011; Paranjape & Harris, 2007). PABP was reported to bind upstream of the conserved 3' SL of DENV and in vitro translation of DENV reporter RNAs in BHK cell extracts was inhibited by the PABP-specific translation inhibitor, PABP-interacting protein 2, indicating that PABP modulates translation efficiency (Polacek et al, 2009). It was noted that some of these proteins have RNA folding activity.
Proteins that can refold RNA structures and assist RNAs in reaching their functionally active states are classified into three categories: RNA chaperones, RNA annealers, and RNA helicases (Rajkowitsch et al, 2007). RNA chaperones can disrupt RNA-RNA interactions and loosen RNA structures but do not require ATP. RNA annealers accelerate annealing of complementary RNAs. RNA helicases utilize energy derived from ATP hydrolysis to resolve or unwind RNA structures and to displace RNA-bound proteins. Some proteins have more than one of the above RNA folding activities. In vitro and in vivo assays for measuring RNA annealing or RNA displacement are available (Rajkowitsch et al, 2007; Semrad, 2011).

Viral and cell proteins with RNA folding activities are known to be required in many steps of the virus replication cycle (Zuniga et al, 2009). For flaviviruses, annealing and resolving of the 5'-3' RNA-RNA interaction of the viral genome RNA and also of the terminal stem-loop structures of 5' and 3' UTRs of viral genome and minus-strand RNAs are essential for efficient virus replication (Friebe & Harris, 2010; Friebe et al, 2011; Zhang et al, 2008a). Recent studies on dengue virus suggest that the NS3 protein not only has ATP-dependent RNA unwinding (helicase) activity similar to that of eukaryotic DEAD box proteins but also ATP-independent annealing activity (Gebhard et al, 2012). The flavivirus capsid protein has been reported to have higher RNA chaperone activity than NS5, but NS3 did not show RNA chaperone activity (Gebhard et al, 2012; Luo et al, 2008; Pong et al, 2011). Some cell RNA helicases have been reported to be essential for the flavivirus replication cycle. For example, the DEAD box RNA helicase DDX6, which binds to the possible pseudoknot structures DB1 and DB2 in the middle of the 3' UTR of dengue viral RNA, was reported to be required for efficient dengue virus particle production (Ward et al, 2011). Also, in WNV-infected cells, knockdown of the DEAD box RNA helicase DDX56 reduced WNV yields but did not affect virus RNA replication, and
the production in progeny virus in the DDX56 knockdown cells was restored by overexpression of active DDX56 protein, suggesting that DDX56 helicase activity is required for WNV viron assembly (Xu et al, 2011; Xu & Hobman, 2012). Three cell proteins, NF90, NF45, and RNA helicase A (RHA) were reported to bind to the 3' SL of the dengue genome RNA, and knockdown of NF90 decreased dengue virus production (Gomila et al, 2011). These three proteins are in the NFAR protein family and the formation of NF90:NF45 heterodimers and NF90:NF45:RHA heterotrimers has been reported (Fierro-Monti & Mathews, 2000). NF90 and RHA contain double-strand RNA binding motifs. RHA has DNA/RNA helicase activity and NF90 is activated by protein kinase R (PKR) (Fierro-Monti & Mathews, 2000). NF90, NF45, and RHA were also reported to bind to the 5' and 3' UTRs of the genome RNA of bovine viral diarrhea virus, a member of another genus in the family Flaviviridae, and viral replication was inhibited by RHA knockdown (Isken et al, 2003).

1.7 Stress granules (SGs)

TIA-1/TIAR are primarily located in the nucleus but shuttle between the nucleus and cytoplasm. One of the functions of TIA-1/TIAR is in SG assembly. In response to environmental stress, one of the four eukaryotic translation initiation factor 2 alpha (eIF2α) kinases, PKR, PERK, GCN2, or HRI, is activated to phosphorylate eIF2α, which leads to the reduction of the ternary complex, eIF2-guanosine triphosphate (GTP)-methionine transfer ribonucleic acid (eIF2-GTP-tRNA^{Met}) required for translation initiation. TIA-1/TIAR interacts with and aggregates mRNAs with stalled preinitiation complexes that lack eIF2-GTP-tRNA^{Met} to form discrete cytoplasmic granules known as SGs that cause translational repression (Anderson & Kedersha, 2002). Indirect immunofluorescence analyses (IFAs) revealed that SGs contain small,
but not large, ribosomal subunits, most translation preinitiation components (eIF3, eIF4E, eIF4G, and PABP), poly(A)$^+$ RNA, and additional RNA-binding proteins, including HuR, GTPase-activating protein SH3 domain-binding protein (G3BP or G3BP1), and tristetraprolin (TTP) (Kedersha & Anderson, 2002; Kedersha et al, 2002). Overexpression of G3BP induces SG formation (Tourriere et al, 2003). G3BP and TIAR/TIA-1 function as SG nucleating proteins needed for initiating SG assembly (Gilks et al, 2004; Kedersha et al, 1999; Tourriere et al, 2003). The stalled untranslated mRNAs in SGs can either be returned to active translation or recruited to processing bodies (P-bodies) for degradation (Anderson & Kedersha, 2009). However, the formation of SGs can also be eIF2α-independent, such as by inhibition of the eIF4A RNA helicase that is required for the ribosome recruitment phase of translation initiation (Dang et al, 2006; Mazroui et al, 2006).

1.8 Interference with SG formation by virus infections

Several types of virus infections can modulate cell proteins involved in SG formation to either induce or block SG formation for their benefit (Lloyd, 2012; White & Lloyd, 2012). Antagonism of SG formation in virus infected cells was assessed by the loss of cellular SG formation in response to oxidative stress induced by arsenite treatment. WNV does not induce SG formation at early times of infection and induces eIF2α phosphorylation and SG formation in only few cells at late times (Courtney et al, 2012; Tu et al, 2012). It was suggested that viral dsRNA is protected from detection by PKR by association with cell membranes which prevents SG formation in WNV infected cells (Courtney et al, 2012). PKR activation was also reported to be blocked by JEV NS2A early during a JEV infection (Tu et al, 2012). In addition, WNV, DENV, and JEV infections inhibit arsenite-induced SG formation (Emara & Brinton, 2007;
Katoh et al, 2013). The interaction of JEV core protein with one of the SG components, Caprin-1 was suggested to inhibit SG formation in JEV-infected cells (Katoh et al, 2013).

Some virus infections induce SG formation to inhibit cellular cap-dependent translation. Poliovirus uses a cap-independent translation mechanism and inhibits cell protein expression by induction of SG formation in an eIF2α-independent manner at an early stage of infection (Mazroui et al, 2006). However, SGs are disassembled by viral 3Cpro-mediated cleavage of G3BP 2-3 h after infection (White et al, 2007). At later times of infection, a type of pseudo-SG containing TIA-1 remains but lacks translation initiation factors and mRNA binding proteins, and contains a low amount of polyadenylated mRNA. The translation apparatus released from SG aggregation is thought to be used to support viral translation (White & Lloyd, 2011). Poliovirus infections also inhibit the formation of canonical SGs in response to arsenite as well as other exogenous stressors (White & Lloyd, 2011).

**GOALS OF THE DISSERTATION**

Most single stranded RNA viruses have small genomes that only encode a few proteins and they use cellular proteins to assist in accomplishing their replication cycle in cells (Ortin & Parra, 2006). RNA viruses encode their own RdRp to synthesize viral RNA. Many studies have shown that cellular proteins facilitate the recruitment and/or positioning of RdRp to viral RNA templates to initiate viral RNA synthesis (Lai, 1998; Nagy & Pogany, 2012). WNV requires cellular proteins to complete its life cycle (Brinton, 2001). Four cellular proteins that bind to the 3' terminal stem-loop (SL) of the WNV minus-strand RNA [WNV 3' (-) SL RNA] were previously reported by our lab (Shi et al, 1996). These cell proteins are likely to be involved in the initiation of WNV plus strand RNA synthesis. One of the cell proteins was identified as
TIAR (Li et al, 2002). This protein is closely related to TIA-1. The production of progeny WNV viral particles was only slightly decreased in TIAR knock-out cells due to the redundant function of TIA-1 (Li et al, 2002). The region of TIAR responsible for binding to the WNV 3' (-) RNA was mapped to the central RNA binding domain, RRM2, and the TIAR/TIA-1 binding sites on the WNV 3' (-) SL RNA were mapped to two single-stranded loops (Emara et al, 2008; Li et al, 2002). Mutations or deletions of the mapped binding sites on the viral RNA in a WNV infectious clone decreased or inhibited virus replication (Emara et al, 2008). These previous data supported the hypothesis that TIAR facilitates viral genome RNA synthesis. However, the interaction of TIAR with the WNV 3' (-) SL RNA in cells had not been shown and the details of the physical interaction between TIAR and the WNV 3' (-) SL RNA were not known. To gain a better understanding of how TIAR/TIA-1 enhances WNV replication, the research of this dissertation further analyzed the in vivo and in vitro interactions between the WNV 3' (-) SL RNA and TIAR/TIA-1.

AIM 1: To analyze the physical interaction between the WNV 3' (-) SL RNA and TIAR/TIA-1 in vitro.

AIM 2: To study the interaction of TIAR with the WNV 3' (-) RNA in infected cells.

AIM 3: To study the involvement of TIAR in enhancing WNV RNA replication in infected cells.

Chapter 2 includes the results of Aim 1. Chapter 3 includes the results of Aims 2 and 3. Chapter 4 contains the results of an additional study of the characteristics of WNV-induced small TIAR foci in human lung adenocarcinoma epithelial A549 cells.
Figure 1.1. Flavivirus replication cycle in a cell.
(A) Virion attachment, entry by endocytosis, and uncoating of the viral genome RNA. (B) Translation of a single viral polyprotein. (C) Polyprotein processing. (D) Minus-strand RNA synthesis. (E) Plus-strand RNA synthesis. (F) Encapsidation of genome and assembly of immature virions. (H) Transport of the immature virions through the Golgi. (I) Virion maturation and exit at the plasma membrane by exocytosis. A complete replication cycle takes about 10-12 h. Modified figure and legend adapted from (Brinton, 2002).
Figure 1.2. Dengue virus replication complex vesicles revealed by electron tomography (ET) and three-dimensional reconstruction. 

(A) Dengue virus-infected cells were analyzed by ET. Virus-induced vesicles (Ve) that are the sites of viral RNA synthesis were seen in the endoplasmic reticulum (ER)-derived membrane close to the nuclear envelope (NE). A pore (white arrowhead) observed between the cytoplasm and a virus-induced vesicle was suggested to be used for newly synthesized viral genome RNA transport out of the vesicle. Virions (black arrowhead) were seen in Golgi. (B) Three-dimensional reconstruction from Z-stack images of ET. (C) A model of dengue virus RNA synthesis, assembly, and virion release. The viral genome associates with the rough ER and viral proteins are translated. NS4A with other viral and perhaps cellular proteins induce invaginations of the ER membrane, leading to the formation of vesicles that are connected to the cytosol via a pore. Viral RNA synthesis occurs in these vesicles. Progeny RNA genomes associate with capsid proteins and an immature viron is assembled and buds through the ER membrane. Individual virions travel toward distal sites of the ER lumen, where they collect in dilated ER cisternae, and then are transported via secretory vesicles to the Golgi complex where the virion E protein is glycosylated. Modified figures and legend adapted from (Paul & Bartenschlager, 2013; Welsch et al, 2009).
Figure 1.3. Molecular regulation of poliovirus RNA synthesis.

(A) Viral replication complex of Poliovirus is assembled and contains RdRp precursor 3CD and host poly(rC)-binding protein 2 (PCBP2) bound to the 5' (+) cloverleaf-like structure and poly(A)-binding protein (PABP) bound to the 3' (+) poly(A) tail. (B) Interaction of PABP and PCBP2 facilitates the cleavage of 3CD and the release of the RdRp 3D\textsuperscript{pol}. 3D\textsuperscript{pol} starts synthesis of minus-strand RNA using viral di-uridylated VpG protein (VpG-UU) as a primer. (C) The newly synthesized replicative form (RF), consisting of hybridized (+) RNA and (-) RNA, is partially unwound by the chaperone activity of the host heterogeneous nuclear ribonucleoprotein C (hnRNP C) and the binding of PCBP2 and 3CD to the 5' (+) RNA. (D) After cleavage of 3CD, the released 3D\textsuperscript{pol} starts synthesis of plus-strand RNA, and produces large amounts of (+) RNA. Modified figure and legend from (Nagy & Pogany, 2012).
CHAPTER 2

In Vitro Binding Analysis of TIAR with the 3’ Stem Loop of the West Nile Virus (WNV) Minus-Strand RNA

INTRODUCTION

T cell intracellular antigen-1 (TIA-1) and its related protein, TIAR, are ~42 kDa RNA binding proteins that regulate RNA splicing and mRNA silencing in eukaryotic cells (Izquierdo & Valcarcel, 2007b; Kedersha et al, 2000). TIAR/TIA-1 contain three RNA recognition motifs (RRMs) at the N-terminus and a glutamine-rich C-terminal auxiliary domain. Two isoforms of TIA-1 and of TIAR are generated by alternative mRNA splicing (Beck et al, 1996). TIA-1a has 11 more amino acids than TIA-1b located in the linker region at the N-terminus of RRM2, and the two isoforms are either equally expressed or the TIA-1a isoform is more abundant than TIA-1b (Beck et al, 1996; Izquierdo & Valcarcel, 2007b). TIARa has 17 more amino acids than TIARb in the RRM1 domain, and TIARb is the major isoform in cells (Beck et al, 1996). TIAR shares 80% amino acid identity with TIA-1 (Dember et al, 1996). There is high sequence identity between TIA-1 a/b and TIAR a/b within the RRM regions (RRM1: 79%; RRM2: 92%; RRM3: 91%) (Kim et al, 2013). Only considering the major isoform TIARb, TIARb RRM1 and TIA-1 a/b RRM1 share 85% identity using the LALIGN sequence alignment web server (http://embnet.vital-it.ch/software/LALIGN_form.html). The C-terminal auxiliary domain is a glutamine-rich prion-related domain. Overexpression of recombinant TIA-1 in cells induces spontaneous stress granule (SG) formation but overexpression of recombinant TIA-1 RRM123 does not. However, substitution of the TIA-1 auxiliary domain with the yeast Sup35 prion domain reconstitutes SG formation, suggesting that the TIA-1 glutamine-rich C-terminal
auxiliary domain has prion-like aggregation activity (Gilks et al, 2004). The C-terminal auxiliary domains of TIA-1 and TIAR share only 51% similarity (Kawakami et al, 1992; Kim et al, 2013).

Using the SELEX method, Dember et al. (1996) showed that TIAR/TIA-1 bind to U-rich RNA sequences. The $K_d$ of the interaction of TIAR with a selected U-rich RNA was determined to be 8 nM by nitrocellulose filter binding assays. Using a similar method, TIAR was also found to bind to C-rich RNA motifs in cellular RNAs. However, the binding activity of TIAR for the C-rich RNA was lower than that for the U-rich RNA (Kim et al, 2007). TIAR was also shown to bind to T-rich ssDNA in UV-crosslinking and biosensor assays (Kim et al, 2013; Suswam et al, 2005).

The RRM domain folds into a $\alpha\beta$ sandwich structure with $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ topology. An RRM domain contains two consensus amino acid sequences, named RNP1 and RNP2, which are located on the $\beta_3$ and $\beta_1$ strands of the RRM domain, respectively. The $\beta$ sheets of the RRM domain form a platform for RNA or ssDNA binding (Maris et al, 2005). The nuclear magnetic resonance (NMR) structures of each of the RRMs of TIAR (PDB ID of TIAR RRM1, RRM2, and RRM3 are 2CQI, 2DH7, and 1X4G, respectively) (Kim et al, 2013), and the NMR and crystal structures of TIA-1 RRM2 (PDB ID: 2RNE and 3BS9, respectively) (Kumar et al, 2008; Kuwasako et al, 2008) have been reported. Even though the three TIAR/TIA-1 RRMs share a common structure topology, each of the RRM domains has a different RNA binding affinity. RRM2 was shown to be sufficient and required for U-rich RNA and T-rich ssDNA binding (Dember et al, 1996; Kim et al, 2013). RRM1 contributes to T-rich ssDNA binding (Suswam et al, 2005). The finding that RRM123 has higher AU-rich binding and lower C-rich binding than RRM12 suggested that RRM3 also contributes to the binding specificity (Kim et al, 2007; Kim et al, 2011). Full-length TIAR was reported to bind U-rich RNA more efficiently than RRM123,
RRM12, RRM23, or RRM2, indicating that the glutamine-rich C-terminal auxiliary domain of this protein also contributes to RNA binding (Dember et al, 1996). A linker region between RRM2 and RRM3 was also shown to be required for RNA and ssDNA binding (Kim et al, 2013).

