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Oxidative stress influences positive strand RNA virus genome synthesis and capping

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ABSTRACT

Flaviviruses are 5′ capped positive-stranded RNA viruses that replicate their genomes within endoplasmic reticulum-derived vesicles. Flaviviruses are well known to induce oxidative stress late in infection but it is unknown if oxidative stress plays a positive role in the viral RNA replication cycle. We therefore examined how oxidation affects flavivirus RNA replication. We found that antioxidant treatment reduced virus production, reduced the viral positive-to-negative strand RNA ratio, and resulted in the accumulation of uncapped positive-sense viral RNAs. Treatment of the NS5 RNA capping enzyme in vitro with oxidizing agents enhanced guanylyltransferase activity, indicating that the guanylyltransferase function of the flavivirus NS5 RNA capping enzyme is activated by oxidative conditions. Antioxidant treatment also reduced alphavirus RNA replication and protein expression while enhancing nsP1 capping activity. These findings suggest that RNA viruses may utilize oxidative stress induced during infection to help temporally control genome RNA capping and genome replication.

Introduction

Flaviviruses, including the Dengue viruses and West Nile virus (WNV), cause significant human disease globally each year and are major causes of morbidity and mortality. It has been estimated that approximately 40% of the world's population live in areas with endemic dengue fever, which results in 50–100 million cases every year (Guzman et al., 2010). The recent emergence of WNV in the Americas (Pesko and Ebel, 2012) and the impending re-emergence of dengue viruses in North America make flavivirus infection a significant domestic public health issue.

How flaviviruses and other RNA viruses regulate the replication of their genome is still unclear, making it imperative that we gain a better understanding of the replication mechanisms used by flaviviruses and other RNA viruses to provide novel targets for development of specific antiviral therapeutics. Flaviviruses are small, single stranded 5′ capped RNA viruses with genomes of approximately 11 kb in length. The viral genome encodes a single polyprotein which is co-translationally cleaved by cellular and viral proteases into three structural proteins C, prM and E and eight nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B and NS5) (Lindenbach et al., 2007). An uncapped negative-strand copy of the genomic RNA is generated early in the replication cycle, which is used as a template for synthesizing 5′ capped positive strand genomes late in infection that support further protein production, interfere with cellular processes, or are packaged into virions (Hussain et al., 2011; Moon et al., 2012; Silva et al., 2010).

Flavivirus genomic RNA replication occurs on modified endoplasmic reticulum (ER) membranes. Invaginations of the ER membrane form structures referred to as vesicle packets or replication compartments in which NS3 and NS5 replication complexes assemble to replicate positive strand viral RNAs (Chu and Westaway, 1992; Gillespie et al., 2010). The interior of the replication compartments is contiguous with the cytoplasm and has generally been thought to be a reducing environment. The outer surface of the replication compartments is exposed to the oxidizing environment of the ER lumen (Csala et al., 2010). The cytoplasm is considered to be a reducing environment, and cellular proteins such as glutathione and superoxide dismutase are responsible for keeping the cytoplasm from becoming too oxidizing and forming aberrant disulfide bonds, although disulfide bonds can form in some instances (Saaranen and Ruddock, 2012). Oxidative stress occurs when insults to the ER, such as

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induction of the unfolded protein response during infection or disruption of ER membrane integrity, causes an imbalance of intracellular reactive oxygen species (ROS) such as oxygen ions and peroxides between the ER and cytoplasm. Maintaining this homeostatic balance between the oxidizing ER and reducing cytoplasm is critical for the well-being of the cell, and oxidative stress or changes in cellular redox conditions can lead to cell death due to damage of cytoplasmic components and induction of apoptosis.

