West Nile Virus-Induced Activation of Mammalian Target of Rapamycin Complex 1 Supports Viral Growth and Viral Protein Expression

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ABSTRACT

Since its introduction in New York City, NY, in 1999, West Nile virus (WNV) has spread to all 48 contiguous states of the United States and is now the leading cause of epidemic encephalitis in North America. As a member of the family Flaviviridae, WNV is part of a group of clinically important human pathogens, including dengue virus and Japanese encephalitis virus. The members of this family of positive-sense, single-stranded RNA viruses have limited coding capacity and are therefore obligated to co-opt a significant amount of cellular factors to translate their genomes effectively. Our previous work has shown that WNV growth was independent of macroautophagy activation, but the role of the evolutionarily conserved mammalian target of rapamycin (mTOR) pathway during WNV infection was not well understood. mTOR is a serine/threonine kinase that acts as a central cellular censor of nutrient status and exercises control of vital anabolic and catabolic cellular responses such as protein synthesis and autophagy, respectively. We now show that WNV activates mTOR and cognate downstream activators of cap-dependent protein synthesis at early time points postinfection and that pharmacologic inhibition of mTOR (KU0063794) significantly reduced WNV growth. We used an inducible Raptor and Rictor knockout mouse embryonic fibroblast (MEF) system to further define the role of mTOR complexes 1 and 2 in WNV growth and viral protein synthesis. Following inducible genetic knockout of the major mTOR cofactors raptor (TOR complex 1 [TORC1]) and rictor (TORC2), we now show that TORC1 supports flavivirus protein synthesis via cap-dependent protein synthesis pathways and supports subsequent WNV growth.

IMPORTANCE

Since its introduction in New York City, NY, in 1999, West Nile virus (WNV) has spread to all 48 contiguous states of the United States and is now the leading cause of epidemic encephalitis in North America. Currently, the mechanism by which flaviviruses such as WNV translate their genomes in host cells is incompletely understood. Elucidation of the host mechanisms required to support WNV genome translation will provide broad understanding for the basic mechanisms required to translate capped viral RNAs. We now show that WNV activates mTOR and cognate downstream activators of cap-dependent protein synthesis at early time points postinfection and that pharmacologic inhibition of mTOR (KU0063794) significantly reduced WNV growth. We used an inducible Raptor and Rictor knockout mouse embryonic fibroblast (MEF) system to further define the role of mTOR complexes 1 and 2 in WNV growth and viral protein synthesis. Following inducible genetic knockout of the major mTOR complex cofactors raptor (TOR complex 1 [TORC1]) and rictor (TORC2), we now show that TORC1 supports flavivirus protein synthesis via cap-dependent protein synthesis pathways and supports subsequent WNV growth.

West Nile virus (WNV) is an enveloped, single-stranded, positive-sense RNA virus in the genus Flavivirus, which includes multiple clinically important viral species such as dengue virus, yellow fever virus, and Japanese encephalitis virus. Since the first North American outbreak in New York City, NY, in 1999 (1), WNV has spread across the continent to become the leading cause of epidemic encephalitis (2). To date, there have been more than 37,000 confirmed cases of WNV disease, 16,000 cases of neuroinvasive disease, and 1,500 fatalities (www.cdc.gov/westnile). Currently, there is no licensed human vaccine or pharmacologic therapy for WNV. Owing to difficulties in predicting the location and timing of WNV outbreaks, insufficient enrollment of WNV-infected patients has complicated human clinical trial design for candidate vaccines and therapeutic interventions. A better understanding of the molecular pathogenesis of flaviviruses and the mechanisms behind how they successfully compete with host messages for access to translational components may reveal broad-spectrum antiflaviviral targets that can be evaluated and licensed for treatment of acute flaviviral infections.

Due to the high mutation rates of RNA viral genomes and their subsequent ability to rapidly generate escape mutations, we evaluated highly evolutionarily conserved host factors that flaviviruses are required to co-opt to support essential functions such as viral RNA translation and genomic replication. We have previously shown that WNV growth is independent of autophagy activation,
but pharmacologic inhibition of phosphatidylinositol 3-kinases (PI3K) significantly decreased WNV growth (3). The class I and III PI3K pathways activate mammalian target of rapamycin (mTOR), a serine/threonine kinase that is highly conserved from yeast to mammals (4–6). In mammals, mTOR forms the catalytic core of two distinct multiprotein complexes known as TOR complex 1 (TORC1) and TORC2, distinguished by the inclusion of raptor and rictor, respectively (6). TORC1 is responsible for the governance of diverse anabolic and catabolic processes such as protein synthesis and autophagy in response to cellular conditions and is functionally inhibited by the macrolide rapamycin. TORC2 is rapamycin insensitive and can activate Akt through S473 phosphorylation (7). To date, TORC2 is not known to directly interact with components of the cellular translation system; however, TORC2 signaling through Akt can lead to additional regulation of TORC1 activity (8, 9). Thus, we examined the roles of TORC1 and TORC2 in the translation of flaviviral RNA in mammalian host cells.

TORC1 is thought to govern cellular translation rates primarily through two means: (i) activation of 40S ribosomal protein S6 kinases S6K1 and S6K2 (10) and (ii) phosphorylation-induced inhibition of elf4E-binding protein (4EBP1-3), which when hypophosphorylated sequesters the host translation factor elf4E, preventing cap-dependent translation initiation. S6K1 has been identified as the primary kinase that phosphorylates the ribosomal protein S6 (11) and appears in cells as two distinct isoforms, p70S6K and p85S6K. p70S6K differs from p85S6K by a 23-amino-acid sequence at the amino terminus that functions as a nuclear localization signal. S6K1 (referred to as p70S6K hereafter) regulates translation through the phosphorylation of ribosomal protein S6 and elf4E. The cellular factor elf4B is an accessory factor to the eukaryotic translation initiation factor elf4F (also known as DDX2) which possesses helicase activity to unwind secondary structure in the 5’ untranslated region (5’ UTR) of mRNA (12). Phosphorylation of elf4B is thought to enhance RNA scanning of the 40S ribosomal subunit, enhance elf4A-mediated melting of secondary structure, or facilitate the translation of a specific subset of cellular RNAs (13, 14).