TIAR was reported to interact specifically with the 3' terminal stem-loop (SL) of the WNV minus-strand RNA [WNV 3' (-) SL RNA] and it was proposed that this interaction facilitates plus strand RNA initiation (Li et al, 2002; Shi et al, 1996). The TIAR RRM2 was shown to bind to the WNV 3' (-) SL RNA by gel mobility shift assay and the binding affinity of TIAR RRM2 for the WNV 3' (-) SL RNA was 10 times higher than the binding of TIA-1 RRM2 to this RNA (Li et al, 2002). To gain information about the requirement of this interaction for virus replication, TIAR-knockout mouse embryo fibroblasts (MEFs) were infected. Only a small decrease in extracellular WNV yield was observed and this was attributed to the higher level of TIA-1 in the TIAR-knockout cells (Li et al, 2002). The expression of TIA-1a is usually higher than that of TIA-1b in MEFs. Although the total amount of TIA-1 was upregulated in TIAR-knockout MEFs, TIA-1b was up-regulated to a greater degree. TIAR was previously reported to down-regulate the expression of TIA-1 (Izquierdo & Valcarcel, 2007a). The binding sites for TIAR on the WNV 3' (-) SL RNA were mapped to two of the three single-stranded loops, loop 1 (L1) and loop 2 (L2) (Emara et al, 2008). Mutations of mapped binding sites in L1 and L2 in a WNV infectious clone inhibited virus replication (Emara et al, 2008). L1 and L2 are complementary to sequences on the side of the 5' (+) SL. Because the MTase of NS5 binds to nucleotide sites on the 5' (+) SL, contacts between this RNA and NS5 have been proposed as the mechanism by which NS5 is recruited to the end of the 3' (+) SL in the context of a flavivirus genome that is cyclized due to 5'-3' RNA interactions (Lodeiro et al, 2009). However, the
majority of the L1 and L2 substitutions did not alter the structure of the 5' (+) SL predicted to be important for NS5 binding (Emara et al, 2008).

To test the hypothesis that the up-regulation of TIA-1b contributed to compensation for the loss of function of TIAR in MEFs, the binding activities of TIARb, TIA-1a, and TIA-1b to the WNV 3' (-) SL RNA were compared. The binding specificity of full-length TIAR and TIAR RRM123 for the WNV 3' (-) SL RNA was further analyzed by comparing the binding activity for the complementary sequence [WNV 5' (+) SL RNA]. In collaboration with Dr. Jin Zhang and Dr. Markus Germann in the Department of Chemistry, GSU, solution binding assays and NMR spectrometry were also done to analyze the interaction of TIAR with the WNV 3' (-) SL RNA in solution.

MATERIALS AND METHODS

2.1 In vitro transcription of 32P-labeled RNA probes and unlabeled RNA transcripts

DNA templates for transcribing the WNV 3'(-) SL RNA (75-nt) and WNV 5' (+) SL RNA (74-nt) were amplified using the previously constructed plasmid pWNV75NCR (Emara et al, 2008) and specific primers. The forward primer for WNV 3' (-) SL RNA was 5'-[T7]CAGCTCGCACCCTGTTAATTGTTG-3' and the reverse primer was 5'-AGTAGTTCGCCTGAGC-3'; the forward primer for WNV 5' (+) SL RNA was 5'-[T7]AGTAGTTTCGCTGTGAGC-3' and the reverse primer was 5'-AGCTCGCACTGTGTTAATTGTTG-3'. The forward primers contained the T7 polymerase promoter sequence (5'-TAATACGACTCACTATAGG-3'). The PCR products of a 50 µl reaction were gel-purified using MiniElute PCR Purification Kit (QIAGEN) and dissolved in 10 µl of RNase free water (Gibco). The 32P-labeled RNAs were in vitro transcribed using a MAXIscript® in vitro Transcription Kit (Ambion). The 20 µl reaction
containing DNA template (0.5 µg), T7 enzyme (30U), [α-32P]GTP (50 µCi; PerkinElmer), 30 µM GTP, and 0.5 mM CTP, UTP, and ATP in Transcription Buffer (Ambion) was incubated at 37°C for 1 h. The template DNA was removed by adding TURBO DNase (1 U; Ambion) for 15 min at 37°C. After addition of an equal volume of 2x Gel Loading Buffer II (Ambion), the reaction mixture was heated at 95°C for 5 min. RNA transcripts were purified by electrophoresis on a 6% denaturing polyacrylamide gel (19:1 acrylamide/bis-acrylamide with 6 M urea). The wet gel was autoradiographed, and the 32P-labeled RNA band was excised. RNA was eluted from the gel slices by rocking overnight at 4 °C in elution buffer (0.5 M NH₄OAC, 1 mM EDTA, and 0.2% SDS). The eluted RNA was filtered through a 0.45 µm cellulose acetate filter unit (Millipore), precipitated with ethanol, resuspended in water, and stored at -80°C. Unlabeled WNV 3'(-) SL RNA (75-nt) and WNV 5'(+) SL RNA (74-nt) were in vitro transcribed using a high yield MEGAscript Kit (Ambion) with the same protocol as 32P-labeled RNA except for the addition of 32P-labeled NTP into the transcription reaction. Transcribed unlabeled RNA was purified in water using NucAway Spin Columns (Ambion) and confirmed by electrophoresis on a 6% denaturing polyacrylamide gel with GelStar™ Nucleic Acid Gel Stain (Lonza). The purified RNA was aliquoted and stored at -80°C.

2.2 Cloning, expression and purification of recombinant TIAR/TIA-1 from E. coli

The constructs of TIAR/TIA-1 that were made and expressed are listed in Figure 2.1. The cDNAs of the human TIARb and TIA-1b were amplified from plasmids pMT2-TIAR and pMT2-TIA-1 (kind gifts from Dr. Anderson, Harvard Medical School, Boston, MA.) and subcloned into pGEX-6P-1 (GE Healthcare) which contains a T7 promoter and expresses a protein fused to an N-terminal glutathione S-transferase (GST)-tag. These plasmids were named
pGEX-TIARb and pGEX-TIA-1b. To construct pGEX-TIA-1a, the appropriate 33-nt sequence (NCBI Reference Sequence: NM_022173) was inserted into the beginning of the RRM2 domain of pGEX-TIA-1b using double-PCR. Plasmids used to express TIARb RRM123, TIA-1a RRM123, and TIA-1b RRM123 were generated by inserting a stop codon at the end of the RRM3 domain in pGEX-TIARb, pGEX-TIA-1a, and pGEX-TIA-1b, respectively. Human TIARb RRM2 cDNA, amplified from pGEX-TIARb with some linker sequences included at both ends was subcloned into pGEX-6P-1 because extra amino acids at both ends were previously suggested to be needed to maintain proper folding of the domain (Kuwasako et al., 2008). Plasmids for expressing TIA-1a RRM2 and TIA-1b RRM2 were constructed from mouse TIA-1 cDNA, but the amino acid sequences of TIA-1a RRM2 and TIA-1b RRM2 are identical between mouse and human. The cDNAs of mouse TIA-1a RRM2 and TIA-1b RRM2 were amplified from pET-TIA-1a and pET-TIA-1b, which was previously constructed from TIA-1 cDNA obtained from C3H/He MEFs, and subcloned into pGEX-6P-1. Similar to pGEX-TIARb, the pGEX-TIA-1b RRM2 insert was also constructed with the same flanking sequences at both ends. The sequences of all clones were verified by restriction enzyme digestion and DNA sequencing.

For protein expression, the validated plasmid DNAs were used to transform Rosetta 2(DE3) pLysS cells (Novagen), which supply rare tRNAs for “universal translation” thus avoiding translational limitations due to alternative codon usage and also carry the T7 lysozyme gene which is a natural inhibitor of T7 RNA polymerase and represses basal expression of target genes under the control of the T7 promoter. Transformed cells were grown in 250 ml of LB medium containing carbencillin (50 µg/ml) and chloramphenicol (34 µg/ml) to an optical density at 600 nm of 0.8 at 37°C. After cooling to room temperature, the cells were induced overnight at
20°C with the lactose analog isopropyl-D-thiogalactoside (IPTG) (0.5 mM). The cells were then centrifuged at 3,000 x g for 15 min and the pellets were stored at -80°C. The GST tagged recombinant proteins were purified by affinity chromatography using a Glutathione Sepharose 4B matrix in the Bulk GST Purification Module (GE Healthcare) according to the manufacturer’s protocol. Briefly, cell pellets were resuspended in 50 ml of lysis buffer [Cellytic Express (Sigma), 1 mM DTT, and complete EDTA-free protease inhibitor cocktail (Roche) in phosphate-buffered saline (PBS) buffer] and incubated at 37°C for 20 min or until the cells were lysed. Cell lysates were centrifuged at 10,000 x g for 20 min and the supernatants were applied to 1 ml of the Sepharose matrix and incubated at 4°C for at least 2 h. The GST-TIA-bound Sepharose were washed three times with PBS buffer and rinsed one time with the PreScission Protease cleavage buffer [50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT]. Then 80 units of PreScission Protease (GE Healthcare) in 1 mL of cleavage buffer was added to the GST-TIA-bound Sepharose and incubated at 4°C for at least 5 h. Cleaved TIAR/TIA-1 proteins and peptides were eluted with cleavage buffer and buffer-exchanged into binding buffer [5 mM HEPES (pH 7.5), 25 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 2mM DTT, and 3.8% glycerol], aliquoted and stored at 4°C for up to a month or at -80°C for longer times. The purified proteins were separated by SDS-PAGE (37.5:1 acrylamide/bis-acrylamide), stained with GelCode Blue Stain Reagent (Thermo Scientific) and analyzed by Western blotting using anti-TIA-1/TIAR (H-120), anti-TIAR (C-18), and anti-TIA-1 (C-20) antibodies (1:5000; Santa Cruz). The secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Cell signaling) or HRP-conjugated anti-goat IgG antibody (Santa Cruz). All antibodies were diluted in 5% nonfat dry milk in TBS plus Tween 20 buffer (50 mM Tris, 150 mM NaCl, pH 8.0, and 0.05% Tween 20).
2.3 **UV crosslinking assay**

*In vitro* synthesized $^{32}$P-labeled RNA probe was diluted in the binding buffer, denatured at 85°C for 10 min and slowly renatured (0.1°C/s) to 20°C. The renatured RNA probe (20,000 cpm; ~580 pM) and 1.1 μM of purified proteins in a final volume of 20 μl of binding buffer were incubated at room temperature for 15 min, and then irradiated at a distance of ~3 cm from the UV light (short wave UV radiation) on ice in GS Gene Linker UV chamber (Bio Rad) for 20 min. After irradiation, the RNA-protein complexes were incubated with RNase A (40 μg) at 37°C for 30 min. After addition of an equal volume of 2x sample buffer (8% SDS, 25% glycerol, 87.5 mM Tris-Cl, pH6.8, 0.02% of bromophenol blue) containing 5% β-mercaptoethanol, the samples were boiled for 5 min, and electrophoresed on a 13.5 % SDS-polyacrylamide gel (37.5:1 acrylamide/bis-acrylamide). The gels were dried and protein-RNA complexes were visualized by autoradiography.

2.4 **Western blotting**

Proteins or protein-RNA complexes in gels were electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in TBST buffer [Tris-buffered saline (TBS) buffer (50 mM Tris and 150 mM NaCl, pH 8.0) plus 0.05% of Tween 20] for 1 h at room temperature, and then incubated with primary antibodies in 5% milk in TBST buffer for 1 h at room temperature. The membranes were then washed with TBST buffer 3 times and incubated with secondary antibodies in 5% milk in TBST buffer for 1 h at room temperature. The membrane was washed with TBST buffer two times and TBS buffer one
time. The primary antibodies used were anti-TIAR (C-18) (1:2000; Santa Cruz) and anti-TIA-1 (C-20) (1:2000; Santa Cruz) antibodies. The secondary antibodies were HRP-conjugated anti-goat IgG antibody (Santa Cruz). The washed membranes were processed for enhanced chemiluminescence with the Super-Signal West Pico detection kit (Pierce) according to the manufacturer’s instructions.

2.5 Gel mobility shift assay and competition gel mobility shift assay

A $^{32}$P-labeled RNA probe diluted in the binding buffer was denatured at 85°C for 10 min and slowly renatured (0.1°C/s) to 20°C. The renatured RNA probe (2,000 cpm; ~58 pM) and different concentrations of purified TIARb (75-1275 nM) or TIARb RRM123 (133-2000 nM) proteins were incubated at room temperature for 30 min in a final volume of 10 µl of binding buffer containing RNase inhibitor and 1 mM of DTT. The RNA-protein complexes were electrophoresed on a 9% nondenaturing polyacrylamide gel (29:1 acrylamide/bis-acrylamide) at 4°C. Gels were dried and visualized by autoradiography or with a phosphoimager (Fuji). To compare the binding activities of the WNV 3' (-) SL RNA and of WNV 5' (+) SL RNA, the same dilutions of a single batch of either purified TIAR or TIAR RRM123 were used for a gel mobility shift assay. For competition gel mobility shift assays, increasing concentrations of specific or non-specific competitor RNA were added to reactions containing constant concentrations of purified TIARb and $^{32}$P-labeled RNA probe. The RNA-protein complexes generated were electrophoresed and analyzed as described for the gel mobility shift assay.
2.6 Hill plot fitting of gel mobility shift assay data

The intensity of the free RNA probe bands in gel mobility shift assays was quantified using Multi Gauge V3.1. The percent of RNA bound was calculated by determining the percent decrease in free RNA probe due to binding to proteins and was plotted against the protein concentration to generate an average theoretical Hill saturation binding curve by “one-site specific binding with Hill slope” using GraphPad Prism 6 and $K_d$ and Hill coefficient ($h$) estimates were determined (Daugherty et al, 2008; Gagnon & Maxwell, 2011; Ryder et al, 2008; Smith, 1998; Yakhnin et al, 2012).

RESULTS

2.7 Detection of a bacterial protein contaminant that efficiently binds to the WNV 3′ (-) SL RNA

A single, fast migrating, shift band was observed when using a partially purified lysate from control E. coli cells (Figure 2.1A). Increased amounts of shifted probe were observed with increasing amounts of lysate. This band was present in the partially purified lysate from TIA-1a-His expressing but not in purified lysates from either control cells or TIA-1a-His expressing cells, unless the amount of sample loaded was increased (Figure 2.1B). A second, broader and slower migrating band near the top of the gel was observed only with lysates from cells expressing TIA-1a-His. Western blotting of the proteins on this gel confirmed that only the upper band contained TIA-1 (Figure 2.1C). In a previously published paper (Emara et al, 2008), the $K_d$ calculated for the TIAR-WNV 3′ (-) SL RNA interaction in gel mobility shift assays included the bacterial protein contaminant band. Since the purified TIA-1a-His still had some of the contaminant, in the present study, TIAR/TIA-1 were expressed with an N-terminal GST-tag and
purified using glutathione sepharose. In addition, the GST-tag was removed after purification of the GST-tagged protein by cleavage. The bacterial contaminant was not detected in gel shift assays done with GST-tagged purified proteins (Figure 2.4 A-F).

2.8 Analysis of GST-tagged and untagged purified TIAR/TIA-1 proteins

To analyze the binding of TIAR/TIA-1 to the WNV 3' (-) SL RNA \textit{in vitro}, plasmids for GST-tagged full-length proteins as well as for the RRM123 and RRM2 peptides of TIARb, TIA-1a, and TIA-1b were constructed, the proteins were expressed in \textit{E. coli} and the tag was removed after protein purification (Figure 2.2). The characteristics of these TIAR/TIA-1 proteins are listed in Table 2.1. The calculated molecular weights of the purified proteins determined from gel migration were consistent with the estimated molecular weights (Figure 2.3A and D). For each of the purified full-length TIAR/TIA-1 protein samples, small amounts of a lower molecular weight protein (~36 kDa) were observed. Attempts to separate this peptide from the correct-size proteins by ion-exchange or sizing column chromatography were not successful. The identity of the purified proteins was confirmed by Western blotting. TIARb was detected by anti-TIAR antibody and TIA-1a and TIA-1b were detected by anti-TIA-1 antibody (Figure 2.3A). The purified TIAR/TIA-1 proteins as well as the RRM123 and RRM2 peptides were able to bind the WNV 3' (-) SL RNA probe in UV crosslinking assays (Figure 2.3C). The same proteins shown in Figure 2.3C without RNA probe were separated in a gel stained with Comassie blue, which is shown in Figure 2.3D. The GST-tag alone was also expressed and shown not to bind to the WNV 3' (-) SL RNA. The crosslinking assay detected additional bands for the full-length and RRM123 proteins which are likely to be multimer complexes (Figure 2.3C).
2.9  Analysis of the gel migration of complexes of untagged TIAR/TIA-1 and WNV 3' (-) SL RNA

To determine whether the complex of WNV 3' (-) SL RNA and untagged TIAR/TIA-1 proteins migrated into the gels, the proteins and RNA-protein complexes on native gels were electrophoretically transferred to membranes and analyzed by Western blotting. A small amount of TIAR was observed in the wells and the amount of protein in the gel increased with increasing protein concentration (Figure 2.4A). In the absence of RNA, TIA-1a and b were detected only close to the well but migrated further into the gel when RNA was present (Figure 2.4B and C). These results indicate that interaction of the positively charged TIAR/TIA-1 proteins with RNA facilitates their migration into gels in the direction of the anode.

2.10  At least two molecules of TIAR/TIA-1 bind to each WNV 3' (-) SL RNA

The binding activities of the purified TIAR/TIA-1 proteins and RRM123 peptides for the WNV 3' (-) SL RNA were analyzed using gel mobility shift assays (Figure 2.5A-F). The mobilities of the RNA-TIAR/TIA-1 complex decreased with increasing protein concentration, suggesting that the binding ratio changed or that an intermediate complex was formed with increasing protein concentration (Hellman & Fried, 2007; Smith, 1998). The data were quantified by calculating the percent of RNA bound (the percent decrease in free RNA probe due to binding to proteins) and followed by analysis using Hill plot fitting (Figure 2.5G and H and Table 2.2). The dissociation constants (K_d) for TIA-1a and TIA-1b are 461 ± 35 nM and 424 ± 72 nM, respectively, indicating that TIA-1a and TIA-1b bind to the WNV 3' (-) SL RNA with similar activities. The K_d for TIARb (296 ± 17 nM) was lower than the K_d for TIA-1a and TIA-1b, indicating that TIARb binds to the WNV 3' (-) SL RNA more efficiently than does TIA-1a/b
The binding data obtained are not consistent with the hypothesis that TIA-1b binds better to the WNV 3' (-) SL RNA than TIA-1a. The Hill coefficient (h), a number that indicates whether the binding of a ligand to a molecule is enhanced or repressed if there are ligands already present on the same molecule, was also calculated from the gel mobility shift assay data. If the h is larger than 1, the binding is enhanced and considered to be positively cooperative. The Hill coefficients calculated for the interactions of the WNV 3' (-) SL RNA with TIARb, TIA-1a, or TIA-1b were 3.2 ± 0.5, 2.9 ± 0.7, and 2.5 ± 1.0, respectively. Also at lower protein concentrations, a faint intensity but faster mobility RNA-protein complex band was detected, while at higher protein concentrations, a series of higher intensity but slower mobility bands were observed. The data indicate that the binding of TIAR/TIA-1 to the WNV 3' (-) SL RNA may occur in two-steps with at least two molecules of full-length of TIAR/TIA-1 binding to each WNV 3' (-) SL RNA. Multiple bands of purified TIAR/TIA-1a/TIA-1b RRM123 peptides were detected in non-reducing SDS-PAGE suggesting that purified RRM123s alone can form an alternative conformation that promotes multimer formation (Figure 2.3B). The gel mobility shift data obtained for TIAR RRM123 showed a four-band shift pattern suggesting various combinations of monomers or dimers can occupy the two binding sites of the RNA (Figure 2.5D). However, the gel shift data for TIA-1a/b RRM123 showed a smear instead of clear multiple bands (Figure 2.5E and F). This may be due to the difference in the charge of TIA-1a/b RRM123 and TIAR RRM123; the estimated pI of TIA-1a/b RRM123 is 7.77, while the pI of TIAR RRM123 is 8.45. The Hill coefficients calculated for all of the full length and RRM123 protein interactions indicate that multiple proteins bind to one molecule of the WNV 3' (-) SL RNA (Figure 2.5G and H).
Previously, the region of TIAR/TIA-1 binding of the WNV 3' (-) SL RNA was mapped to the RRM2 domain (Li et al, 2002). Gel mobility shift assays were next used to analyze the interaction of TIAR RRM2 with the WNV 3' (-) SL RNA. However, the charge of TIAR RRM2 (pI = 9.65) is positive under the conditions for the gel mobility shift assays. The TIAR RRM2 peptide itself was not able to migrate into the native gel and some of the RRM2-RNA complexes had difficulty migrating into the gel. Hence, the estimated K_d of RRM2 obtained from gel shift assays is likely not to be accurate (Figure 2.6). In order to more accurately analyze the physical interaction between RRM2 and the WNV 3' (-) SL RNA, a solution binding assay, isothermal titration calorimetry (ITC), was used. These assays were done by Drs. Zhang and Germann in the Department of Chemistry at GSU. The preliminary results indicated that two or three molecules of TIAR RRM2 bind to one molecule of WNV 3' (-) SL RNA (data not shown).

2.11 The C-terminal prion domain of TIAR contributes to the binding specificity for the WNV 3' (-) SL RNA

To determine whether the C-terminal prion domain of TIAR contributes to the binding activity for the WNV 3' (-) SL RNA, the RNA binding activities of the full length TIAR/TIA-1 proteins were compared to those of their respective RRM123 peptides using gel mobility shift assays. TIARb bound better to the WNV 3' (-) SL RNA than did TIA-1a/b. However, the binding activities of TIARb RRM123 and TIA-1a/b RRM123 for the WNV 3' (-) SL RNA were similar (Figure 2.5G and H and Table 2.2). Although TIARb bound to the WNV 3' (-) SL RNA with higher affinity than to the WNV 5' (+) SL RNA (Figure 2.7C), its RRM123 peptide without the prion domain bound to the WNV 3' (-) SL RNA only slightly better than to the WNV 5' (+) SL RNA.
RNA (Figure 2.7D). These data strongly suggest that the C-terminal prion domain of TIAR/TIA-1 contributes to the binding specificity for the WNV 3' (-) SL RNA.

2.12 TIAR specifically binds the WNV 3' (-) SL RNA and preferentially binds to U-residues in vitro

The nucleotides of the WNV 5' (+) SL RNA are complementary to those of the WNV 3' (-) SL RNA. However, the structures of the two SLs differ due to G-U base pairs. To determine whether the binding of TIAR is specific for the WNV 3' (-) SL RNA, the binding activities of TIAR for the WNV 3' (-) SL RNA and WNV 5' (+) SL RNA were compared in gel mobility shift assays. Binding of TIARb to the WNV 5' (+) SL RNA was observed at a concentration of 300 nM while binding of TIARb to the WNV 3' (-) SL RNA was observed starting at a concentration of 225 nM (Figure 2.7A and B), indicating that TIAR binds better to the WNV 3' (-) SL RNA than to the WNV 5' (+) SL RNA. The RNA binding specificity of TIAR was further analyzed using different RNA competitors in competition gel mobility shift assays. The specific competitors, cold WNV 3' (-) SL RNA and 20U RNA, were able to efficiently compete with the $^{32}$P-labeled WNV 3' (-) SL RNA binding to TIARb (Figure 2.8A). In contrast, the non-specific competitor, tRNA, competed inefficiently with the $^{32}$P-labeled WNV 3' (-) SL RNA (Figure 2.8A). Both specific competitors, cold WNV 5' (+) SL RNA and 20U RNA, and the non-specific competitor tRNA efficiently competed with $^{32}$P-labeled WNV 5' (+) SL RNA binding to TIARb (Figure 2.8B). These data indicate that TIAR specifically binds to the WNV 3' (-) SL RNA but not to the WNV 5' (+) SL RNA.

To further analyze the nucleotide binding preference of TIAR, competition gel mobility shift assays were next done with polyA, polyU, polyC, or polyG competitors. PolyU at 3.1 ng/µl
efficiently competed with the WNV 3' (-) SL RNA (Figure 2.9A). PolyC was the next most efficient at competing with the probe. PolyA and polyG competed with lower efficiency (Figure 2.9B). The results indicated that TIAR preferentially binds to Us in the WNV 3' (-) SL RNA. Similar results were obtained using purified TIA-1a protein.

**DISCUSSION**

Previously published data showed a slight decrease in WNV yield and upregulation of TIA-1b in TIAR knockout cells. *In vitro* binding assay data showed that the TIAR RRM2 binds to the WNV 3' (-) SL RNA 10 times better than TIA-1 RRM2 (Li et al, 2002). TIARb is the major isoform in cells. Hence, we hypothesized that TIA-1b, with a length similar to TIARb, binds to the WNV 3' (-) SL RNA more efficiently than TIA-1a. In this study, the full-length TIARb protein was found to bind 1.5-fold better to the WNV 3' (-) SL RNA than either isoform of TIA-1 but the binding activities of TIA-1a and TIA-1b for the WNV 3' (-) SL RNA were found to be similar. TIA-1a and TIA-1b were also reported to have similar RNA binding activities to the cellular RNA msl-2 and to Fas 5'-splice site regions (Izquierdo & Valcarcel, 2007a). These data suggest that the up-regulated TIA-1b in TIAR-knockout cells did provide more efficient binding to the WNV 3' (-) SL RNA. Instead, the overall increase in TIA-1 was able to compensate for the loss of TIAR function in TIAR knockout cells so that the reduction of WNV replication in TIAR knockout cells was small (Li et al, 2002). In order to further analyze the effect of TIAR on WNV replication, TIAR-overexpressing cells were used and the results obtained are discussed in Chapter 3.

The WNV 3' (-) SL RNA is complementary to the 5' (+) SL RNA sequence, which was reported as a promoter to be required for minus-strand RNA synthesis in the context of a
cyclized genome RNA template (Lodeiro et al., 2009). Comparison of the binding activity of TIAR for the WNV 3' (-) SL and the 5' (+) SL RNA indicated that TIAR binds specifically to the WNV 3' (-) SL RNA but not to the WNV 5' (+) SL RNA.

The observation that the mobility of the RNA-TIAR/TIA-1 shift bands decreased with increasing protein concentration and that the Hill coefficients for the TIAR/TIA-1 interactions with the WNV 3' (-) SL RNA were larger than 1 suggested that multiple TIAR/TIA-1 proteins bind cooperatively to each WNV 3' (-) SL RNA. The TIAR/TIA-1 binding sites on the WNV 3' (-) SL RNA were previously mapped to L1 and L2 (Emara et al., 2008). It is likely that one molecule of TIAR/TIA-1 binds to L1 and another binds to L2. However, it is also possible that one or more TIAR/TIA-1 molecules could interact with the initial RNA-protein complex through protein-protein interactions since the calculated Hill coefficients indicate there are about 3 proteins interacting with 1 RNA. Preliminary binding data obtained with ITC assays for the interaction of TIARb and a linear RNA containing only the single-stranded L1 and L2 region of the WNV 3' (-) SL RNA (20-nt) fit best to a two-step binding model. The first step $K_d$ was 60.6 nM and the second step $K_d$ was 23.6 nM, indicating that the RNA-TIAR interaction is a positively cooperative interaction (Zhang and Germann, unpublished data). However, although the 20-nt RNA contains the binding sites, they are not in the context of the SL structure. Further ITC studies of TIAR interacting with a complete or nearly complete WNV 3' (-) SL RNA are needed. Overall, the gel shift data and the previous mapping data suggest a two-site cooperative binding model for the TIAR/TIA-1- WNV 3' (-) SL RNA interaction.

A model of the interaction of TIAR with the WNV 3' (-) SL RNA is proposed based on a tertiary structure of WNV 3' (-) SL RNA predicted by the 3d RNA web server (http://biophy.hust.edu.cn/3dRNA/3dRNA.html; Zhao et al., 2012). L2 of WNV 3' (-) SL RNA
forms an open loop while L1 is embedded in the double-stranded stem helix. Also, L1 and L2 are located on opposite sides of the structure (Figure 2.10A). Previous mapping data showed that TIAR-binding to a C-substituted L2 was weaker than that to C-substituted L1 (Emara et al, 2008), suggesting that the L2-TIAR interaction is required for complete TIAR binding to the L1. We propose that L2 is more easily assessable for initial TIAR binding and then L1 becomes available only after a conformational change due to the L2-TIAR interaction. Recent ITC data also suggested that flexibility in the stem structure of the WNV 3' (-) SL RNA is required for efficient binding to TIAR (Zhang and Germann, unpublished data). Previous data showed that deletion of either L1 or L2 in a WNV infections clone produced no detectable virus. Single nucleotide mutations or substitutions in the UA-rich sequence of L1 reduced or inhibited WNV replication, but single nucleotide mutations or substitutions in L2 did not significantly affect virus replication (Emara et al, 2008). The data suggest that TIAR/TIA-1 binding at both L1 and L2 is necessary for efficient WNV replication. According to the proposed model, one TIAR molecule binds to L2 of the WNV 3' (-) SL RNA which leads to a conformational change in the RNA that facilitates binding of another TIAR molecule to L1 (Figure 2.10B). The prion domain may enhance protein folding that is optimal for interaction with the WNV 3' (-) SL RNA.
<table>
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<tr>
<th>Protein</th>
<th>Amino acids</th>
<th>pI</th>
<th>MW. (Da)</th>
<th>Extinction Coefficient&lt;sup&gt;1&lt;/sup&gt;</th>
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<tbody>
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<td>380</td>
<td>7.74</td>
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<td>7.74</td>
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<td>9.70</td>
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<td>19480</td>
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<sup>1</sup> Extinction coefficient was calculated based on assuming that all Cys residues are reduced.
Table 2.2. Dissociation constants and Hill coefficients of purified TIAR/TIA-1 proteins and their truncations determined using a WNV 3' (-) SL RNA probe in gel shift assays

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_d$ (nM)$^1$</th>
<th>Hill Coefficient (h)$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIARb</td>
<td>296 ± 17</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>TIA-1a</td>
<td>461 ± 35</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td>TIA-1b</td>
<td>424 ± 72</td>
<td>2.5 ± 1.0</td>
</tr>
<tr>
<td>TIARb RRM123</td>
<td>382 ± 56</td>
<td>2.9 ± 1.0</td>
</tr>
<tr>
<td>TIA-1a RRM123</td>
<td>372 ± 24</td>
<td>3.8 ± 0.8</td>
</tr>
<tr>
<td>TIA-1b RRM123</td>
<td>322 ± 9</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>TIAR RRM2</td>
<td>1804 ± 60</td>
<td>2.6 ± 0.2</td>
</tr>
</tbody>
</table>

$^1$The equilibrium dissociation constant ($K_d$) and Hill coefficient (h) were determined from gel shift assays as Hill plot fitting curve using GraphPad Prism 6. The $K_d$ is the protein concentration that binds to half of the RNA at equilibrium. Hill coefficient greater than 1.0 indicates positively cooperative binding.
Figure 2.1. A bacterial protein contaminant in previous preparations of purified TIAR binds efficiently to the WNV 3' (-) SL RNA.

(A) Gel mobility shift assays done with increasing concentrations of an *E. coli* lysate partially purified on a cobalt column and a 32P-WNV 3' (-) SL RNA probe. (B) Gel mobility shift assays done with partially purified or purified *E. coli* lysates from cells expressing or not expressing TIA-1a-His and a 32P-WNV 3' (-) SL RNA probe. The complexes were analyzed on nondenaturing polyacrylamide gels (5% 29:1 acrylamide/bis-acrylamide). The images were detected with a Phosphoimager. Lane 1: probe only, Lane 2: partially purified *E. coli* lysate (1X), Lane 3: purified *E. coli* lysate (4X), Lane 4: partially purified TIA-1a-His (0.1X), Lane 5: partially purified TIA-1a-His (1X), Lane 6: purified TIA-1a-His (1X), Lane 7: purified TIA-1a-His (4X).

(C) Western blot analysis of the proteins in the gel shown in panel B. After gel shift assay, the proteins were transferred to a membrane and detected using anti-TIA-1 (C-20) antibodies. FP, free probe.
Figure 2.2. Schematic diagrams of the purified TIA peptides.
Human TIARb, TIA-1a, and TIA-1b coding sequences were used, with the exception of the TIA-1a RRM2 and TIA-1b RRM2 sequences that were from mouse. However, the amino acid sequences of the TIA-1 RRM2s in mouse are the same as in human. TIAR (orange) and TIA-1 (blue) amino acid sequences have 80% similarity. All peptides were fused to an N-terminal GST tag and expressed in *E. coli*.
Figure 2.3. Purified TIA proteins and truncated peptides bind to the WNV 3' (-) SL RNA using UV crosslinking assays.

(A) Purified TIARb, TIA-1a, TIA-1b proteins were treated with β-mercaptoethanol and boiled for 5 min and then analyzed by SDS-PAGE, stained with Coomassie blue or blotted using anti-TIAR antibody (C-18, Santa cruz) or anti-TIA-1 antibody (C-20, Santa cruz). (B) Purified TIA proteins and truncated peptides that were not treated with reducing reagents or boiled were analyzed by SDS-PAGE, and the gel was stained with Coomassie blue. (C) UV crosslinking assays done with purified TIA proteins or truncated peptides and the 32P-labelled WNV 3' (-) SL RNA probe. RNase treated RNA-protein complexes were treated with β-mercaptoethanol and boiled for 5 min and then analyzed by SDS-PAGE. The image was detected by autoradiography. (D) Coomassie blue staining of the proteins in the gel shown in panel C.
Figure 2.4. TIAR/TIA-1 protein - WNV 3' (-) SL RNA complexes efficiently migrate into gels.

Gel shift assays were done with WNV 3' (-) SL RNA and increasing amounts of purified (A) TIARb, (B) TIA-1a or (C) TIA-1b and the complexes were electrophoresed on non-denaturing polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane and detected with anti-TIAR antibody or anti-TIA-1 antibody. The probe used in panel A was $^{32}$P- WNV 3' (-) SL RNA (2000 cpm, 58 pM) and in panel B and C, cold WNV 3' (-) SL RNA (30 nM) was used.
Figure 2.5. More than one TIAR/TIA-1 protein binds to the WNV 3' (-) SL RNA in a positively cooperative manner.