TORC1 also governs cellular cap-dependent translation initiation rates through the hyperphosphorylation of the elf4F-binding protein 1 (4EBP1). In its hypophosphorylated state, 4EBP1 binds to elf4E, a key component of the eukaryotic preinitiation complex responsible for binding the 5’ RNA cap structure. As it is progressively phosphorylated at T37, T46, S65, and T70 by TORC1, 4EBP1 dissociates from elf4E, freeing elf4E to bind with elf4F and form the elf4F preinitiation complex, which is then capable of recruiting the mRNA 5’-terminal m’G(5’)-ppp(5’)-N cap (15, 16). In this manner, TORC1 is able to regulate cellular cap-dependent translation initiation events by fine-tuning different aspects of initiation.

While there is precedent for viral modulation of mTOR activity in order to facilitate viral replication, it has primarily been characterized in DNA virus models (17). As positive-sense RNA virus genomes must compete directly with host messages for access to translational components, further understanding of this mechanism may provide insight into the molecular basis of RNA virus genomic translation. Direct interference with this pathway has been observed at multiple points by RNA viruses such as hepatitis C virus, which enhances mTOR and S6K activity in infected hepatocytes (18, 19). Additionally, inhibition of TORC1 function by the macrolide temsirolimus reduced Andes virus protein synthesis but not host protein synthesis (20), implying that RNA viruses can utilize mTOR activity independent of normal cellular function to successfully compete with host messages for translational components despite the vast overabundance of host mRNA compared to viral genomes.

To this end, we specifically examined the Akt–mTOR–p70S6K pathway under cellular conditions of WNV infection. As the WNV genome contains a 5’-terminal m’G(5’)-ppp(5’)-N cap structure but no polyadenylation signal of the 3’ end of the genome, we focused primarily on factors that govern translation initiation events: mTOR and p70S6K. To monitor the activation of this pathway under various cellular conditions, we used phospho-specific antibodies to track the activity of these translational components. Due to the neurotropic nature of WNV, we made use of primary neuronal culture systems (21) and an ex vivo organotypic brain slice culture model (22) coupled with pharmacologic inhibition of mTOR to screen for effects of mTOR inhibition on WNV growth in clinically relevant cell types. Advances in oncology have provided novel second-generation mTOR inhibitors that inhibit the catalytic site of mTOR (23), thereby inhibiting both complexes and their downstream signaling cascades. To evaluate the role of each mTOR complex in isolation during WNV infection, we made use of a recently described inducible genetic knockout of the mTOR cofactors raptor (TORC1) and rictor (TORC2) to determine the relative contribution of each complex independently during WNV infection (24). By inactivating each complex independently in this manner, we demonstrate that loss of raptor expression and subsequent inactivation of TORC1 results in a significant WNV growth defect, which is associated with significant reductions in viral protein expression but continued formation of replication complexes and no significant difference in cell-associated viral genome copies. We demonstrate for the first time in a flavivirus model that WNV infection manipulates components of the mTORC1 pathway that are directly tied to cellular protein synthesis, specifically at the level of translation initiation, in support of WNV growth and viral gene expression.

### MATERIALS AND METHODS

**Ethics statement.** Animal work was done in strict accordance with all Office of Laboratory Animal Resources (OLAR) and Institutional Animal Care and Use Committee (IACUC) guidelines at the University of Colorado Denver Anschutz Medical Campus.

**Virus propagation, titration, and UV inactivation.** West Nile virus stocks were obtained from 385-99 (NY99) strain (derived from a clone) and propagated in C6/36 Aedes albopictus (ATCC CRL-1660) cells as previously described (25). Syrian golden hamster kidney cells (BHK-21; ATCC C-13) were used to measure viral titer by a standard plaque assay (3). For infection, cells were inoculated with WNV and incubated at 37°C and 5% CO2 for 1 h. All time points were measured from the end of the 1-h absorbance period and reported as hours postadsorption (hpa). WNV was UV irradiated to render virus replication incompetent by subjecting 50-μl aliquots of viral stock in a 96-well plate format to UV irradiation (UV-Stratalinker 1800; Stratagene) at 20 mJ for 20 min at room temperature. UV inactivation of WNV stock was confirmed through standard plaque assay. Inoculation volumes were based on the original viral titer of stock to ensure equivalent inoculation between live and UV-irradiated virus.

**Cell lines.** All cell lines were maintained at 37°C and 5% CO2. Vero (ATCC CCL-81) and BHK-21 cells were maintained in Dulbecco’s modified Eagle medium supplemented with 1% penicillin-streptomycin (1% Penn-Strep), 10% heat-inactivated fetal bovine serum (FBS), 1% nones-

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sential amino acids, and 1% sodium pyruvate. Murine embryonic fibroblasts (MEFs) (gifts from Michael Hall) were maintained in minimal essential medium (MEM) supplemented with 1% Penn-Strep and 10% heat-inactivated fetal bovine serum.

**Serum starvation assays.** For serum starvation assays, cells were plated and allowed to adhere to 6 h. The medium was aspirated, and the wells were washed with phosphate-buffered saline (PBS) to remove residual serum. The cells were incubated with serum-free medium for 12 h prior to infection. After a 1-h viral adsorption period in serum-free inoculum, serum-free medium was added to the wells, and infection was allowed to progress until the indicated times for harvest.

**Primary neuronal culture generation.** Generation of primary neuronal cultures has been described at length elsewhere (21). In brief, mice of genotypes described are used. Briefly, embryonic day 15 (E15) Swiss Webster mouse (Harlan Laboratories) was euthanized in accordance with OLAR and IACUC regulations. Brains were removed from individual embryos, dissected to isolate tissue composed of striatal medium spiny neurons (MSNs) and cortical neurons (CORT), and tissues were placed in Ca2+-/Mg2+-free Hank's balanced salt solution with 10 mM HEPES. Tissues were minced with a razor blade and treated with 10% trypsin and 3 mg/ml DNase I (Sigma-Aldrich) for 30 min. After pelleting at 1,000 × g for 5 min and suspension in neuronal plating medium (1× MEM with Earle's salts, 10 mM HEPES, 10 mM sodium pyruvate, 0.5 mM glutamine, 12.5 mM glutamate, 1% FBS, 0.6% glucose, 1% B27 supplement), neurons were then counted and plated in neuronal plating medium at a density of 700,000 neurons/well in a six-well plate format. MSN cultures were generated as a 2:3 coculture of MSN and CORT cells. Twenty-four hours after plating, the medium was removed and exchanged with glia-conditioned medium (neurobasal plating medium supplemented with 2% B27 and 2.5% neuronal). Neuronal cultures were then allowed 6 days to mature prior to infection. Organotypic brain slice cultures (BSCs) were made as previously described (22). BSCs were allowed to mature to culture ex vivo for 24 h prior to use for experiments. BSCs were cultured with four slices per well, each tissue culture well representing one brain replicate from the same brain regions starting from a single brain slice cultures (BSCs) were made as previously described (21).

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**Pharmacologic inhibitors and insulin.** Three-plated with mitogen (DMSO) at 10,000× stock concentration of 10 mM and diluted in cell culture medium to a working concentration of 10 μM. Rapamycin (Tocris) was diluted in DMSO to a 1-mM stock concentration and diluted in cell culture medium to a working concentration of 1 μM. Inhibitors were added as indicated in the figures. Prior to inoculation of pretreated cells, inhibitor-containing medium was aspirated, and the wells were washed with PBS to remove residual free inhibitor. After aspiration of inoculum, the wells were washed with PBS to remove residual virus, and the medium was added back with the indicated inhibitor or DMSO control until harvest. Insulin was diluted to a 1,000× stock concentration of 100 M and diluted in medium to a working concentration of 100 M.

**MTT cellular viability assay.** To determine cellular viability after treatment with mTOR inhibitors, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl) reagent diluted to 5 mg/ml in PBS was added to cell medium to a final concentration of 500 μg/ml and allowed to incubate at 37°C for 2 h. The medium was aspirated, and the resulting formazan crystals were solubilized in 1 ml of 0.1 M HCl in anhydrous isopropanol. The resulting solution was plated in a clear-bottom 96-well plate (200 μl/well), and absorbance at 570 nm was obtained on a Victor XS spectrophotometer (PerkinElmer). Samples from WNV-infected cells were normalized to mock-infected, untreated controls at each time point. A cell-free medium control was also included to account for background due to phenol red in the medium.

**Inducible Raptor/Rictor murine embryonic fibroblasts.** Inducible knockout Raptor and Rictor (iRapKO and iRicKO, respectively) murine embryonic fibroblasts (MEFs) were gifts from Michael Hall and have been previously described in detail (24).

For induction, 2 mM 4-hydroxystamoxifen (Sigma-Aldrich) suspended in 70% ethanol was diluted in media to a final concentration of 1 μM. At 24 h postinduction, the cells were trypsinized, counted, and plated at 80,000 cells/well in a 6-well plate format or 10,000 cells/well in an 8-well chamber slide format. The cells were allowed 48 additional hours before experimental manipulation to allow for full induction of Raptor or Rictor gene knockout. The cells were then infected with 1 × 10^5 PFU WNV/well (6-well plate format) or 1 × 10^4 PFU WNV/well (8-well chamber slide format) for 1 h at 37°C and 5% CO2.

**Western blots.** The cells were harvested at various times postinfection in 1× PBS, pelleted at 3,000 × g for 5 min, resuspended in lysis buffer consisting of 1% Triton-X, 10 mM triethanolamine-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM methanesulfonoyl fluoride, 1× Halt protease, and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL), and disrupted using an ultrasonic processor VCPX130 (Sonic & Materials). Whole-cell extracts were run on standard SDS-polyacrylamide gels (Criterion system; Bio-Rad, USA). The separated proteins were electrically transferred to polyvinylidene fluoride 0.45-μm-pore membranes (Millipore, USA). For all Western blot (WB) analysis, membranes were activated for 10 to 15 s in methanol and blocked for 1 h with 5% blocking grade buffer (Bio-Rad) after the transfer and probed with primary antibodies: mouse monoclonal antibody to WNV envelope (Novus) and phospho-Akt (S473) (Cell Signaling); rabbit monoclonal antibody to ribosomal protein S6, raptor, rictor, 4EBP1, phospho-4EBP1 (T37/46), phospho-mTOR (S2448), mTOR, and β-actin; rabbit polyclonal antibody to Akt, phospho-p70 S6 kinase (T389), phospho-ribosomal protein S6 (S235/236), p70 S6 kinase, phospho-eIF4B (S422), and eIF4B (all Cell Signaling). Additional polyclonal rabbit WNV NS3 antibody was a generous gift from Aaron Brault (CDC, Fort Collins, CO). After the membranes were washed, they were probed with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA), and images were obtained after incubation with Western Lightning ECL Pro (PerkinElmer, MA) and visualized with ChemiDoc XRS+ system (Bio-Rad, Hercules, CA).

**Immunohistochemistry and confocal microscopy.** All images were acquired on an Olympus FV1000 confocal microscope. Following infections as outlined above, cells of interest were fixed using 4% paraformaldehyde (PFA) in PBS for 10 min. Staining was performed as previously described (3) using antibodies previously described above. An additional mouse polyclonal IgM double-stranded RNA (dsRNA) antibody was from Roy Hall.