Gel mobility shift assays done with a $^{32}$P-WNV 3' (-) SL RNA probe and increasing concentrations of purified (A) TIARb, (B) TIA-1a, (C) TIA-1b, (D) TIARb RRM123, (E) TIA-1a RRM123, and (F) TIA-1b RRM123. Complexes were analyzed on non-denaturing polyacrylamide gels (9% 29:1 acrylamide/bis-acrylamide). The images were detected with a phosphoimager. Each experiment was repeated at least twice, and representative data are shown. (G) and (H) The percent of RNA bound in each experiment was quantified for samples from two to four experiments using Multi Gauge V3.1 and plotted against the concentration of protein to generate an average theoretical Hill saturation binding curve using GraphPad Prism 6. Bars indicate standard error. The predicted overall $K_d$ of each purified protein is listed in Table 2.2.
Figure 2.6. The binding of TIAR RRM2 to the WNV 3' (-) SL RNA is positively cooperative.

(A) Gel mobility shift assays done with a $^{32}$P-WNV 3' (-) SL RNA probe and increasing concentrations of purified TIAR RRM2. Complexes were analyzed on non-denaturing polyacrylamide gels. The image was detected with a phosphoimager. The experiment was repeated at least twice, and representative data are shown. (B) The percent of RNA bound in each experiment was quantified using Multi Gauge V3.1 and plotted against the concentration of proteins to generate an average theoretical Hill saturation binding curve using GraphPad Prism 6. Bars indicate standard error. The predicted overall $K_d$ of TIAR RRM2 is listed in Table 2.2.
Figure 2.7. TIAR interacts more efficiently with the WNV 3' (-) SL RNA than the WNV 5' (+) SL RNA.

(A) Gel mobility shift assays done with the $^{32}$P-WNV 5' (+) SL RNA probe (2000 cpm) and increasing amounts of purified TIARb (75-1275 nM) or TIARb RRM123 (133-2000 nM). (B) Gel mobility shift assays done with the $^{32}$P-WNV 3' (-) SL RNA probe and increasing amounts of purified TIARb or TIARb RRM123. Complexes were analyzed on non-denaturing polyacrylamide gels. The images were detected by autoradiography. FP, free probe. PD, probe dimer. (C) and (D) The percent of RNA bound to the probes in the gels shown in (A) and (B) was quantified using Multi Gauge V3.1 software and plotted against the concentration of TIARb or TIARb RRM123 to generate a theoretical Hill saturation binding curve using GraphPad Prism 6. The same batch of purified protein and dilutions were used in one experiment to compare protein interactions with the two RNAs. Each experiment was repeated twice and representative data are shown.
Figure 2.8. The interaction of TIAR with the WNV 3' (-) SL RNA is specific.

(A) Competition gel mobility shift assays done with a $^{32}$P-labeled WNV 3' (-) SL RNA probe. (B) Competition gel mobility shift assays done with a $^{32}$P-labeled WNV 5' (+) SL RNA probe. Increasing concentrations of a specific competitor RNA [cold probe or a 20 nucleotide polyU (20rU)] or a nonspecific competitor RNA (yeast tRNA) were added to reactions containing $^{32}$P-labeled RNA (2000 cpm; 58 pM) and 500 nM of TIARb. Complexes were analyzed on non-denaturing polyacrylamide gels. The images were detected by autoradiography. Each experiment was repeated at least twice and representative data are shown.
Figure 2.9. TIAR preferentially binds to U residues.
Competition gel mobility shift assays were done using a $^{32}$P-WNV 3' (-) SL RNA probe. Increasing concentrations of (A) cold WNV 3'(-) SL RNA, polyA or polyU, or (B) tRNA, polyC or polyG were added to reactions containing $^{32}$P-labeled RNA (2000 cpm; 58 pM) and 700 nM of purified TIARb protein. RNA-protein complexes were analyzed on non-denaturing polyacrylamide gels. The images were detected by autoradiography.
Figure 2.10. Proposed model of the interaction of TIAR with the WNV 3' (-) SL RNA. (A) Tertiary structure of the WNV 3' (-) SL RNA predicted using the 3d web server (Zhao et al., 2012) and displayed using Pymol software. (B) Two-step model of TIAR binding to the WNV 3' (-) SL RNA. One molecule of TIAR binds to the exposed loop (L2) of the RNA through the RRM2 domain. This results in a conformational change in the RNA that facilitates another TIAR binding to L1.
CHAPTER 3
TIAR Interacts with the 3’ Stem Loop of the West Nile Virus (WNV) Minus-Strand RNA in Infected Cells and Facilitates WNV RNA Replication

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne flavivirus. The virus is endemic in Africa, the Middle East, parts of Europe and the former Soviet Union, south and central Asia, Australia, and recently North America (Mackenzie et al, 2004). Most WNV infections cause asymptomatic or mild disease in humans but can cause severe diseases, including meningitis, encephalitis, and poliomyelitis. There are no specific anti-viral therapies and only supportive treatment is currently available for treating WNV infections. No WNV vaccines are available for humans (Kramer et al, 2007). WNV and other members of the genus *Flaviviruses*, such as Japanese encephalitis virus (JEV), dengue virus (DENV), and yellow fever virus have a similar genome organization and share common replication mechanisms. The genome of flaviviruses is about 11 kb in length, and is a single-stranded, positive-sense RNA with a type I cap at the 5’ end and a highly structured 3’ untranslated region (UTR) without a polyA tail. The viral replication cycle occurs in the cytoplasm of infected cells. Virions enter cells by endocytosis and the genome then enters the cytoplasm by fusion of the viron and endosomal membranes. After the viral genome is released, it is translated and then serves as a template for minus-strand RNA synthesis (Lindenbach et al, 2013). Initially, equal amounts of plus-strand and minus-strand RNAs are produced and the genome RNAs switch back and forth between translation and transcription functions. Later after a sufficient amount of viral proteins have been produced and they have induced membrane remodeling, there is an exponential increase in the production of plus strand RNA (Chu & Westaway, 1985; Cleaves et al, 1981). Evidence from electron tomography experiments
indicates that the exponential viral genome RNA synthesis occurs in virus-induced ER membrane invaginations. The viral genome and structural proteins are assembled at the ER membrane and viral particles bud into the lumen of the ER (Miorin et al, 2013; Welsch et al, 2009). Nascent viral particles are transported through the Golgi and exit at the cell surface by fusion of viron containing vesicles with the plasma membrane. A complete WNV replication cycle takes around 10-12 hours (Chambers et al, 1990).

Sequences located in the 5' UTR as well as in the capsid coding region and in the 3' UTR of the viral genome form conserved secondary stem-loop RNA structures (Thurner et al, 2004). Deletions or mutations within the 5' terminal stem loop (SL), SLA, in a WNV and DENV infectious clone or of the 3' terminal SL in a WNV infectious clone inhibited virus replication (Cahour et al, 1995), but did not affect viral RNA translation (Davis et al, 2007; Emara et al, 2008; Filomatori et al, 2006; Lodeiro et al, 2009). Viral RNA synthesis is initiated \textit{de novo} from the 3' end of the template by the viral RNA-dependent RNA polymerase (RdRp), NS5, without an RNA primer (Choi & Rossmann, 2009). NS5 does not bind specifically to the 3' SL of viral genome RNA but instead binds specifically to the 5' SL through its methyltransferase domain (Dong et al, 2008; Zhang et al, 2008b). The 5'-3' RNA cyclization and adjacent sequences of flavivirus genomes have been shown to be required for viral RNA synthesis but not for viral translation. The 5'-3' interaction brings the NS5 protein bound on the 5' SL in proximity to the 3' end of the genome facilitating minus-strand initiation (Friebe & Harris, 2010; Friebe et al, 2011; Zhang et al, 2008a). The mechanism regulating viral RNA cyclization is not known and little is known about the mechanism regulating plus-strand RNA synthesis (Lindenbach et al, 2013; Saeedi & Geiss, 2013). The 3' terminal sequence of the viral minus-strand RNA is required for
initiation of viral plus-strand (genome) RNA synthesis and is complementary to the sequence of the 5' end of the viral genome RNA.

Like most single stranded RNA viruses, the WNV genome encodes a small number of proteins and evidence suggests that these viruses use cellular proteins to facilitate most phases of their replication cycle in cells (Ortin & Parra, 2006). RdRp alone was able to copy most primed RNA templates in vitro in cell extracts (Tan et al, 1996) and it has been proposed that cellular proteins facilitate RdRp being specifically recruited and/or positioned on to viral RNA templates for viral RNA synthesis (Lai, 1998). Multiple cellular proteins have been reported to be involved in viral RNA synthesis by plus-strand RNA viruses including RNA binding proteins (hnRNPs, PCBP2, PABP, PTB, TIAR, La), translation factors (EF-1α, β, γ, eIF-3, and ribosomal proteins), cytoskeletal proteins (tubulin and actin), and RNA and protein chaperones (helicases and heat-shock proteins) (Lai, 1998; Nagy & Pogany, 2012). However, for only a few of these have functional studies been done.

Previous in vitro binding studies done in our lab showed that different sets of cellular proteins bind to the WNV 3' terminal SLs of the genomic and minus-strand RNAs (Blackwell & Brinton, 1995; Shi et al, 1996). One of the three proteins that binds to the 3' (+) SL was identified as elongation factor-1 alpha (eEF-1α) (Blackwell & Brinton, 1997). The interaction between the 3' (+) SL RNA and eEF-1α positively regulates WNV minus-strand RNA synthesis (Davis et al, 2007). Four proteins were detected binding to the WNV 3' (-) SL RNA and one was identified as T-cell intracellular antigen-1 (TIA-1) related protein (TIAR) (Li et al, 2002; Shi et al, 1996). TIAR and the closely related protein TIA-1 are AU-rich element (ARE) binding proteins. The N terminal region of TIAR/TIA-1 contains three RNA recognition motif (RRM) domains and the C-terminal region contains a prion-related domain (PRD) (Dember et al, 1996;

WNV progeny particle production decreased slightly in TIAR knock-out cells compared to wild type cells but there was no difference in WNV production in TIA-1 knock-out cells (Li et al, 2002) indicating that these two proteins provide redundant functions for WNV replication. Mutation of the mapped TIAR/TIA-1 binding sites in the 3' (-) SL RNA in a WNV infectious clone reduced or inhibited virus replication (Emara et al, 2008), suggesting that these cell proteins facilitate WNV genome RNA replication in infected cells. In vitro analysis of RNA-protein interactions showed that TIAR bound better than TIA-1 to the WNV 3' (-) SL RNA. In this part of the current study, we analyzed the interaction of TIAR and WNV viral RNA in WNV-infected cells and the effects of TIAR overexpression on WNV genome RNA synthesis. Using a more sensitive immunofluorescence assay, proximity ligation assay (PLA), which can detect two molecules located < 40 nm apart inside cells, we showed colocalization of TIAR with viral dsRNA in perinuclear regions of infected cells. Immunoprecipitation of RNA-protein complex experiments detected the interaction of TIAR with the WNV 3' (-) SL RNA in WNV-infected cells. An increased ratio of viral plus-strand RNA levels to minus-strand RNA levels was observed in the WNV-infected cells overexpressing TIAR. These results provide additional evidence that TIAR binds to the 3' terminus of WNV minus-strand RNA in infected cells and is involved in enhancing WNV genome RNA synthesis.
MATERIALS AND METHODS

3.1 Cell lines  

Baby hamster kidney 21 strain W12 (BHK) cells and C3H/He mouse embryo fibroblasts (MEFs) were maintained in minimal essential medium (MEM; Gibco) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and 10 µg/ml of gentamicin at 37°C in a 5% CO₂ atmosphere. A monkey kidney cell line, MA104, was maintained in MEM supplemented with 10% FBS and 10 µg/ml of gentamicin at 37°C in a 5% CO₂ atmosphere. A human lung adenocarcinoma epithelial cell line (A549) was maintained in F-12K Nutrient Mixture (Kaighn’s) (Gibco) supplemented with 10% FBS and 100 units/ml of penicillin and 100 µg/ml of streptomycin at 37°C in a 5% CO₂ atmosphere. Clonal A549 cell lines stably expressing TIAR were established by transfecting cells with the pEF6-HA-TIARb plasmid (EF-1α promoter) using Lipofectamine 2000 (Invitrogen), followed by cell selection with blasticitin (10 µg/ml). Single cell clones were selected with cloning rings and maintained in drug-containing media. The level of recombinant protein expression for each clone was analyzed by Western blotting. The clones, A549-TIAR-CL1, 2, and 5 were used for experiments.

3.2 Viruses  

A stock of WNV strain Eg101 was prepared by infecting a monolayer of BHK cells at a multiplicity of infection (MOI) of 0.1 and harvesting culture fluid at 32 h after infection. Clarified culture fluid (1x10⁸ PFU/ml) was aliquoted and stored at -80°C.
### 3.3 Indirect immunofluorescence assay (IFA)

Cells grown to ~80% confluency on 12 mm diameter coverslips (Fisher Scientific) in 24-well plates were infected with WNV at an MOI of 5. At 24 h after WNV infection or mock-infection, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS, washed with PBS, and blocked with 5% horse serum in PBS for 1 h at room temperature. Cells were then incubated with primary antibodies in PBS plus 5% horse serum for 1 h at room temperature. Viral replication complexes (containing predominantly genome (+) RNA products being copied from minus-strand templates) were detected with mouse anti-dsRNA antibody (1:1000; English & Scientific Consulting, Szirak, Hungary). TIAR was detected with goat anti-TIAR antibody (C-18) (1:200; Santa Cruz) or rabbit anti-TIAR antibody (1:100; Cell signaling). TIA-1 was detected with goat anti-TIA-1 antibody (C-20) (1:200; Santa Cruz). Rabbit anti-calnexin antibody (1:400; Cell Signaling) was used to detect the endoplasmic reticulum (ER). Rabbit anti-GAPDH antibody (1:2000; Sigma) was used to detect a soluble cytoplasmic protein. After incubation with primary antibodies, cells were washed three times for 10 min with PBS and then incubated with appropriate secondary antibodies (Alexa Fluor® 488 or 594 conjugated donkey anti-mouse or anti-goat IgG antibodies from Invitrogen) and 0.5 µg/ml of Hoechst 33258 dye (Invitrogen) in PBS plus 5% horse serum for 1 h at room temperature and then washed three times in PBS. Coverslips were mounted onto glass slides using ProLong Gold antifade reagent (Invitrogen). Images were obtained using a confocal laser scanning microscope, LSM 510 (Zeiss), or a widefield fluorescent microscope, Axio Observer Z1 microscope (Zeiss), with a 63x or 40x oil immersion objective. For protein colocalization analysis, image stacks from the widefield microscope were recorded at 200 nm.
intervals and subjected to “iteractive deconvolution” using Volocity software (PerkinElmer). The images presented were linearly contrast enhanced to increase clarity.

3.4 Proximity ligation assay (PLA)

Cells grown to ~80% confluency on 10 mm diameter coverslips (Ted Pella) in 24-well plates were infected with WNV at an MOI of 5. At 24 h after infection, cells were fixed with 1% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS, washed with PBS, and blocked with 5% horse serum in PBS for 30 min at 37°C. Cells were incubated with two primary antibodies in 5% horse serum in PBS for 30 min at 37°C and then washed with Duolink wash solution (Olink Bioscience). The primary antibodies were used as described for IFA, except that the antibody concentrations used were 2 fold lower. Cells were then incubated with two corresponding PLUS and MINUS oligonucleotide-labelled PLA probes diluted in Duolink In Situ Antibody Diluent (Olink Bioscience) for 1 h at 37°C and washed with Duolink wash solution. Cells were next treated with ligation and rolling-circle amplification reactions according to the manufacturer’s protocol. Cells were subsequently immunostained with appropriate secondary Alexa Flour antibodies in PBS for 30 min at 37°C. Cells were stained with Hoechst dye in PBS for 5 min at 37°C and coverslips were mounted onto glass slides. Images were taken using a widefield fluorescent microscope as described for IFA. The number of PLA punctae in the cytoplasm per cell was quantified using Volocity software (PerkinElmer) (Jung et al, 2013). The PLA punctae were identified by the “find objects using standard deviation (SD) intensity,” then separated into individual punctae using the “separate touching objects” tool, and filtered based on size. The punctae located in the nucleus was
excluded from the calculation. Twenty to forty representative cells were analyzed. The data was plotted using GraphPad Prism 6.