**RNA isolation and qPCR.** Cells were harvested at the time postinfection indicated in the figures, and RNA was isolated using the RNeasy minikit (Qiagen) per the manufacturers' instructions and stored at −70°C until cDNA library generation. cDNA libraries were generated from total RNA isolates using the Scriptscript III first-strand synthesis system (Life Technologies) per the manufacturer's protocol using random hexamer primers. Quantitative PCR (qPCR) was accomplished using primer/probe sets to the WNV 3’ untranslated region per established protocols to quantify WNV genome copy number (26), and 18S RNA levels were quantified as an internal standard for normalization of WNV genomic copies per established protocols (27).

**Statistical analysis.** All data were analyzed using Prism software (GraphPad Prism6). One-way analyses of variance (ANOVA) were used to determine statistical differences in viral titer. Differences in viral genome copy were determined by unpaired t test with Welch's correction.

**RESULTS**

WNV activates mTOR. First, we determined the activation status of the PI3K-Akt signaling pathway following WNV infection. Vero cells fed with 10% serum (fetal bovine serum [FBS]) were inoculated with WNV P991 (derived from the 385-99 infectious
clone; New York, 1999) (28) at a multiplicity of infection (MOI) of 3 or mock infected, followed by biochemical inhibition of Akt (10 μM Akt inhibitor 1/2; Calbiochem) at 0 h postadsorption. The Akt1/2 inhibitor functions by binding the pleckstrin homology domain, making this a specific inhibitor for Akt but not other members of the AGC family of kinases lacking this domain (29, 30). Cells were harvested at 24 h hours postadsorption (hpa) for protein lysate processing and Western blot analysis. Western blot analysis revealed decreased activation of both Akt and mTOR following pharmacologic treatment with Akt inhibitor (Fig. 1A). Under serum-fed conditions, WNV activates Akt at 24 hpa, while mTOR exhibited reduced activity compared to mock-infected cells; inhibition of Akt prevented activation of both Akt and mTOR during WNV infection.

Next, we determined the activation status of the PI3K-Akt-mTOR-p70S6K signaling pathway following WNV infection. Serum, nutrients, and amino acids robustly activate mTOR (6, 31); therefore, we utilized Vero cells subjected to 12-h serum starvation prior to infection to better detect the effect of WNV virus infection on mTOR activation status. Our previous work showed that inhibition of PI3K with 3-methyladenine (3MA) or wortmannin decreased WNV growth independent of autophagy activation (3). Thus, we determined the role of PI3 kinase in WNV-induced mTOR activation. Vero cells were serum starved for 12 h prior to infection, inoculated with WNV or mock infected, treated with the PI3 kinase inhibitor 3MA at 0 hpa, and cells were harvested at 3 hpa for Western blot analysis. We detected evidence of increased phospho-p70S6K expression following WNV infection, and this activation was blocked by the PI3 kinase inhibitor 3MA (Fig. 1B).

Given this data, we next evaluated mTOR activation status following WNV infection in serum-starved Vero cells, as serum starvation effectively inhibits mTORC1 and p70S6K activation. Vero cells were serum starved for 12 h prior to infection and inoculated with WNV (MOI of 3) or mock infected. Compared to mock-infected, serum-starved cells, WNV-infected, serum-starved Vero cells exhibit enhanced levels of p-p70S6K (T389), indicating activation of the TOR-dependent translation initiation pathway (Fig. 1C). In general, we use phosphorylated p70S6K (p-p70S6K) (T389) as a marker of TORC1 activity (reviewed in reference 5). As expected, at 3 hpa, mock-inoculated, serum-starved Vero cells exhibit marked downregulation of p-p70S6K compared to mock-infected fed cells (Fig. 1C).

Next, we determined mTOR/S6K activation in a different cell type using WNV-infected serum-starved Syrian golden hamster kidney (BHK-21) cells coupled with insulin treatment as a positive control for mTORC1-directed p70S6K activation (32). This approach highlights the necessity of serum starvation to observe ac-
activation of p70S6K, as the stimulating effects of insulin treatment on mTOR and p70S6K can be visually appreciated only under starvation conditions. We found that p-p70S6K (T389) is increased in WNV-infected BHK cells compared to that in mock-inoculated cells at 2 and 3 h postadsorption (Fig. 1D). To quantify the level of p70S6K activation, densitometry analysis was completed for four replicate Western blots of mock- and WNV-inoculated, serum-starved BHK cells at 3 hpa. Densitometry data show enhancement of phosphorylated p70S6K in WNV-infected BHKs compared to mock-inoculated samples (P/H11005 0.05) (Fig. 1F).

Next, we inoculated ex vivo-cultured primary mouse cortical neurons that had been deprived of B27 supplement for 2 h prior to inoculation with WNV (MOI of 3) and harvested at 6 hpa to define the activation status of mTORC1 in a terminally differentiated model of neuronal infection. Western blot analysis for p-p70S6K (T389) exhibits increased expression of p-p70S6K following WNV inoculation in primary neurons (Fig. 1E).

Enhanced mTOR/S6K activity colocalizes with WNV infection. Since WNV-induced TOR activation may support viral RNA translation, we determined whether TOR activity was activated in virus-infected cells. We inoculated serum-starved Vero cells with WNV or UV-inactivated WNV (MOI of 3) and fixed the cells at 3 hpa for immunocytochemistry analysis using antibodies to WNV envelope (ENV) or dsRNA as a marker of viral infection. In dsRNA-positive, serum-starved Vero cells, we found increased mTOR expression compared to mock-inoculated, serum-starved cells (Fig. 2). Increased expression of mTOR was not observed in serum-starved cells inoculated with UV-inactivated WNV, indicating that infectious viral RNA is required for activation of TOR signaling. As expected, total mTOR is robustly expressed in the cytoplasm in uninfected, serum-fed Vero cells, while serum starvation completely abrogates mTOR signal (Fig. 2).