3.5 Immunoprecipitation of intracellular RNA-protein complexes

RNA-TIAR complexes were immunoprecipitated using anti-TIAR antibody and an RNA-binding protein immunoprecipitation kit, Magna RIP™ (Millipore). Confluent monolayers of C3H/He MEFs in T-150 flasks were mock infected or infected with WNV at an MOI of 5 for 9, 12, or 16 h. Cells were washed twice with ice-cold PBS and scraped into 5 ml of ice-cold PBS. Cell lysates were transferred to a centrifuge tube and centrifuged at 1,500 rpm for 5 min at 4°C. Cells were resuspended in a volume (~100 µl) of complete RIP Lysis Buffer (Millipore) equal to the cell pellet volume and mixed by pipetting. The cells were incubated on ice for 5 min to allow the hypotonic RIP buffer to swell the cells and then stored at -80°C. Cell lysates were thawed and clarified by centrifugation at 14,000 rpm for 10 min at 4°C. At least 210 µl of supernatant was used for each immunoprecipitation experiment. Ten µl was used as the input, 100 µl was incubated with 5 µg of the control anti-goat IgG antibody-conjugated protein A/G magnetic beads and 100 µl was incubated with 5 µg of anti-TIAR antibody-conjugated protein A/G magnetic beads in RIP Immunoprecipitation Buffer (Millipore) at 4°C overnight. The bead samples were washed 6 times with RIP Wash Buffer (Millipore). A magnetic separator was used to recover the beads. The beads and the saved input sample were then incubated at 55°C for 30 min in Proteinase K Buffer (Millipore) and the beads were removed. RNA was extracted using TRI Reagent (Molecular Research Center), dissolved in 20 µl of RNase-free water and stored at -80°C. The terminal regions of the viral plus and minus strand RNAs as well as a negative control RNA, GAPDH 3' UTR, were amplified by RT-PCR using the SuperScript III One Step RT-PCR
system with Platinum Taq (Invitrogen). Primer sequences are listed in Table 3.1. One specific primer and 1 µl of RNA were added in the enzyme mix for the RT reaction done at 50°C for 30 min, and after inactivation of the RT enzyme at 94°C for 10 min, the reverse primer was added and a PCR of 25 cycles was done. RT-PCR products were analyzed on agarose gels and the band intensity was quantified using Multi Gauge V3.1 software. The relative band intensity levels in each sample were calculated by setting the intensity of the 10% input band at 100.

3.6 Plaque assay

Viral infectivity titers were determined by infecting monolayers of BHK cells in duplicate wells of a six-well plate with serial 10-fold dilutions of a virus sample. After adsorption for 30 min at 37°C, the virus inoculum was removed and the cells were overlaid with MEM containing 2.5% FBS and 0.5% SeaKem ME agarose (Bio-Whittaker Molecular Applications), and incubated for 72 h at 37°C in a 5% CO₂ atmosphere. After removal of the agarose plugs, cells were stained with 0.05% crystal violet in 10% ethanol.

3.7 Western blotting

Confluent (~95 %) monolayers of A549 or A549-TIAR cells in six-well plates were mock infected or infected with WNV at an MOI of 0.1 for 16, 24, or 32 h. Cells were washed with PBS and scraped in 150 µl of cell lysis buffer (1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate and protease/phosphatase inhibitor in PBS). After addition of an equal volume of 2x sample buffer (8% SDS, 25% glycerol, 87.5 mM Tris-Cl, pH6.8, 0.02% of bromophenol blue) containing 5% β-mercaptoethanol, the cell lysates were boiled for 5 min. Proteins in the lysate
samples were separated by 10% SDS-PAGE and then electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in TBST buffer [Tris-buffered saline (TBS) buffer (50 mM Tris and 150 mM NaCl, pH 8.0) plus 0.05% of Tween 20] for 1 h at room temperature, and then incubated with primary antibodies in 5% milk in TBST buffer for 1 h at room temperature, except for the membrane with anti-p-eIF2α antibody which was blocked and incubated in 5% bovine serum albumin in TBST buffer. The membranes were then washed with TBST buffer 3 times and incubated with secondary antibodies in 5% milk in TBST buffer for 1 h at room temperature. The membrane was washed with TBST buffer two times and TBS buffer one time. The primary antibodies used were anti-NS3 (1:1000; R&D Systems), anti-HA (1:1000; Convance), anti-eIF2α (1:1000; Cell Signaling), anti-p-eIF2α S51 (1:1000; Cell Signaling), and anti-β-actin (1:5000; Abcam) antibodies. The secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG antibodies (Cell signaling) and HRP-conjugated anti-goat IgG antibody (Santa Cruz). The washed membranes were processed for enhanced chemiluminescence with the Super-Signal West Pico detection kit (Pierce) according to the manufacturer’s instructions.

3.8 Real-time quantitative (q) RT-PCR

Confluent (~95%) monolayers of A549 or A549-TIAR overexpressing cells in six-well plates were mock infected or infected with WNV at an MOI of 0.1 for 2, 16, 24, or 32 h. Cells were washed with PBS and then 1 ml of TRI Reagent (Molecular Research Center) was added to the well to lyse the cells. Total intracellular RNA was extracted according to the manufacturer’s instructions. Specific RNAs were quantified using a TaqMan one-step RT-PCR master mix reagent kit (Applied Biosystems) on an Applied Biosystems 7500 Fast real-time PCR system
according to the manufacturer’s protocol. WNV Eg101 RNA was analyzed using previously designed WNV Eg101 NS1 region-specific primers and probe (Sigma) (Scherbik et al, 2006). β-actin mRNA was detected using human β-actin primers and probe (Applied Biosystems) and used as the endogenous control. The cycling parameters were reverse transcription at 48°C for 30 min, AmpliTaq activation at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Each sample was assayed in triplicate. The Ct values obtained for each sample were normalized to those for β-actin in the same sample and presented as the relative fold change compared to the 2 h infected A549 cell sample in relative quantification (RQ) units using the comparative Ct (ΔΔCt) method of the SDS Applied Biosystems software which also applied statistical analysis to the data. Error bars represent the standard error of the mean (SEM) and indicate the calculated minimum (RQMin) and maximum (RQMax) of the viral RNA expression levels based on a RQMin/Max of the 95% confidence level. The viral RNA levels obtained for two cell lines were considered statistically different (p<0.05) when the error bars did not overlap. The data were exported and plotted in Microsoft Excel.

For the specific detection of either viral plus or minus-strand RNA, T7-tagged primer qRT-PCR was performed as previously described (Davis et al, 2007) using a TaqMan RNA-to-Ct 1-Step kit (Applied Biosystems). Total cell RNA was extracted from monolayers of A549 or A549-TIAR cells infected with WNV Eg101 at an MOI of 5. The specific primers and probe were designed to the WNV Eg101 NS1 region. For viral plus-strand RNA detection, the T7-NS1-Rev primer: 5'-[T7]CTCCGATTGTGGTTGCTTC-3' was used for the reverse transcriptase (RT) reaction at 48°C for 30 min and the T7 primer: 5'-GCGTAATACGACTCACTATA-3', the NS1-For primer: 5'-GGCGGTCCTGGGTGAAGTCAA-3' and the NS1 probe: 5'-6FAM-
TGCACTTGGCCTGAAACGCACACTTTGT-TAMRA-3’ were added for the real time PCR step after inactivation of RT at 95°C for 15 min. For viral minus-strand RNA detection, the T7-NS1-For primer: 5’-[T7]GGCGGTCTGGGTGAAGTC-3’ was used for the RT reaction and the T7 primer, the NS1-Rev primer: 5’-CTCCGATTGTGGTGCTTCGT-3’ and the NS1 probe were used for the real time PCR step. Real time PCR was performed as follows: 40 cycles of 95°C for 15 s and 60°C for 1 min. To evaluate the increase in viral plus-strand RNA levels, the amount present at 2 h was subtracted from the amounts obtained at subsequent times after infection. To evaluate the increase in viral minus-strand RNA levels, the amount present at 12 h was subtracted from the amounts obtained at subsequent times after infection. The ratio of viral plus-strand RNA levels to minus-strand RNA levels was then calculated. The significance of the difference between ratios obtained for the two cell lines was analyzed by the Student’s t-test (p<0.05) using Microsoft Excel.

RESULTS

3.9 TIAR colocalizes with viral dsRNA in replication complexes in the WNV-infected cells

Previously our lab reported that TIAR relocalized from the nucleus to the cytoplasm and completely colocalized with viral dsRNA in the perinuclear region in WNV-infected BHK cells using Texas red- and FITC-labeled secondary antibodies (Emara & Brinton, 2007). However, in recent studies, the majority of TIAR was observed to localize in the nucleus with some in the cytoplasm in mock-infected BHK cells and no significant change in the location of TIAR was observed in the WNV-infected BHK cells when both anti-TIAR and anti-dsRNA antibodies were used with Alexa Fluor-conjugated secondary antibodies (Figure 3.1A). TIAR was also found to
be primarily located in the nucleus in WNV-infected BHK cells using anti-TIAR antibody alone and an Alexa Fluor-conjugated secondary antibody. These results suggest that the previously reported results were a technical artifact caused by an interaction between the secondary antibodies used. TIAR localization was next analyzed in additional cell lines after WNV infection using the Alex Fluor antibodies. In MEFs, similar to BHK cells, TIAR was primarily located in the nucleus in mock-infected MEF cells and no obvious change was observed in the WNV-infected MEF cells (Figure 3.1B). However, in about 50% of infected human lung A549 and monkey kidney MA104 cells after 24 h WNV infection, TIAR was enriched in regions of the perinuclear region where viral replication complexes were located and some of the cytoplasmic TIAR appeared to be in small foci (Figure 3.1C-D). Colocalization was observed between some of the adjacent TIAR foci and viral replication complexes. The same pattern of TIAR distribution in infected A549 cells was observed when using anti-TIAR antibody either alone or with anti-dsRNA antibody and the Alexa-Fluor-conjugated secondary antibodies indicating that there was no technical artifact (Figure 3.2). The other 50% of the infected cells did not show a detectable change in TIAR location but contained comparable numbers of replication complexes as the cells with small cytoplasmic TIAR foci. About 5-10 percent of the infected cells contained larger TIAR granules in the cytoplasm that were likely SGs and the number of these cells did not increase with time after infection. The WNV-induced small foci in A549 cells were further characterized and the data are discussed in Chapter 4.

If only a few molecules of TIAR are required in each replication vesicle to enhance WNV genome RNA synthesis because they recycle functionally, and some TIAR is already localized in the cytoplasm in mock-infected cells, a change in TIAR localization would not be detectable by IFA. To further analyze the colocalization of TIAR and dsRNA in infected cells, a more
sensitive immunofluorescence microscopy assay, PLA, was used. This assay is able to detect close association of two molecules (<40 nm apart) in cells even though the amount of the molecules present is limited (Soderberg et al, 2006). In both WNV-infected A549 cells with small TIAR foci in the cytoplasm and ones with an undetectable change in TIAR location, a similar increase in the number of anti-TIAR/anti-dsRNA PLA punctae (white) in the cytoplasm were detected compared to the number of punctae in the cytoplasm of mock-infected cells (Figure 3.3A). An increase in the number of punctae in infected cells was also seen when PLA was done with antibody to an ER marker, anti-calnexin (Figure 3.3B). PLA was also done with anti-GAPDH antibody and anti-dsRNA antibody. GAPDH is a soluble cytoplasmic housekeeping protein. The majority of the anti-GADPH/anti-dsRNA punctae were located in the nucleus in both infected and mock-infected cells (Figure 3.3C).

The average number of PLA punctae in the cytoplasm per cell was quantified (Figure 3.3D). The number of anti-TIAR/anti-dsRNA PLA punctae in infected cells was much higher than the number in mock-infected cells. However, in the majority of infected cell, the number of anti-calnexin/anti-dsRNA punctae was lower than the number of anti-TIAR/anti-dsRNA punctae. This is likely due to calnexin having a broader distribution on the ER membrane than the areas of the ER membrane involved in virus-induced vesicles. The soluble cytoplasmic protein GAPDH was used as a negative control. Even though no change in TIAR location was detected in WNV-infected BHK cells, an increase in the number of the anti-TIAR/anti-dsRNA PLA punctae in the cytoplasm were detected in these cells (Figure 3.4A) while the number of anti-GAPDH/anti-dsRNA PLA punctae in infected cells increased slightly but was still at background levels (Figure 3.4B). The average number of PLA punctae in the cytoplasm per cell was quantified as previously described (Figure 3.4C). The results obtained were similar to those from A549 cells.
and indicate that TIAR and viral dsRNA are located close together in the perinuclear regions of WNV-infected cells.

3.10 TIAR interacts with the WNV 3' (-) SL RNA in the WNV-infected cells

To determine whether TIAR interacts with the WNV 3' (-) SL RNA in infected cells, the presence of TIAR-RNA complexes was analyzed by RNA-protein immunoprecipitation. C3H/He MEF lysates from mock-infected or WNV-infected cells were immunoprecipitated using TIAR antibody or a control IgG, and RNA in the complexes was extracted and amplified by RT-PCR using primers specific for the viral 3' minus (-), 5' plus (+), 5' (-), or 3' (+) terminal RNAs, or the GAPDH mRNA 3'UTR. Consistent with data from a previous study (Cleaves et al, 1981), the amount of minus-strand viral RNA detected was 10 times less than that of the plus-strand viral RNA. The intensity of the band obtained with the 10% input sample was set at 100. At 9 h after WNV infection, the relative band intensity of the WNV 3' (-) RNA pulled down by anti-TIAR antibody was 78 and increased to more than 200 at 12 and 16 h after infection (Figure 3.5). In contrast, the relative band intensities of WNV 5' (+) or 3' (+) RNAs after anti-TIAR pull-down were not enriched. The intensity of the negative control GAPDH 3'UTR band was also not enriched. The IgG control antibody pulled down very low amounts of RNA. These results indicate that TIAR is recruited to the WNV 3' (-) SL RNA and this interaction can be detected by 9 h after infection and increases with time after infection.

3.11 Overexpression of TIAR enhances WNV genome RNA production in the cells

To determine whether TIAR facilitates WNV replication, viral RNA replication was analyzed in three stable cell clones A549-TIAR-CL1, -CL2, and -CL5, each expressing different
levels of recombinant TIAR. The recombinant TIAR expression level was detected by Western blotting with anti-HA antibody and was the highest in A549-TIAR-CL5 while the levels in A549-TIAR-CL1 and -CL2 were lower (Figure 3.6A). To determine the localization of the recombinant TIAR in cells, wild type (wt) A549 and A549-TIAR-CL5 cells were infected with WNV at an MOI of 5 and samples were collected at 24 h after infection for IFA. The expression of recombinant TIAR in the A549-TIAR-CL5 clone cells was clearly detected using an anti-HA antibody (Figure 3.7A). The recombinant TIAR colocalized with the endogenous TIAR in both mock-infected and WNV-infected cells (Figure 3.6A). A previous study reported that TIA-1 overexpression induced spontaneous SG formation in monkey kidney COS7 cells (Gilks et al, 2004). However, in the A549-TIAR-CL5 cells, the number of cells with SGs (TIAR aggregation in the cytoplasm) was low and similar to that in control infected and mock-infected A549 cells (data not shown). The shapes, sizes, and growth rates of the A549-TIAR clone cells were also similar to those of the wt A549 cells. In ~50% of the A549-TIAR-CL5 cells, WNV replication complexes were asymmetrically distributed but they extended over a larger region of the cytoplasm at late times of infection compared to the wt A549 cells and many were larger than those seen in WNV-infected wt A549 cells (compare data in Figure 3.1C and 3.6B).

The effects of TIAR overexpression on extracellular virus yields and intracellular viral protein and viral RNA production in WNV-infected cells were analyzed. A549-TIAR-CL1, -CL2, -CL5 and wt A549 monolayers were infected with WNV at a low MOI (0.1) and samples were collected at mock, 2, 16, 24, and 48 h after infection. In wt A549 cells, viral protein levels detected with anti-NS3 antibody at 24 h after infection were the highest but only increased slightly by 48 h (Figure 3.7A). In contrast, NS3 expression levels were lower in A549-TIAR clone cells at 24 h but increased by 48 h. The NS3 levels observed at 48 h in A549-TIAR-CL2
and CL5 cells were higher than the maximum level in wt A549 cells. Also, in the A549-TIAR clone cells, eIF2α phosphorylation levels were not increased in mock-infected cells and increased at late times after infection to levels similar to what was seen in wt A549 cells (Figure 3.7A), indicating that TIAR overexpression in A549 cells does not induce a cellular stress response. Extracellular virus progeny yields were titered by plaque assay. At 48 h after infection, virus production by the three TIAR clones was significantly higher than that from wt A549 cells with the CL5 cells showing the highest yield (Figure 3.7B). Intracellular viral RNA was quantified using qRT-PCR. The viral RNA levels in wt and TIAR clone cells were similar at 16 and 24 h but the RNA levels in the three TIAR clones were significantly higher than in wt cells at 48 h (Figure 3.7C). The increases in viral protein, RNA and yield observed were TIAR dose-dependent at late times after a low-MOI infection.

To determine whether TIAR overexpression enhances WNV replication at earlier times, a higher MOI (MOI of 5) was used to synchronize the infection. Culture fluid and cell lysate samples were collected from WNV-infected cells at 2, 5, 8, 12, 16, and 24 h after infection and from mock-infected cells. Similar levels of extracellular virus and intracellular viral protein for wt A549 and the TIAR clone cells were observed at all times after infection (Figure 3.8A and B). Strand-specific qRT-PCR was used to detect intracellular viral plus-strand RNA levels. Intracellular viral plus-strand RNA levels were higher in the three TIAR clones than in the wt cells at 24 h after infection (Figure 3.8C). To further confirm that TIAR overexpression was enhancing viral genome RNA synthesis, the ratio of viral plus-strand to minus-strand RNA levels was determined at each time after infection. Only the A549-TIAR-CL5, which had the highest level of recombinant TIAR expression, was used for this study. Intracellular RNA samples were collected at 12, 16, 20, 24, and 28 h after infection. Viral plus-strand and minus-strand RNA
levels and control β-actin RNA levels were quantified by qRT-PCR. The Ct values for the control β-actin RNA were essentially identical for the two types of cells at each time point, indicating that the amount of RNA from the two cell types in the assay was comparable. The ratio of plus-strand to minus-strand RNA was then calculated. The plus/minus RNA ratio was similar in wt A549 and A549-TIAR-CL5 cells at 12 and 16 h, but significantly higher in A549-TIAR-CL5 cells at 20, 24, and 28 h after infection (Figure 3.8D). The results indicate that TIAR enhances viral genome RNA synthesis at later times of WNV infection.