Next, we determined the role of WNV-induced mTOR activity in infected cells by evaluating the expression and localization of p70S6K as a downstream marker of mTORC1 activity. Serum-starved Vero cells were inoculated with WNV or UV-inactivated WNV (MOI of 3). Cells were fixed at 3 hpa and labeled for immunocytochemistry using antibodies to p70S6K and dsRNA as described above. In serum-starved, WNV-inoculated Vero cells, p70S6K expression is increased almost exclusively in the cytoplasm (Fig. 3). We see no evidence of p70S6K expression in serum-starved Vero cells inoculated with UV-inactivated WNV, indicating that infectious viral RNA is required to increase p70S6K expression (Fig. 3). This is consistent with a model where WNV enhances activity of key cytoplasmic proteins to support viral gene expression, as WNV replicates exclusively in the cytoplasm. As expected, p70S6K was robustly expressed in the cytoplasm and nuclei of serum-fed, uninfected Vero cells, and expression was downregulated following serum starvation of mock-infected Vero cells (Fig. 3).

Pharmacologic inhibition of Akt or mTOR prevents WNV-induced activation of p70S6K. Flavivirus endocytosis transiently activates Akt through a PI3K-dependent mechanism that can be detected as early as 1 h postinfection (33–36) and therefore may be important in activating p70S6K in support of viral gene expression. To determine the significance of WNV-induced mTOR-
p70S6K activation, we used a biochemical inhibitor of mTOR (KU00063794 [KU]). KU inhibits mTOR by binding directly to the mTOR catalytic site, thereby inactivating mTOR’s Ser/Thr kinase function and preventing signaling to downstream effectors such as p70S6K or 4EBP1 (37).

As WNV and other flaviviruses are known to cause clinically important neuroinvasive disease, we determined the effect of mTOR inhibition on WNV growth in a primary striatal neuronal culture model of WNV infection. Striatal medium spiny neurons (MSN) and cortical neurons (CORT) were isolated from E15 Swiss-Webster pups (Harlan Laboratories) and cultured in glia-conditioned MSN growth medium as previously described (21).

Neuronal cultures were pretreated with KU or DMSO vehicle control for 30 min prior to infection. After 1-h adsorption of virus (MOI of 3), medium was added containing the specified pharmacologic inhibitor. We found that KU effectively inhibits serum- and virus-induced mTOR activity in both MSN and CORT primary neuronal culture types over a 48-h period as measured by Western blotting of whole-cell lysates for activated p-p70S6K (T389) (TORC1) and p-Akt (S473) (TORC2) (Fig. 4A and B).

WNV growth is dependent on mTOR activity in primary neuronal cultures. We next used an ex vivo organotypic brain slice culture (BSC) model coupled with WNV infection to determine the contribution of mTOR activity to WNV replication in a model containing multiple neuronal subtypes. We infected BSCs with 10^4 PFU/slice of WNV, and the BSC medium was treated with rapamycin (1 μM) or KU (10 μM) at 0 hpa. At 72 hpa, BSCs treated with the TORC1-specific inhibitor rapamycin exhibited a 5-fold decrease in WNV titer (3.3 × 10^6 ± 6.5 × 10^5 PFU/ml) compared to vehicle-treated WNV-infected BSCs (1.8 × 10^7 ± 3.6 × 10^6 PFU/ml; P = 0.01) (Fig. 5A). WNV-infected BSCs treated with broad-spectrum mTOR inhibitor KU exhibited a significant 300-fold decrease in viral titer (7.9 × 10^4 ± 4.6 × 10^4 PFU/ml) compared to vehicle-treated, WNV-infected BSCs (2.5 × 10^7 ± 8/1 × 10^6 PFU/ml; P = 0.01) (Fig. 5B).

We next determined the role of mTOR in support of WNV growth in distinct neuronal subtypes. Primary MSN and CORT neurons were treated with KU and inoculated with WNV as described above. Following WNV infection and pharmacologic inhibition of mTOR, supernatants were collected at the time points indicated in the figures, and viral growth was determined using the standard plaque assay. Treatment with KU significantly reduced WNV growth 4-fold in MSNs at 48 hpa (7.28 × 10^5 ± 3.1 × 10^5 PFU/ml; P = 0.008) compared to WNV-infected vehicle-treated cells (2.83 × 10^6 ± 2.3 × 10^5 PFU/ml; P = 0.0326; n = 5) compared to untreated, WNV-infected CORT neurons (3.6 × 10^6 ± 9.84 × 10^5 PFU/ml; n = 3) (Fig. 5D). This reduction in viral titer corresponds to the observed sustained inhibition of p70S6K and Akt under these conditions as demonstrated in Fig. 4A and B.

Due to the high sensitivity of neuronal cells to both pharma-
cologic treatment and viral infection, we determined the effect of mTOR inhibition following WNV infection on neuronal viability using an MTT-based \textit{in vitro} assay kit (Sigma-Aldrich). Both MSN and CORT primary neurons were assayed at 12, 24, and 48 h postinfection. A blank control was generated in cell-free wells containing neuronal medium to control for background, and absorbance was normalized to control samples generated in untreated, uninfected neuronal cultures. Treatment of WNV-infected MSN (Fig. 6E) or CORT (Fig. 6F) cultures with KU did not prevent WNV-induced decreases in neuronal viability. Treatment with DMSO and KU inhibitor decreased survival of MSNs at all time points observed. However, the same treatments had little effect on cortical neuronal survival at 12 and 24 hpa, indicating that decreased viral growth in cortical neurons is likely to be related to inhibition of TOR and not TOR inhibitor-induced neuronal toxicity.

\textbf{Inducible knockout of mTOR cofactor raptor or rictor prevents WNV-induced mTOR activation.} Due to the observation that KU treatment alone can result in decreases in neuronal viability, especially in our MSN cultures, we next utilized an inducible knockout mouse embryonic fibroblast (MEF) system to eliminate raptor expression (inactivating TORC1, referred to as iRapKO) or rictor expression (inactivation TORC2, referred to as iRicKO) to define the roles of the two distinct mTOR complexes, as previously described (24). After a 72-h induction period with 4-hydroxytamoxifen (4OHT) (2 mM stock in 70% ethanol; working concentration, 1 \mu M) to allow for full deletion of the target gene product, we found specific deletion of \textit{Raptor} or \textit{Rictor} expression and found reduced activation of their downstream effectors p-p70S6K (T389) and p-Akt (S473), respectively, but not in the total levels of p70S6K and Akt (Fig. 6A). Serum starvation of noninduced control MEFs resulted in loss of p-p70S6K signal. In serum-starved MEF cells, we found evidence of increased p-p70S6K in WNV and UV-irradiated WNV at early time points, 0 hpa and at 3 hpa (Fig. 6B). In mock- and WNV-inoculated, 4OHT-induced iRapKO cells, p70S6K activation was reduced over a 48-h period (Fig. 6C). In mock- and WNV-inoculated, 4OHT-induced iRicKO cells, Akt activation but not p-p70S6K was abrogated over a 48-h period (Fig. 6D). These data demonstrate that WNV-induced activation of p-p70S6K is dependent on the presence of raptor and therefore dependent on TOR complex 1 activity and that the inducible knockdown of mTOR complex 1 and 2 function remains stable for the duration of our experimental conditions with or without WNV inoculation.

\textbf{Raptor expression and TORC1 activity support WNV growth.} Next, we determined the individual roles of TORC1 and TORC2 in support of WNV growth. iRapKO MEF cells were induced with 4-hydroxytamoxifen or ethanol vehicle for 72 h in a 6-well plate format, then removed from puromycin selection and induction media, and inoculated with WNV. WNV-infected, 4OHT-induced iRapKO cells, p70S6K activation was reduced over a 48-h period (Fig. 6A). In mock- and WNV-inoculated, 4OHT-induced iRapKO cells, p70S6K activation was reduced over a 48-h period (Fig. 6C). In mock- and WNV-inoculated, 4OHT-induced iRapKO cells, Akt activation but not p-p70S6K was abrogated over a 48-h period (Fig. 6D). These data demonstrate that WNV-induced activation of p-p70S6K is dependent on the presence of raptor and therefore dependent on TOR complex 1 activity and that the inducible knockdown of mTOR complex 1 and 2 function remains stable for the duration of our experimental conditions with or without WNV inoculation.
12) compared to WNV-infected, vehicle-induced iRapKO MEFs (1.55 ± 2.51 × 10^6 PFU/ml; P < 0.00001; n = 15) (Fig. 7A). Similarly, iRicKO MEF cells were induced with 4OHT or ethanol vehicle for 72 h, removed from puromycin selection and induction media, and inoculated with WNV for viral titer assay of supernatants at the time points indicated in the figure. We found no significant difference in WNV growth in WNV-infected, 4OHT-induced iRicKO MEFs compared to WNV-infected, vehicle-induced iRicKO MEFs (Fig. 7B). However, WNV-infected, 4OHT-induced iRicKO MEFs exhibited a nonsignificant decrease in WNV growth at 12 hpa (5.5-fold; 7.19 ± 2.39 × 10^5 PFU/ml; n = 9), 24 hpa (3.2-fold; 2.35 ± 7.85 × 10^4 PFU/ml; n = 12), 36 hpa (2.8-fold; 1.04 ± 5.18 × 10^4 PFU/ml; n = 12), and 48 hpa (2.2-fold; 5.40 ± 1.45 × 10^4 PFU/ml; n = 9) compared to vehicle-induced controls. This trend indicates that TORC2 may contribute to WNV growth, but this effect may be secondary to TORC2-induced activation of Akt, a known activator of mTORC1 (7), as WNV growth approaches equivalence in induced and iRicKO cells by 48 h postadsorption.

Next, we determined the numbers of viral genome copies in the same treatment groups using quantitative reverse transcription-PCR (qRT-PCR) to determine the effect of Raptor knockdown on WNV genome replication. At 3 and 24 hpa, we harvested cell pellets from control and induced iRapKO cells inoculated with WNV. Cell pellets were used to provide a readout of plus-strand synthesis of viral replication complexes within the cell. To ensure specificity for WNV genomes, we utilized established protocols for the quantitation of WNV plus-strand RNA using a primer-probe set to the WNV 3’ noncoding region (26) and normalized WNV genome copy numbers to the levels of 18S RNA in host cells as previously described (27). At 3 hpa, there is no statistical difference in WNV genome copy between the two conditions when
normalized to 18S RNA expression levels (control, 0.2775 \pm 0.08897 \ [n = 4]; induced, 0.4664 \pm 0.2349 \ [n = 4]; P = 0.1451) (Fig. 7C). By 24 hpa, WNV-inoculated, control cells show a non-significant 4-fold increase in genome copy compared to WNV-induced 4OHT-induced raptor knockout MEF cells (control, 2.254 \pm 0.6819 \ [n = 4]; induced, 0.5611 \pm 0.2077 \ [n = 4]; P = 0.0818) (Fig. 7C). These data show that induction of the raptor knockout and subsequent inactivation of mTORC1 activity results in a 20-fold decrease in viral growth and a nonsignificant 4-fold reduction in viral genome copies.