**DISCUSSION**

Multiple cellular proteins were previously reported to interact with the 3' terminal sequences of various flaviviral genomic RNA, such as NF90, PTB, DDX6, p100, La and eEF-1α (Anwar et al, 2009; Davis et al, 2007; Gomila et al, 2011; Lei et al, 2011; Vashist et al, 2009; Ward et al, 2011). TIAR and La proteins are the only two proteins reported to date to bind to the 3' terminal sequences of the flaviviral minus-strand RNA (Li et al, 2002; Yocupicio-Monroy et al, 2003). The possible involvement of most of these proteins in virus replication was assayed only by analyzing the effect of knocking them down (Anwar et al, 2009; Gomila et al, 2011; Vashist et al, 2009; Ward et al, 2011). For only eEF-1α and TIAR/TIA-1 were the binding sites on the protein mapped on the viral RNA and additional studies done to further analyze their role in virus replication (Davis et al, 2013; Davis et al, 2007; Emara et al, 2008; Li et al, 2002). In the present study, data from multiple types of assays supported the hypothesis that TIAR facilitates WNV genome RNA initiation from the minus-strand template. However, this has not yet been directly demonstrated using an in vitro RdRp assay. The membrane association of four of the six flavivirus replication complex proteins has so far prevented reconstitution of functional in vitro
replication complexes. For many plant plus-strand RNA viruses, like TBSV, *in vitro* RdRp assays have been successfully established with membrane-bound replication complexes (Nagy & Pogany, 2012). These plant virus genomes can replicate in yeast. Systematically generated gene knock-out yeast cells are available, and membrane-bound replication complexes can be obtained from extracts of yeast cells with a particular cell protein knocked out (Nagy & Richardson, 2012). Using an *in vitro* TBSV RdRp assay consisting of recombinant viral replication proteins and cell-free yeast extracts, GAPDH was shown to be involved in TBSV plus-strand RNA synthesis (Huang & Nagy, 2011; Wang & Nagy, 2008). Previously using *in vitro* flavivirus RdRp assays with extracts of flavivirus-infected cells containing RdRp and endogenous viral templates, with nuclease treated cell extracts and exogenous RNA template, or with purified NS5 and exogenous RNA template, the labeled products represented elongation from already initiated templates, terminal nucleotide addition on the template, or elongation from a hairpin or primer (Ackermann & Padmanabhan, 2001; Bartholomeusz & Wright, 1993; Chu & Westaway, 1985; Chu & Westaway, 1987; Grun & Brinton, 1986; Guyatt et al, 2001; Steffens et al, 1999; Tan et al, 1996; You & Padmanabhan, 1999; Yu et al, 2007). Recently, it was shown that NS5 could initiate *de novo* when the template contained the 5' SL of the viral genome which contains binding sites for the NS5 MTase (Filomatori et al, 2006). To date, an *in vitro* RdRp system consisting of flavivirus membrane-bound replication complexes similar to the *in vitro* TBSV RdRp assay has not been successfully developed.

The roles of host factors involved in flavivirus plus-strand RNA synthesis are not yet well understood. The cellular protein TIAR was previously reported to interact with the WNV 3' (-) SL RNA and mutations of the mapped TIAR binding sites in the 3' (-) SL of a WNV infectious clone inhibited or reduced virus replication, suggesting that TIAR facilitates WNV genome RNA
replication (Emara et al., 2008; Li et al., 2002). In this study, colocalization of TIAR with viral RNA at a distance of less than 40 nm was detected in perinuclear regions on the ER membrane in infected cells using the sensitive immunofluorescence assay, PLA. Also, an RNA-protein immunoprecipitation assay confirmed that TIAR interacts with the WNV 3' (-) SL RNA in infected cells. Extracellular virus titers as well as intracellular viral proteins and viral RNAs increased in a dose-dependent manner in the TIAR-overexpressing A549 cells infected at a low-MOI at late times after WNV infection. The observation of an increased ratio of viral plus-strand RNA to minus-strand RNA levels starting at 16 h after infection confirmed that TIAR functions to enhance viral plus-strand RNA synthesis and that this effect is greatest at later times when asymmetric production of viral plus-strand RNA occurs. The observation that WNV replication complexes extended over a larger region in the cytoplasm as well as many larger replication complexes in TIAR-overexpressing A549 cells also supported the hypothesis that TIAR facilitates rapid reinitiation of genome RNA synthesis from the minus-strand template.

A cellular protein that facilitates viral genome RNA synthesis could be part of the RdRp holoenzyme, and/or it could directly bind to viral genome RNA and recruit RdRp to the viral RNA (Lai, 1998). It has been shown that active poliovirus and Qβ phage RdRp complexes contain several cellular proteins. Host factor 1 (HF1) in the Qβ RdRp complex binds to the 3' terminal sequences of Qβ phage genome RNA and facilitates minus-strand RNA synthesis. Based on the data obtained to date, TIAR likely binds to the 3' (-) SL and recruits the RdRp. Previously, we reported that NS3 was co-precipitated with TIAR in WNV-infected cells (Emara & Brinton, 2007). However, NS3 is known to interact with NS5 and direct interaction between TIAR and NS3 or NS5 has not yet been tested. It is possible that TIAR may only interact with the viral RdRp when it is bound to the 3' (-) SL RNA. The interaction of purified TIAR with
purified viral non-structural proteins needs to be studied in the presence and absence of the 3' (-) SL RNA to determine whether TIAR directly interacts with replication components. Three additional cell proteins, p50, p60, and p108, were previously reported to bind to the 3' (-) SL RNA (Shi et al, 1996). It is possible that a complex consisting of four cell proteins and the viral 3' (-) SL RNA is what is required to recruit the RdRp. Identification of the three additional proteins and analysis of their functions is needed to elucidate the mechanism of efficient viral plus-strand RNA initiation. In addition, it has been reported that the C-terminal prion domain of TIAR directly binds to hnRNP C1/C2 (41/43 kDa) through protein-protein and RNA-mediated interaction (Izquierdo, 2010). hnRNP C1/C2 was also reported to interact with the 3' end of poliovirus minus-strand RNA and the poliovirus RdRp precursor 3CD, and to enhance poliovirus plus-strand RNA synthesis (Brunner et al, 2010; Brunner et al, 2005; Ertel et al, 2010). The possible involvement of hnRNP C1/C2 in flavivirus replication has not yet been studied.
Table 3.1. Primer sequences used for immunoprecipitation assays of RNA-protein complexes

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1[T7]: TAATACGACTCACTATAGG
Figure 3.1. Formation of WNV-induced TIAR foci in the perinuclear regions of ~50% of infected human lung A549 cells and monkey kidney MA104 cells but not in rodent BHK and MEF cells.

(A) BHK, (B) C3H/He MEF, (C) A549, and (D) MA104 cells were either mock-infected or infected with WNV (MOI of 5). At 24 h after infection, cells were fixed and analyzed by IFA. Viral replication complexes (predominantly genome RNA being copied from minus strand templates) were detected with anti-dsRNA antibody and Alexa Fluor 594-conjugated anti-mouse IgG. TIAR protein was detected with anti-TIAR antibody and Alexa Fluor 488-conjugated anti-goat IgG. Images were acquired using a widefield microscope and deconvolved. Scale bar, 11 µm. Representative cells are shown.
Figure 3.2. An anti-TIAR antibody alone was used to confirm the formation of WNV-induced TIAR foci in infected A549 cells.

A549 cells were either mock-infected or infected with WNV (MOI of 5). At 24 h after infection, cells were fixed and analyzed by IFA. Viral replication complexes were detected with anti-dsRNA antibody and Alexa Fluor 594-conjugated anti-mouse IgG. TIAR protein was detected with anti-TIAR antibody and Alexa Fluor 488-conjugated anti-goat IgG. Top two sets of panels- anti-TIAR antibody alone. Third set of panels- anti-dsRNA antibody alone. Bottom set of panels- both anti-TIAR and anti-dsRNA antibodies. Images were acquired using a widefield microscope and deconvolved. Scale bar, 90 µm. Representative cells are shown.
Figure 3.3. TIAR and viral dsRNA colocalization on the ER membranes of infected cells was detected by a proximity ligation assay (PLA) in A549 cells. A549 cells were either mock-infected or infected with WNV (MOI of 5) and cells were analyzed at 24 h after infection. (A) Anti-dsRNA/anti-TIAR PLA punctae (white) in mock-infected or WNV-infected cells. Two representative infected cells were shown, one with no detectable TIAR location change and one with TIAR foci in the cytoplasm. (B) Anti-dsRNA/anti-calnexin and (C) anti-dsRNA/anti-GAPDH PLA punctae (white) in mock-infected or WNV-infected cells. Images were acquired using a widefield microscope. Scale bar, 5.6 μm. (D) The number of PLA punctae in the cytoplasm per cell was quantified using Volocity software and plotted using GraphPad Prism 6. Dots indicate individual cells, horizontal lines indicate the mean value, and error bars indicate standard deviations (SD).
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C. 

![BHK_cells_PLA_punctae_in_the_cytoplasm_per_cell](bhk_cells_pla_punctae_in_the_cytoplasm_per_cell.png)
Figure 3.4. TIAR and viral dsRNA colocalization in infected BHK cells was detected by a proximity ligation assay (PLA).

BHK cells were either mock-infected or infected with WNV (MOI of 5) and cells were analyzed at 24 h after infection. (A) Anti-dsRNA/anti-TIAR or (B) anti-dsRNA/anti-GAPDH PLA punctae (white) in mock-infected or WNV-infected cells. Images were acquired using a widefield microscope. Scale bar, 90 µm. Representative cells are shown. (C) The number of PLA punctae in the cytoplasm per cell was quantified using Volocity software and plotted using GraphPad Prism 6. Dots indicate individual cells, horizontal lines indicate the mean value, and error bars indicate SD.
Figure 3.5. TIAR binds to the WNV 3' (-) SL RNA in infected cells.
C3H/He MEFs were collected and lysed at 9, 12, and 16 h after WNV infection. RNA-protein complexes were immunoprecipitated with anti-TIAR antibody. RNA was then extracted from the pellet and analyzed by RT-PCR. 3' (-), 5' (+), 5' (-), and 3' (+) RNAs were detected separately; the forward primer was added in the RT reaction and the reverse primer was added prior to 25 cycles of PCR after the RT enzyme was inactivated. GAPDH is an abundant cell mRNA and its 3' UTR was targeted for RT-PCR as a negative control (Mazan-Mamczarz et al., 2006). The RT-PCR products were analyzed on 1% agarose gels. Band intensity was quantified using Multi Gauge V3.1 software and the relative levels at each time were calculated based on setting the intensity of the 10% input band at 100. The experiment was performed at least twice and representative data are shown.
Figure 3.6. WNV replication complexes extend further into the cytoplasm and are larger in TIAR-overexpressing A549-CL5 cells.

(A) A549 or A549-TIAR-CL5 cells were either mock-infected or infected with WNV (MOI of 5). Cells were fixed and analyzed by IFA at 24 h after infection with anti-TIAR and anti-HA antibodies. Images were acquired using a widefield microscope and deconvolved. Scale bar, 90 μm. (B) IFA done with anti-TIAR and anti-dsRNA antibodies in A549-TIAR-CL5 cells infected (MOI of 5) for 8, 16, 24 or 32 h. Images were acquired using a laser scanning confocal microscope. Scale bar, 20 μm. Representative cells are shown.
Figure 3.7. Increased virus yields, intracellular viral protein and viral RNA levels were observed at late times after a low MOI infection in A549 cells overexpressing TIAR. (A) Wild type A549 cells and A549 clone cells that stably express HA-tagged TIAR (TIAR-CL1, -CL2, and -CL5) were either mock infected or infected with WNV (MOI of 0.1). Expression of recombinant TIAR in different cell clones was detected by Western blotting using anti-HA antibody. Intracellular viral protein levels were detected using anti-WNV NS3 antibody. Total eIF2α, p-eIF2α, and β-actin were detected by specific antibodies. (B) Extracellular virus yields were measured by plaque assay on BHK cells. (C) Intracellular viral RNA levels were assessed by qRT-PCR. The viral RNA levels were normalized to β-actin mRNA in the same sample and are shown as fold change over the amount of viral RNA at 2 h after infection. *, statistical significance compared to the wt A549 sample was determined at a 95% confidence interval (P< 0.05). Each experiment was performed at least twice, the results showed the same trend, and representative data are shown.
A. 

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B. 

Virus titer (log_{10} PFU/ml) vs. Time after infection (h) for A549, A549-TIAR-CL1, A549-TIAR-CL2, and A549-TIAR-CL5.

C. 

Viral plus/minus RNA ratio vs. Time after infection (h) for A549, A549-TIAR-CL1, A549-TIAR-CL2, and A549-TIAR-CL5.

D. 

Viral plus/minus RNA ratio vs. Time after infection (h) for A549 and A549-TIAR-CL5.
Figure 3.8. An increased ratio of viral plus-strand to minus-strand RNA levels was detected after high MOI infection in A549 cells overexpressing TIAR.

(A) Wild type A549 cells and A549 clone cells that stably express HA-tagged TIAR (TIAR-CL1, -CL2, and -CL5) were either mock infected or infected with WNV (MOI of 5). Expression of recombinant TIAR in different cell clones was detected by Western blotting using anti-HA antibody. Intracellular viral protein levels were detected using anti-WNV NS3 antibody. eIF2α, p-eIF2α, hsp70, and β-actin were detected by specific antibodies. (B) Extracellular virus yields were measured by plaque assay on BHK cells. (C) Intracellular viral plus-strand RNA levels were assessed by qRT-PCR. The viral RNA levels were normalized to β-actin mRNA in the same sample and are shown as fold change over the amount of viral RNA at 2 h after infection. (D) Wild type A549 cells and A549-TIAR-CL5 cells were either mock infected or infected with WNV (MOI of 5). At 12, 16, 20, 24, and 28 h after infection, intracellular viral RNA levels were assessed by qRT-PCR using strand specific primers. The ratio of viral plus-strand RNA level to minus-strand RNA level was calculated as described in Materials and Methods. *, statistical significance compared to the wt A549 sample was determined at a 95% confidence interval (P<0.05). Each experiment was performed at least twice, the results showed the same trend, and representative data are shown.
CHAPTER 4

Characterization of WNV-Induced TIAR Foci in A549 cells

INTRODUCTION

When environmental stress occurs, one of the four eukaryotic translation initiation factor 2 alpha (eIF2α) kinases, PKR, PERK, GCN2, or HRI, is activated to phosphorylate eIF2α, which leads to mRNAs with stalled preinitiation complexes. This leads to the formation of discrete stress granules (SGs) in the cytoplasm and translational repression (Anderson & Kedersha, 2002). The RNA binding proteins, T cell intracellular antigen-1 (TIA-1) and its related protein TIAR and GTPase-activating protein SH3 domain-binding protein (G3BP or G3BP1) are known to nucleate SG assembly (Gilks et al, 2004; Kedersha et al, 1999; Tourriere et al, 2003). Other cell proteins are also included in SGs, including small, but not large, ribosomal subunits, most translation preinitiation components (eIF3, eIF4E, eIF4G, and PABP), poly(A)+ RNA, and additional RNA-binding proteins, including HuR and tristetraprolin (TTP) (Kedersha & Anderson, 2002; Kedersha et al, 2002). Once the cells recover from stress, SGs in the cytoplasm disappear and the SG mRNAs either go back to active translation or are recruited to processing bodies (P-bodies) for degradation (Anderson & Kedersha, 2009).

In response to many virus infections, cells generate SGs to protect themselves. On the other hand, viruses that use a cap-independent mechanism to translate their own RNA take advantage of the shutdown of cap-dependent translation to outcompete cellular mRNA translation (Lloyd, 2012). Poliovirus uses cap-independent, IRES-mediated translation and poliovirus infection induces SG formation at early times but inhibits SGs during the mid-phase of the infection cycle (Mazroui et al, 2006; White et al, 2007). The poliovirus-induced SGs contain unique components compared to SGs induced by other stresses such as arsenite treatment.
The flavivirus genome has a type I cap structure at the 5' end and uses cap-dependent translation initiation similar to cellular mRNAs. WNV does not induce SG formation at early times of infection and induces SG formation in only few cells at late times (Courtney et al., 2012). WNV, dengue virus, and Japanese encephalitis virus infections also inhibit arsenite-induced SG formation (Emara & Brinton, 2007; Katoh et al., 2013).