Since changes in viral protein synthesis will inevitably have an effect on viral genome replication, we next determined the roles of raptor and rictor in viral protein synthesis. Whole-cell lysates from WNV-inoculated iRapKO and iRicKO MEFs were analyzed using Western blot analysis for WNV envelope protein and non-structural protein 3 (NS3) to determine whether the reduction in WNV titer in 4OHT-induced iRapKO MEFs was related to decreased viral protein expression. These two viral proteins were chosen due to their different fates: WNV ENV is incorporated into assembled virions, and WNV NS3 is an important component of the replication complex. Following WNV inoculation of iRapKO MEFs, WNV ENV and NS3 protein expression were reduced compared to WNV-inoculated, noninduced control MEF cells (Fig. 8A and C). Interestingly, in the iRicKO cell lines, WNV ENV and NS3 expression levels are low at early time points (12 and 24 hpa) but continue to increase until they are nearly equivalent to expression levels observed in the noninduced control MEF cells (Fig. 8B and D). Additionally, the time course of protein expression in iRicKO MEF cells correlates with earlier titer data (Fig. 7B), as the induced iRicKO MEFs produce nearly equivalent titers of WNV (5.40 \times 10^6 \pm 1.45 \times 10^6 \text{ PFU/ml}; n = 9) compared to noninduced control iRicKO MEFs by 48 hpa (1.2 \times 10^7 \pm 2.51 \times 10^6 \text{ PFU/ml}; n = 9).

Our data suggest that raptor and TORC1 activity support viral protein expression and have a limited effect on genome replication while having a larger effect on viral growth. Thus, we determined the role of TORC1 activity on the formation of viral replication complexes as labeled with an antibody to dsRNA and on the production of ENV protein. Serum-fed, control (noninduced MEF cells) and serum-fed, 4OHT-induced raptor knockout MEF cells were inoculated with WNV and harvested at 24 hpa for immunocytochemistry analysis. Compared to noninduced cells, WNV-inoculated, 4OHT-induced iRapKO cells exhibit loss of raptor expression associated with a loss in ENV expression (Fig. 9A). We next determined the effects of raptor deletion and TORC1 inactivation on the formation of replication complexes as defined by dsRNA-positive cells. 4OHT-induced iRapKO MEF cells and noninduced control MEF cells were inoculated with WNV and harvested at 24 h postadsorption as previously described for immunocytochemistry analysis. Cells were labeled with antibodies to total mTOR and p70S6K to track mTORC1 activity and antibodies to dsRNA to label viral replication complexes. In WNV-inoculated, 4OHT-induced iRapKO MEF cells, we found loss of
Flaviviruses have limited genomic coding capacity and have evolved mechanisms designed to usurp host cellular machinery to support viral gene expression and genome replication. Arboviruses such as dengue virus and WNV must successfully translate their genomes in evolutionarily distant vertebrate and invertebrate species using highly conserved cellular mechanisms between disparate cell types to support viral gene expression and genome replication (38). Flaviviral modulation of the highly conserved TOR pathway would be advantageous for viral growth, as this system regulates multiple important cellular functions, including autophagy, translation, cell growth, and cell survival in all eukaryotes from yeast to mammals (5, 6).

We have shown for the first time in a flavivirus model of viral infection that West Nile virus infection of serum-starved mammalian cells activates mTOR complex 1 in support of viral growth and viral protein expression at time points as early as 3 h postinfection. In a primary neuronal culture model, we found that pharmacologic inhibition of mTOR significantly decreased viral growth by 4- to 7-fold in ex vivo neuronal cultures and up to 300-fold in an organotypic brain slice culture (BSC) model. Due to the fact that KU treatment alone reduced viability of neurons, we used an inducible Raptor and Rictor gene knockout MEF cell line to identify the mechanism responsible for the WNV growth defect under conditions of mTOR inhibition. These studies allowed us to define the specific role of flavivirus-induced mTOR complexes 1 and 2 in the viral life cycle. Deletion of Raptor and subsequent loss of TORC1 activity as measured by loss of p70S6K phosphorylation resulted in a 16- to 34-fold reduction in viral protein expression, we also found a nonsignificant 4-fold decrease in viral genome copies compared to WNV-inoculated, noninduced MEF cells.

WNV genome replication is clearly dependent on viral protein expression to form replication complexes, and it is difficult to tease these events apart in this experimental system. Despite significant reduction in viral protein expression, we also found continued formation of dsRNA-positive viral replication complexes. Given these data and the important role of TOR activity in cap-dependent translation events, we suspect that TOR is a cellular pathway targeted by flaviviruses to support viral RNA translation.

As the positive-sense RNA is the template for both genomic replication and viral translation, it must be actively recruited for each function independently but cannot occur concurrently on the same template. Therefore, the deletion of raptor and inactivation of mTORC1 may be impacting WNV growth in various ways: it may directly inhibit viral translation through the inhibition of elongation processes; it may inhibit recruitment of the 40S preinitiation complex and initiation of positive-sense RNA scanning or inhibit the recruitment of viral RNA into polysomes. Additional studies of TOR-dependent translation initiation during flaviviral infection will reveal the host cellular translation mechanisms downstream of TORC1 required for viral gene expression.

Since mTOR acts as a primary control of cap-dependent protein translation, we found it interesting that WNV was able to form dsRNA replication complexes and still produce viral particles that result in a plaque. These findings are consistent with prior

**FIG 7** WNV growth is dependent on raptor expression. (A) Control, EtOH-induced MEF cells and 4OHT-induced iRapKO MEF cells were inoculated at 72 h postinduction with 1 × 10^6 PFU/well of WNV (MOI of ~1). Supernatants were harvested at the indicated times, and the viral titer was determined by a standard plaque assay. Values that were significantly different (P < 0.005) are indicated by an asterisk. (B) Control, EtOH-induced MEF cells and 4OHT-induced iRicKO MEF cells were inoculated after induction with 1 × 10^6 PFU/well of WNV (MOI of ~3). Values were not significantly different (ns) (P = 0.1) (nine or more replicate experiments). (C) Control, EtOH-induced MEF cells and 4OHT-induced iRapKO MEF cells were inoculated as described above. Cell pellets were harvested at the indicated time points for qRT-PCR analysis of WNV genome copy number normalized to 18S RNA expression levels (four replicate experiments for each condition).

DISCUSSION

signal for both mTOR expression and p70S6K expression (Fig. 9B and C) but continued expression of dsRNA-labeled cells. These data suggest that loss of raptor expression and TORC1 activity primarily impacts WNV protein expression with less of an effect on formation of viral replication centers.
studies with dengue virus showing that the 5’ and 3’ UTRs of dengue virus were able to initiate viral genome translation in the absence of cap-dependent translation and without an internal ribosomal entry site (IRES) (39). These studies imply a potential common mechanism by which flaviviruses induce mTOR activity to support recruitment and supply of specific initiation factors independent of their normal cellular functions, therefore allowing viral RNA to effectively outcompete host messages for these translational components. This also suggests that flaviviruses may have the capacity to regulate other initiation factors such as eIF4B that result in redundant activities with viral proteins, such as the helicase activity of NS3. This model would provide a definitive competitive advantage for flavivirus RNA translation in a cellular environment in which viral RNA initially represents the minority of total RNAs species.

When Rictor was deleted, resulting in loss of TORC2 activity as evident by the loss of Akt phosphorylation at S473, WNV growth was somewhat diminished, but differences in viral titer values did not reach statistical significance despite 9 to 12 replicate experiments at each time point. It was recently found that WNV capsid induces PI3 kinase-dependent activation of Akt by phosphorylation at S473 and that Akt activation was found to delay WNV-induced caspase-3-dependent apoptosis (36). We evaluated the role of S473 phosphorylation on Akt by mTOR in our rictor knockout studies. mTORC2 is known to regulate the Akt pathway and may play an important role in regulation of apoptosis as well. Additional work to evaluate the role of TORC2 in WNV-induced S473 phosphorylation of Akt and the subsequent effect on apoptosis may help identify the underlying mechanism responsible for the mild growth defect in TORC2 knockout MEF cells. While Akt was previously shown to play an important role in flavivirus growth in multiple other models, our data in rictor knockout MEFs show that WNV can grow in the absence of TORC2-mediated Akt activation.

The cellular mechanisms required to preferentially initiate flavivirus RNA translation over host messages are not completely understood. For DNA viruses such as cytomegalovirus and herpesvirus, TORC1 activation supports viral protein translation and has been extensively characterized (40, 41). For RNA virus infections, much less is known about how mTOR function impacts viral translation. Hepatitis C virus (HCV) nonstructural protein 5A was shown to activate mTOR independent of PI3K and Akt activation by direct binding of the mTORC1 cofactor FKBP38 (FK506-binding protein 38) (19), and infection of hepatocytes with HCV (JFH1 genotype) leads to downregulation of TSC1/2 (tuberous sclerosis 1 or 2) expression and subsequent activation of mTOR and p70S6K (18). Another study with HCV (JFH1 genotype 2a) was found to downregulate mTORC1 activity via an Akt-dependent mechanism at later time points in serum-fed cells in order to activate autophagy and establish persistence in infected hepatocytes (42). As some Flaviviridae members are not known to establish persistence, this late-stage modulation of mTOR to support autophagy by HCV appears to be unique to the Hepacivirus genus within the family, as autophagy induction has not been found to support WNV replication (3). Further evidence supporting the model of RNA viruses usurping host translational pathways via noncanonical mechanisms has been observed with Andes virus, as the TORC1 inhibitor tanseriolimus reduced viral protein synthesis but had no impact on translation of host messages (20). However, the role of mTOR in the early round of viral protein translation, replication, and flavivirus growth is not currently known. On the basis of our prior work showing that WNV is able to grow independently of autophagy activation (3), we do not suspect that changes in WNV growth and WNV protein expression downstream of mTOR manipulation are altered by TOR-induced changes in autophagy activation. However, further studies need to be completed to better define the respective roles of flavivirus-induced mTOR complexes in autophagy activation and flavivirus replication and growth.

While our work does not describe the mechanism by which WNV activates mTORC1 signaling and does not define the specific components of host RNA-binding proteins that are required to support flaviviral protein translation, further studies to define the mTOR-dependent, cap-dependent cellular translation machinery required by flaviviruses will continue to reveal fundamental host-virus interactions required to support viral gene expression, viral replication, and viral growth. These
specific and unique mechanisms can then be targeted and developed into therapeutic approaches for acute viral infection. Further studies are under way to elucidate both the upstream and downstream effectors of TORC1 that are impacted during WNV infection and how TORC1 function potentially supports flaviviral RNA translation.

Given the highly conserved and centralized activity of TOR in cellular protein synthesis, viral-induced TOR activation may be utilized in the wide range of host cells in which WNV must replicate. Further examination of the TOR pathway in the pathogenesis of WNV may help explain the wide host range of flaviviruses.

Following a blood meal, *Aedes aegypti* mosquitoes upregulate TOR-dependent effector mechanisms in support of nutritional and anautogeny (43, 44), implying a role for mTOR activity in the flaviviral enzootic transmission cycle. We are currently expanding our studies to identify the role of a blood meal and subsequent TOR activation in mosquito vectors in the growth and spread of WNV to mammalian and avian hosts.

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**FIG 9** Deletion of Raptor inhibits WNV protein translation but not genomic replication. (A) EtOH-induced, control MEF cells and 4OHT-induced iRapKO MEF cells were inoculated with 1 × 10⁷ PFU WNV per well at 72 h postinduction and analyzed with immunocytochemistry at 24 hpa using antibodies to Raptor (FITC [green]) and WNV envelope (Cy3 [red]). (B) Under the same conditions as described above, cells were fixed and labeled with antibodies to mTOR (FITC [green]) and dsRNA (Cy3 [red]). (C) Under the same conditions as described above, cells were fixed and labeled with antibodies to p70S6K (FITC [green]) and dsRNA (Cy3 [red]). All panels are representative of the images from three independent experiments. DAPI (blue) was used as a nuclear marker in all panels.