The data obtained in Chapter 3 showed that WNV infections induce small TIAR foci in about 50% of primate A549 and MA104 cells but not in rodent BHK and MEF cells. TIAR/TIA-1 aggregation in the cytoplasm is one of the markers of SG formation. Hence, the WNV-induced small TIAR foci in A549 cells were further characterized and compared to canonical SGs induced by arsenite. The number of infected A549 cells containing small TIAR foci increased with time after infection up to ~50%. The perinuclear localization of the small TIAR foci in the WNV-infected A549 and MA104 cells differed from the localization of the SGs induced by arsenite. Additional SG components including G3BP, poly(A)-binding protein (PABP), and eukaryotic translation initiation factor 3 subunit A (eIF3A) as well as cell mRNAs were found to colocalize with the WNV-induced TIAR foci in A549 cells. However, the SG component HuR did not. Although an increase in the levels of phosphorylation of eIF2α was observed in some cells with WNV-induced TIAR foci, this did not correlate with lower viral protein accumulation. The results suggest that the small perinuclear TIAR foci in WNV-infected A549 cells are not canonical SGs but might be pre-SGs.
MATERIALS AND METHODS

4.1 Cell lines

A549 cells, a human lung adenocarcinoma epithelial cell line, were maintained in F-12K Nutrient Mixture (Kaighn’s) (Gibco) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml of penicillin and 100 µg/ml of streptomycin at 37°C in a 5% CO₂ atmosphere. Baby hamster kidney 21 strain WI2 (BHK) cells were maintained in minimum essential medium (MEM; Gibco) supplemented with 5% FBS and 10 µg/ml of gentamicin at 37°C in a 5% CO₂ atmosphere.

4.2 Viruses

A stock of West Nile virus (WNV), strain Eg101, was prepared by infecting a monolayer of BHK cells at a multiplicity of infection (MOI) of 0.1 and harvesting culture fluid at 32 h after infection. Clarified culture fluid (1 x 10⁸ PFU/ml) was aliquoted and stored at -80°C.

4.3 Indirect immunofluorescence assay (IFA)

Cells grown to ~80% confluency on 12 mm diameter coverslips (Fisher Scientific) in 24-well plates were infected with WNV at an MOI of 5. At 24 h after WNV infection or mock-infection, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS, washed with PBS, and blocked with 5% horse serum in PBS for 1 h at room temperature. Cells were incubated with primary antibodies in PBS plus 5% horse serum for 1 h at room temperature. Virus-infected cells were detected with mouse anti-dsRNA antibody (1:1000; English & Scientific Consulting, Szirak, Hungary) or goat anti-
NS3 antibody (1:400; R&D Systems). Components of canonical SGs were detected with goat anti-TIAR antibody (C-18) (1:200; Santa Cruz), rabbit anti-TIAR antibody (1:100; Cell signaling), chicken anti-G3BP antibody (1:800; Sigma), rabbit anti-G3BP antibody (1:400; Sigma), goat anti-HuR antibody (1:100; Santa Cruz), mouse anti-PABP antibody (1:400), and rabbit anti-eIF3A antibody (1:400; Cell Signaling). Components of P-bodies were detected with rabbit anti-Dcp1a antibody (1:1000) (a gift from J. Lykke-Anderson, University of Colorado, Boulder, CO) and rabbit anti-Lsm1 antibody (1:200; Santa Cruz). eIF2α was detected using rabbit anti-eIF2α (1:400; Cell Signaling) and eIF2α phosphorylation was detected with rabbit p-eIF2α S51 (1:200; Cell Signaling). After incubation with primary antibodies, cells were washed three times for 10 min with PBS and then incubated with corresponding secondary antibodies (Alexa Fluor® 488, 594, or 647 conjugated donkey anti-mouse, anti-rabbit, or anti-goat antibodies from Invitrogen) and 0.5 µg/ml of Hoechst 33258 dye (Invitrogen) in PBS plus 5% horse serum for 1 h at room temperature and then washed three times for 10 min with PBS. Coverslips were mounted onto glass slides using ProLong Gold antifade reagent (Invitrogen). Images were obtained using a widefield fluorescent microscope, Axio Observer Z1 microscope (Zeiss), with a 63x or 40x oil immersion objective. For colocalization analyzes, image stacks were recorded at 200 nm intervals and subjected to “iteractive deconvolution” using Volocity acquisition software (PerkinElmer). Because no detectable signal for dsRNA or NS3 was obtained in mock-infected slides, these images were not deconvolved to prevent enhancing background signals. Images shown were linearly contrast enhanced to increase clarity. The mean intensities of p-eIF2α and NS3 signals in each cell in an undeconvolved image were quantified using Volocity software and imported into Microsoft Excel. The values were analyzed using GraphPad Prism6. t-test (unpaired, two-tailed) was used to determine statistical differences in p-
eIF2α levels between virus-infected and mock-infected cells. A Pearson coefficient (two-tailed) analysis was used to analyze the correlation between p-eIF2α and NS3.

### 4.4 Fluorescence in situ hybridization (FISH)

Cells grown to ~80% confluency on 12 mm diameter coverslips (Fisher Scientific) in 24-well plates were infected with WNV at an MOI of 5. At 24 h after WNV infection or mock-infection, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, washed twice with PBS, and permeabilized with 70% ethanol at 4°C overnight. Cells were washed with PBS three times, blocked in hybridization buffer [2x saline-sodium citrate (SSC) buffer, 20% formamide, 0.2% bovine serum albumin (BSA) (2 mg/ml), 1mg/ml yeast tRNA] at 37°C for 15 min, and then incubated with 5 ng/ul of Alexa Fluor 647 conjugated oligo dT(50) (Invitrogen) in hybridization buffer at 37°C for 1 h. Cells were washed at 37°C for 5 min twice with 2x SSC containing 20% formamide, twice with 2x SSC, and once with 1x SSC. Cells were then processed for IFA using a modified protocol. Briefly, cells were washed with PBS and permeabilized by incubation with 0.1% Triton X-100 in PBS at room temperature for 10 min. Cells were then incubated with primary antibodies in PBS containing 0.2% BSA (2 mg/ml) at 37°C for 30 min, washed, and incubated with secondary antibodies and Hoechst dye in PBS containing 0.2% BSA at 37°C for 30 min. After washing, coverslips were mounted onto slides. Images were acquired and analyzed as described for IFA. The white signal for poly(A) obtained with Alexa Fluor 647-conjugated oligo dT(50) was colored red for easier visualization of colocalization with TIAR.
RESULTS

4.5 Numbers of WNV-infected cells containing small TIAR foci in the perinuclear region increase with times after infection

A previous study from our lab showed that TIAR forms small foci in the perinuclear region of WNV-infected A549 and MA104 cells in areas where viral replication complexes are located. To determine whether these small TIAR foci increase with time after WNV infection, infected A549 cells harvested at different times after infection were analyzed. A549 cells were either mock-infected or infected with WNV Eg101 for 8, 16, 24, or 32 h. The cells were fixed, permeabilized, and analyzed by IFA using anti-TIAR and anti-dsRNA antibodies and Alex Fluor conjugated secondary antibodies. In WNV-infected A549 cells, no detectable change in TIAR location was observed at 8 h after infection (Figure 4.1A). By 16 hr, in some infected cells, small TIAR foci formed in the perinuclear region. Colocalization of the small TIAR foci with adjacent edges of viral dsRNA replication complexes was observed by 24 h (Figure 4.1B). The numbers of infected cells with no detectable change in TIAR location, with TIAR foci, and/or with SGs were quantified. Small TIAR foci in the perinuclear regions were observed in 17.9 ± 10.5%, 49.3 ± 5.2%, and 54.2 ± 12.0% of the infected cells at 16, 24, and 32h after infection, respectively (Figure 4.1B). The remainder of the infected cells did not show a detectable change in TIAR location. About 5-10% of the infected cells contained larger TIAR granules in the cytoplasm that were likely SGs but the number of these cells did not increase with time after infection (Figure 4.1B).
4.6 The patterns and sizes of WNV-induced TIAR foci in A549 cells are different from those of arsenite-induced SGs

To determine if the WNV-induced TIAR foci are SGs, the localization to these foci and of canonical SGs induced by arsenite in A549 cells was compared. A549 cells were either mock-infected or infected with WNV and the cells were treated with or without arsenite 30 min before fixation. At 24 h after infection, the cells were fixed, permeabilized, and analyzed by IFA. The size of WNV-induced TIAR foci was smaller than that of arsenite-induced SGs detected by TIAR (Figure 4.2A). The WNV-induced TIAR foci were primarily localized to the perinuclear region of A549 cells, although in some cells, some of the TIAR foci were also scattered in the cytoplasm. In contrast, the arsenite-induced SGs were located primarily towards the outer regions of the cytoplasm (Figure 4.2A).

4.7 Some additional SG components, but not HuR, are included in WNV-induced TIAR foci

To determine whether the WNV-induced TIAR foci contain the same cell components as SGs, the presence of individual SG components in these foci and in canonical SGs induced by arsenite in A549 cells was analyzed. G3BP, another self-aggregating protein that assists in the nucleation of SGs (Tourriere et al, 2003), was first analyzed. Colocalization of G3BP with TIAR in both WNV-infected and arsenite-treated A549 cells was analyzed by triple staining with anti-G3BP, anti-TIAR, and anti-dsRNA (Figure 4.2A). A diffuse cytoplasmic distribution of G3BP was observed in mock-infected A549 cells, while in some WNV-infected cells, G3BP was concentrated in the perinuclear regions where viral replication complexes were located and colocalized with TIAR foci. The relocalization of G3BP in WNV-infected cells was confirmed
using anti-G3BP antibody alone (Figure 4.2B). The data indicate that the WNV-induced small TIAR foci contain G3BP.

To further characterize the TIAR foci in infected A549 cells, the presence of the additional SG proteins, PABP, eIF3A, and another ARE-binding protein, HuR, were analyzed. PABP was evenly distributed in the cytoplasm in mock-infected cells but concentrated in areas where replication complexes were located in some WNV-infected A549 cells (Figure 4.3A). PABP colocalized with G3BP and TIAR in the perinuclear foci of infected A549 cells (Figure 4.3A and B). PABP and G3BP also colocalized as expected in arsenite-induced SGs in the cytoplasm (Figure 4.3A). Some eukaryotic translation initiation factors, such as eIF3, eIF4E and eIF4G, are known to be recruited to SGs (Kedersha et al, 2002). The presence of eIF3A in WNV-induced TIAR foci was analyzed. eIF3A also was concentrated in the perinuclear region in some WNV-infected A549 cells while it had a diffuse distribution in the cytoplasm of mock-infected cells (Figure 4.4A). Triple staining of WNV-infected A549 cells with anti-eIF3A, anti-TIAR, and anti-dsRNA antibodies showed that eIF3A formed foci that colocalized with TIAR, within perinuclear areas where viral replication complexes were located. However, some eIF3A foci did not colocalize with TIAR foci or the area where replication complexes were located. In arsenite-treated cells, eIF3A and TIAR concentrated and colocalized only in SGs (Figure 4.4B). Because two preinitiation complex components, PABP and eIF3A, were detected in the WNV-induced foci in infected A549 cells, the presence of cellular mRNAs was next analyzed. Cellular mRNAs were detected by a fluorescence in situ hybridization assay using an Alexa fluor-labeled oligo dT probe. The cells were subsequently stained with anti-dsRNA and anti-TIAR antibodies. Cellular mRNAs colocalized with TIAR both in arsenite-induced SGs and in WNV-induced TIAR foci in A549 cells (Figure 4.5). Colocalization of the SG component, HuR, another ARE-
binding protein, to the TIAR foci was also analyzed. In mock-infected cells, HuR was primarily located in the nucleus but relocated to the cytoplasm and colocalized with TIAR in SGs in arsenite-treated cells. However, HuR did not colocalize with TIAR or G3BP in perinuclear foci in infected A549 cells (Figure 4.6A and B). To determine whether P-body proteins are included in the WNV-induced TIAR foci in the A549 cells, the cellular locations of Dcp1 and Lsm1 were analyzed. P-bodies are small foci distributed throughout the cytoplasm in uninfected cells that are the sites of mRNA turnover. Previous studies showed that although the number of P-bodies decreases in WNV-infected cells, their size increases (Courtney et al, 2012). Dcp1 and Lsm1 were detected only in P-body foci in infected and mock-infected A549 cells and did not colocalize with TIAR foci (Figure 4.7). These data indicate that TIAR foci in WNV-infected A549 cells contain TIA-1, G3BP, PABP, eIF3A and cellular mRNAs, but not HuR or the P-body components, Dcp1 and Lsm1.

4.8 Viral protein NS3 accumulation is not affected by eIF2α phosphorylation

The data obtained indicate that WNV-induced TIAR foci in the infected A549 cells contain many SG components and cell mRNAs. The presence of cell mRNAs in the TIAR foci suggested that eIF2α phosphorylation might be increased in cells with TIAR foci, resulting in translational suppression. The levels of the viral protein NS3 were analyzed using anti-NS3 antibody and the levels of p-eIF2α were analyzed with anti-p-eIF2α antibody in mock-infected and 24 h WNV-infected A549 cells. Similar high levels of NS3 were detected in infected cells with and without WNV-induced TIAR foci (Figure 4.8A) and in infected cells with increased eIF2α phosphorylation (Figure 4.8B). The mean intensities of the NS3 and p-eIF2α antibody signals in individual cells were quantified and compared to the intensities measured in mock-
infected cells (Figure 4.8C). The mean background NS3 intensity (Alexa488) in mock-infected cells was ≤ 369.87. The mean intensity of eIF2α in the mock-infected cells (Alexa594) was ≤ 327.71. Increased eIF2α phosphorylation (mean intensity > 327.71) was detected in about half (54/115; 47.0 %) of the NS3-positive infected cells. Although in cells with the highest levels of p-eIF2α, NS3 was lower and in cells with the highest intensity of NS3, eIF2α levels were lower (Figure 4.8C), for the majority of infected cells with p-eIF2α there was not an inverse correlation between the levels of p-eIF2α and NS3. Increased intensities for both NS3 and p-eIF2α were observed in most cells (Pearson correlation coefficient (r) is 0.2313, p<0.05). The assay used provided only a snap shot of eIF2α phosphorylation and NS3 accumulation in a cell at a single time and did not measure the rate of protein synthesis.

**DISCUSSION**

SGs and P-bodies are cytoplasmic RNA granules that repress mRNA translation and degrade mRNA under stress, respectively (Anderson & Kedersha, 2009). It was previously shown that WNV infections do not induce SG formation at early times after infection and induce minimal amounts of SGs at late times (Courtney et al, 2012). WNV, dengue virus, and Japanese encephalitis virus infections also repress arsenite-induced SGs (Emara & Brinton, 2007; Katoh et al, 2013). TIAR/TIA-1 are SG nucleating proteins that regulate SG assembly and are often used as SG markers. TIAR was shown to be recruited to flavivirus replication complexes and to enhance of genome RNA synthesis from minus-strand templates. Colocalization of TIAR and viral dsRNA was detected by PLA in the perinuclear regions of multiple types of WNV infected mammalian cell lines. However, TIAR also forms small cytoplasmic foci in WNV-infected primate A549 and MA104 cells but not in infected rodent BHK and C3H/He cells. These data
suggest that the TIAR/TIA-1 protein functioning to enhance viral plus-strand synthesis is not present in the foci.

In WNV-infected A549 cells, it was observed that the TIAR foci contain the additional SG components, G3BP, PABP, and eIF3A. However, P-body components and HuR, a canonical SG component, did not colocalize with the TIAR foci. HuR is an AU-rich element (ARE) binding protein that stabilizes ARE-containing mRNAs (Fan & Steitz, 1998). Similar to TIAR/TIA-1, HuR contains RRM domains for RNA binding. However, TIAR and HuR have different RNA binding activities (Kim et al, 2007; Kim et al, 2011). HuR interacts with the 3' terminal sequences of alphavirus genome RNAs, including Sindbis virus, Ross River virus, and Chikungunya virus, and relocates from the nucleus to the cytoplasm in alphavirus infected cells but not in dengue virus or measles virus infected cells (Dickson et al, 2012; Sokoloski et al, 2010). WNV infection also did not induce HuR relocation in A549 cells.

Consistent with previous data (Emara & Brinton, 2007), WNV infection inhibited arsenite-induced SG formation in WNV-infected A549 cells. In the arsenite-treated A549 cells, small TIAR foci were observed in the perinuclear regions where viral dsRNA was located but discrete large granules formed in the cytoplasm primarily in cells without detectable virus infection. Previous studies have shown that at the early stage of SG formation induced by arsenite, small granules first appear in the cytoplasm and then increase in size and decrease in number (Kedersha & Anderson, 2002; Kedersha et al, 2002). Our data suggest that WNV-induced TIAR foci in A549 cells are not canonical mature SGs but may represent an early stage of SG assembly. The recruitment of TIAR/TIA-1 to WNV replication complexes may trigger a more robust accumulation of TIAR/TIA-1 in the cytoplasm of A549 and MA104 cells than is
needed which leads to stalling of cell mRNAs and recruitment of additional SG proteins to assemble early stage SGs. However, these granules do not mature in infected cells.

SGs induced by different stresses or viruses have been reported to contain unique components. Hsp27 is included in SGs formed after heat shock but not in response to oxidative stress or UV irradiation (Kedersha et al, 1999). SGs induced by sodium selenite, a potential candidate for cancer chemotherapy, differ from canonical SGs in their morphology, composition and mechanism of assembly (Fujimura et al, 2012). Poliovirus infection induces SG formation at early times and the induced SGs are disassembled at late times (White et al, 2007). However, another study showed that pseudo-SGs lacking many canonical SG components were present at late times of poliovirus infection (White & Lloyd, 2011). Also, Sam68 is a unique component of poliovirus-induced SGs at both times that is not present in heat shock or oxidative stress induced SGs (Piotrowska et al, 2010).

eIF2\(\alpha\) phosphorylation stalls translation preinitiation complexes on capped cellular mRNAs and then SG proteins assemble and aggregate to form SGs (Kedersha et al, 2002; Kedersha et al, 1999). Cellular mRNA translation is not inhibited by a flavivirus infection. It has also been observed that eIF2\(\alpha\) is not phosphorylated in WNV-infected rodent cells (Elbahesh et al, 2011) and eIF2\(\alpha\) phosphorylation detectable by Western blotting only begins at late times after WNV or JEV infections (Courtney et al, 2012; Tu et al, 2012). In A549 cells, it was observed that the levels of eIF2\(\alpha\) phosphorylation were higher in some WNV-infected cells than in mock-infected cells. However, viral protein accumulation was not negatively affected by eIF2\(\alpha\) phosphorylation in most infected cells. The data suggest that eIF2\(\alpha\) phosphorylation in most WNV-infected A549 cells seems to not reach the threshold required to inhibit cap-dependent translation.
A previous study showed that many eukaryotic translation initiation factors were down-regulated and some proteins which induce cap-dependent translation were dephosphorylated at 48 hr after dengue virus infection, suggesting that dengue virus may switch to a cap-independent translation process at later stages of the infection cycle (Villas-Boas et al, 2009). Another study showed that dengue genome RNA translation can still occur when cap-dependent translation is inhibited and suggested that an unknown noncanonical cap-independent and an internal ribosome entry site-independent mechanism may be used (Edgil et al, 2006). Also, a study on vesicular stomatitis virus showed that cap-dependent viral protein synthesis was unaffected by eIF2α phosphorylation and the formation of SG-like structures but the mechanism of this “resistance” is not known (Dinh et al). How viral protein translation escapes inhibition by eIF2α phosphorylation in A549 cells with WNV-induced TIAR foci needs to be further elucidated.
A. Hoechst  dsRNA  TIAR  Merge  Enlarged

Mock

WNV 8 h

16 h

24 h

32 h
Figure 4.1. The number of WNV-infected A549 cells containing small TIAR foci increases with time after infection.

(A) A549 cells were either mock-infected or infected with WNV (MOI of 5). At 8, 16, 24 and 32 h after infection, cells were fixed and analyzed by IFA. Viral replication complexes were detected with anti-dsRNA antibody and Alexa Fluor 594-conjugated anti-mouse IgG. TIAR protein was detected with anti-TIAR antibody and Alexa Fluor 488-conjugated anti-goat IgG. Images were acquired using a laser scanning confocal microscope. Scale bar, 20 µm. Representative cells are shown. (B) Numbers of infected cells in with TIAR in different locations were counted. Error bars indicate SD. RC- replication complex.
Figure 4.2. Differential distribution and size of WNV-induced TIAR foci and arsenite-induced SGs in the cytoplasm of A549 cells.
A549 cells were either mock-infected or infected with WNV Eg101 (MOI of 5). At 24 h after infection, cells were fixed, permeabilized, and analyzed by IFA. Some cells were treated with arsenite 30 min before harvest. (A) Cells were stained with anti-G3BP (red), anti-TIAR (green), and anti-dsRNA (white) antibodies. (B) Cells were stained with anti-G3BP antibody (red). Cells were then stained with appropriate Alexa Fluor secondary antibodies and Hoechst (blue). Images were acquired using a widefield microscope and deconvolved except for dsRNA in mock-infected slides. Scale bar, 11 µm.
Figure 4.3. WNV-induced TIAR foci in A549 cells contain poly(A)-binding protein (PABP). A549 cells were either mock-infected or infected with WNV Eg101 (MOI of 5). At 24 h after infection, cells were fixed, permeabilized, and analyzed by IFA. Some cells were treated with arsenite 30 min before harvest. (A) Cells were stained with anti-PABP (green), anti-G3BP (red), and anti-NS3 (white) antibodies. (B) Cells were stained with anti-PABP (red) and anti-TIAR (green) antibody. Cells were then stained with appropriate Alexa Fluor secondary antibodies and Hoechst (blue). Images were acquired using a widefield microscope and deconvolved except for NS3 in mock-infected slides. Scale bar, 11 μm.
Figure 4.4. WNV-induced TIAR foci in A549 cells contain eukaryotic translation initiation factor 3A (eIF3A).

A549 cells were either mock-infected or infected with WNV Eg101 (MOI of 5). At 24 h after infection, cells were fixed, permeabilized, and analyzed by IFA. Some cells were treated with arsenite 30 min before harvest. (A) Cells were stained with anti-eIF3A antibody (green). (B) Cells were stained with anti-eIF3A (red), anti-TIAR (green) and anti-dsRNA (white) antibodies. Cells were then stained with appropriate Alexa Fluor secondary antibodies and Hoechst (blue). Images were acquired using a widefield microscope and deconvolved except for dsRNA in mock-infected slides. Scale bar, 11 µm.
Figure 4.5. WNV-induced TIAR foci in A549 cells contain cellular mRNAs.
A549 cells were either mock-infected or infected with WNV Eg101 (MOI of 5). At 24 h after infection, cells were fixed, permeabilized, and analyzed by fluorescence in situ hybridization (FISH) followed by IFA. Some cells were treated with arsenite 30 min before harvest. Cellular mRNA detected by FISH using Alexa-fluor labeled oligo dT(50). Cells were then stained with anti-TIAR (green) and anti-dsRNA (white) antibodies and followed by appropriate Alexa Fluor secondary antibodies and Hoechst (blue). Images were acquired using a widefield microscope and deconvolved except for dsRNA in mock-infected slides. Scale bar, 11 μm. The right-hand panels contain enlargements of the indicated regions.
Figure 4.6. WNV-induced TIAR foci in A549 cells do not contain one of the canonical SG components, HuR, an ARE-binding protein.

A549 cells were either mock-infected or infected with WNV Eg101 (MOI of 5). At 24 h after infection, cells were fixed, permeabilized, and analyzed by IFA. Some cells were treated with arsenite 30 min before harvest. (A) Cells were stained with anti-HuR antibody (green), anti-TIAR (red), and anti-dsRNA (white) antibodies. (B) Cells were stained with anti-HuR (green) and anti-G3BP (red) and anti-dsRNA (white) antibodies. Cells were then stained with appropriate Alexa Fluor secondary antibodies and Hoechst (blue). Images were acquired using a widefield microscope and deconvolved except for dsRNA in mock-infected slides. Scale bar, 11 µm.
Figure 4.7. WNV-induced TIAR foci do not colocalize with P bodies.
A549 cells were either mock-infected or infected with WNV Eg101 (MOI of 5). At 24 h after infection, cells were fixed, permeabilized, and analyzed by IFA. Cells were stained with anti-dsRNA antibody (white), anti-TIAR antibody (green), and (A) anti-Dcp1 antibody (red) or (B) anti-Lsm1 antibody (red). Cells were then stained with appropriate Alexa Fluor secondary antibodies and Hoechst (blue). Images were acquired using a widefield microscope and deconvolved except for dsRNA in mock-infected slides. Scale bar, 11 µm. The right-hand panels contain enlargements of the indicated regions.
A.

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<table>
<thead>
<tr>
<th>Merge</th>
<th>Hoechst</th>
<th>NS3</th>
<th>dsRNA</th>
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Mock

WNV 24 h
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B.

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<table>
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<th>p-eIF2α</th>
<th>Merge</th>
</tr>
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<tbody>
<tr>
<td></td>
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Mock

WNV 24 h
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C.

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Mean p-eIF2α intensity

Mean NS3 intensity
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- WNV 24h
- Mock
Figure 4.8. Analysis of eIF2α phosphorylation and NS3 accumulation in WNV-infected A549 cells.

A549 cells were either mock-infected or infected with WNV Eg101 (MOI of 5). At 24 h after infection, cells were fixed, permeabilized, and analyzed by IFA. (A) Cells were stained with anti-NS3 (green), anti-dsRNA (red), and anti-TIAR (white) antibodies and Hoechst (blue). (B) Cells were stained with anti-NS3 (green) and anti-p-eIF2α (red) antibodies. Cells were then stained with appropriate Alexa Fluor secondary antibodies and Hoechst (blue). Images were acquired using a widefield microscope. Scale bar, 11 µm. (C) The mean intensities of the p-eIF2α and NS3 signals per cell were quantified for mock-infected (n=48) and 24 h WNV-infected cells (n=144) using Volocity software, and the data were plotted using Microsoft Excel. The correlation of the p-eIF2α and NS3 data was analyzed using GraphPad Prism6. The Pearson correlation coefficient (r) was 0.2313, p < 0.05, indicating p-eIF2α and NS3 tend to increase or decrease together.
CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

It was previously proposed that four host proteins binding to the WNV 3' (-) SL RNA facilitate WNV genome (+) RNA synthesis in infected cells. TIAR was identified as one of the proteins binding to the WNV 3' (-) SL RNA, and the RRM2 domain of TIAR was found to be responsible for the binding. The TIAR binding sites on the WNV 3' (-) SL RNA were mapped to AU sequences in L1 and L2. Mutations or deletions of the binding sites on the viral RNA in a WNV infectious clone reduced or inhibited WNV replication. However, WNV replication was only slightly reduced in TIAR-knockout cells due to compensation by upregulation of TIA-1 in these cells. In this dissertation, we further analyzed the interaction of TIAR and the WNV 3' (-) SL RNA in vitro and in cells, and the involvement of TIAR in enhancing virus replication. The in vitro binding assay data indicated that TIAR specifically binds to the WNV 3' (-) SL RNA but not to the complementary 5' (+) SL RNA and that the C-terminal prion domain of TIAR may contribute to the interaction specificity. Hill coefficients from gel mobility shift assays suggest that multiple TIAR molecules bind to one WNV 3' (-) SL RNA. A model for the interaction of TIAR and the WNV 3' (-) SL RNA was proposed based on the binding data and a predicted tertiary structure of the viral RNA. According to this model, one TIAR molecule first binds to the larger exposed loop (L2) of the 3' (-) SL RNA which leads to a conformational change that exposes the embedded loop (L1) of the RNA. A second TIAR molecule can then bind to L1.

The interaction of TIAR and the WNV 3' (-) SL RNA was also analyzed in cells. Close colocalization of TIAR and viral dsRNA in the perinuclear region of WNV-infected cells was shown using an in situ interaction assay, PLA. RNA immunoprecipitation assay data showed that TIAR is recruited to the WNV 3' (-) SL RNA in cells. In TIAR-overexpressing cells, increased
intracellular viral protein and viral RNA levels as well as extracellular virus yields were observed at late times after a low MOI WNV infection, and an increased ratio of viral plus-strand RNA to minus-strand RNA was detected at late times after a high MOI WNV infection. These data suggest that TIAR is involved in enhancing genome RNA synthesis during the asymmetric RNA synthesis phase at late times of infection.

A schematic of the phases of WNV RNA synthesis in infected cells and the role of TIAR is shown in Figure 5.1. The methyltransferase domain of NS5 binds to the 5' SL of the genome (+) RNA (Dong et al, 2008; Ray et al, 2006). 3'- 5' RNA-RNA interactions result in genome cyclization which brings the 5' (+) and 3' (+) ends of the genome close together and the RdRp domain of NS5 close to the 3' (+) end (Dong et al, 2008). The cellular protein eEF1α binds to three sites on the 3' (+) SL RNA, and may facilitate the opening of the bottom of this stem prior to the 3'- 5' RNA-RNA interaction (Davis et al, 2013). Minus-strand RNA synthesis is initiated resulting in the formation of the double-stranded replicative form (RF) RNA. How the 3' end of the newly synthesized minus-strand is released from the plus-strand template is not known. This would be required for initiation of plus-strand synthesis. During the early stages of the replication cycle, equal amounts of (+) and (-) viral RNAs are produced at low levels. RNA synthesis occurs in the cytoplasm. TIAR/TIA-1 do not appear to be involved at this stage. Once viral replication complex vesicles are formed in the ER, asymmetric and exponential production of plus-strand RNA (genome) occurs (Welsch et al, 2009). The data suggest that the interaction of TIAR/TIA-1 and likely the other three cell proteins (p50, p60, and p108) with the 3' (-) SL RNA form a complex that efficiently recruits NS5 leading to rapid reinitiation of plus-strand synthesis. Newly synthesized plus-strand RNA would be expected to be released from the
template (-) RNA due to the interaction of the NS5 methyltransferase with the 5' SL of the nascent plus-strand RNA during the capping process.

Current results of isothermal titration calorimetry (ITC) analyzed by our collaborators, Drs. Zhang and Germann in the Department of Chemistry, GSU, support the proposed interaction model of multiple TIAR molecules binding to each WNV 3' (-) SL RNA. The RNA-TIAR interaction will continue to be studied. Based on previous mapping data, we proposed a two-molecule binding model, but the ITC data for the binding of TIAR RRM2 to a 20-nt RNA containing the L1 through L2 sequence of the SL RNA suggests that three protein molecules bind to each WNV 3' (-) SL RNA. There are four base pairs between L1 and L2 in the SL RNA. According to the model, the binding of TIAR to L2 is predicted to alter the stem structure so that the 4 base pairs between L1 and L2 are disrupted and this may create an additional RNA binding site. Alternatively, the binding of TIAR to the SL RNA may alter protein conformation to induce a third TIAR molecule to bind to the two TIAR molecules already bound to the RNA through a protein-protein interaction. Hence, binding assays of wild type TIAR and various mutant RNAs will be analyzed using ITC to determine if there is an additional TIAR binding site other than L1 and L2 on the RNA. Furthermore, the contact site(s) for the WNV 3' (-) SL RNA on the TIAR RRM2 will be determined by NMR spectroscopy using an $^{15}$N and/or $^{13}$C labeled TIAR RRM2 domain and the 20-nt RNA containing the L1 through L2 sequence of the SL RNA. The binding of RRM2 with the 3' (-) SL RNA will also be analyzed utilizing bimolecular docking software Haddock.

To further understand the initiation mechanism for WNV plus-strand RNA synthesis, the three additional cell proteins previously shown to bind to the 3' SL of the (-) template RNA will be identified using immobilized RNA pull-down followed by candidate protein identification by
mass spectrometry. To determine whether NS5 RdRp can bind directly and specifically to the 3' (-) SL RNA, gel mobility shift assays and competition gel mobility shift assays will be done. To determine whether TIAR and/or the three additional cell proteins facilitate the binding of NS5 on the WNV 3' (-) SL RNA, gel mobility shift assays will be done in the absence or presence of TIAR and/or additional cell proteins. The binding site(s) on the 3' (-) SL RNA for NS5 or the complex of NS5/TIAR and/or additional cell proteins will be analyzed by footprinting assays.

A coronavirus study showed that the sites in infected cells detected by anti-dsRNA antibody did not correspond with the sites of active viral RNA synthesis detected by click chemistry, a method that labels newly synthesized RNA with uridine analogs in cells (Hagemeijer et al, 2012). To confirm that TIAR localizes with newly synthesized viral RNA, click chemistry could be used to detect the localization of newly synthesized viral RNAs with time after WNV infection in cells. Also, live-cell imaging could be used to further analyze the interaction and colocalization of TIAR with viral RNA in infected cells. In order to observe TIAR in live cells without fixation, overexpression of TIAR protein fused to a fluorescence tag, such as green fluorescence protein (GFP) and a method for viral RNA detection in live cells, such as transfection of multiply-labeled tetravalent RNA imaging probes (MTRIPs) (Santangelo et al, 2009), will be required.
Figure 5.1. Model of the role of TIAR in WNV RNA synthesis.

(A) The NS5 methyltransferase domain binds to sites on the 5' SL of the genome (+) RNA and cellular eEF1α binds to sites on the 3' (+) SL. The eEF1α–3' (+) SL interaction may facilitate opening of the bottom part of the SL in preparation for pairing with nucleotides at the 5' of the genome. (B) Genome cyclization brings the 5' (+) and 3' (+) ends close together, and positions NS5 bound to the 5' SL close to the 3' end of the genome, facilitating initiation of minus-strand RNA synthesis. (C) Minus-strand RNA synthesis results in the formation of double-stranded replicative form (RF) RNA. (D) Early in the infection cycle, equal low amounts of (+) and (-) viral RNAs are produced. RNA synthesis occurs in the cytoplasm. (E) Late in the infection cycle, RNA synthesis occurs in virus-induced vesicles in the ER. The 5' SL of the newly synthesized plus-strand RNA is released from the (-) template RNA during addition of a cap by the NS5 methyltransferase. Late plus-strand RNA synthesis is asymmetric and exponential. Efficient plus-strand reinitiation is facilitated by the complex formed between the 3' SL of the minus-strand template, TIAR and also likely some or all of the other three cell proteins.


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