Flaviviruses are known to induce oxidative stress in infected cells both in culture and in vivo (Kumar et al., 2009; Liao et al., 2002; Lin et al., 2004, 2000; Raung et al., 2001; Verma et al., 2008; Yang et al., 2010), but induction of oxidative stress has been generally thought to be merely a byproduct of infection. Several studies have demonstrated that modulating oxidative stress can alter flavivirus replication. Treatment of infected cells with antioxidants to relieve oxidative stress in mammals or mosquitoes appears to improve disease outcomes (Chen et al., 2012; 2011; Nazmi et al., 2010; Pan et al., 2012). Conversely, oxidation by the nitric oxide donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) attenuates infection and appears to suppress negative strand RNA synthesis in vitro (Charnsilpa et al., 2005; Takhampunya et al., 2006). These data suggest that the intracellular redox balance is important for viral RNA replication, but the mechanism by which oxidative stress induced by flavivirus infection plays an active role in viral RNA replication is unknown.

In this manuscript we examine how oxidative stress affects flavivirus RNA replication during infection. We observed that oxidative stress positively affects viral RNA replication. Conversely, we demonstrate that antioxidant treatment can significantly impair viral RNA replication and alters the amount of capped viral RNA present in cells. Oxidation significantly enhances the guanylyltransferase activity of the flavivirus NS5 RNA capping enzyme which appears to be partially attributable to cysteine residues and disulfide bond formation in the capping enzyme. Oxidation of the conserved methionine residue Met 219 also appears to be involved in modulating NS5 guanylyltransferase activity in vitro and during viral replication. Alphavirus replication also showed reduced viral replication in the presence of antioxidant and enhanced alphavirus nsP1 capping enzyme activity in the presence of oxidizing agent. These findings suggest that positive sense RNA viruses may use oxidative stress during infection to help regulate their genomic RNA replication.

Results

Antioxidants suppress virus-induced oxidative stress and limit viral RNA replication

To explore whether changes in the oxidative environment during infection affects flaviviral replication, we first assessed if antioxidant treatment could affect flavivirus-induced oxidative stress. Kunjin virus (KUNV) infected BHK cells were treated with the antioxidant agent butylated hydroxyanisole (BHA) or mock treated, then incubated with the redox sensitive dye CM-H_2DCFDA to monitor changes in the cellular redox state. Using fluorescence microscopy we observed that KUNV infection significantly increased CM-H_2DCFDA fluorescence as expected, while treatment with BHA in KUNV infected cells exhibited little to no fluorescence signal (Fig. 1A), indicating that BHA blocked KUNV-induced oxidative stress.

We then monitored how BHA treatment affected KUNV release from the cell. KUNV infected baby hamster kidney (BHK) cells were treated with increasing amounts of BHA and at 48 h post-infection, media was collected and KUNV RNA detected by qRT-PCR. We observed that viral RNA levels (expressed as PFU equivalents based on a standard curve) dropped by approximately 1.5 logs at 200 μM BHA and by over 3 logs at 300 μM BHA (Fig. 1B), indicating that BHA treatment suppressed virus release from cells. To determine if BHA would alter virus release from other species (and because KUNV is an arthropod-transmitted virus), we infected human (Huh7), or mosquito (C6/36) cells with KUNV in the presence of 200 μM BHA. At 72 h post infection we collected media and determined viral titers using plaque assays (Fig. 1C). We observed that BHA treatment reduced the amount of KUNV released from each cell line by approximately 3 logs, indicating that BHA treatment caused a significant reduction in viral propagation regardless of cell type in mammalian and mosquito systems.

To determine if antioxidant treatment affects viral RNA replication, we tested the effect of BHA on the replication of a DENV-2 replicon encoding *Renilla* luciferase in BHK cells that we routinely use for antiviral testing (Stahla-Beek et al., 2012; Whitby et al., 2005). After 48 h of treatment with different concentrations of BHA, *Renilla* luciferase expression from the DENV-2 replicon was assessed as a measure of DENV-2 RNA replication. We multiplexed these assays with the CellTiter-Glo Luminescent Cell Viability system to monitor cellular viability and ensure that changes in *Renilla* luciferase activity were not due to BHA toxicity (Stahla-Beek et al., 2012). BHA treatment reduced DENV-2 replicon luciferase signal dramatically at 200 μM BHA, indicating that BHA treatment interfered with viral RNA replication (Fig. 1D). Importantly, we observed minimal cell toxicity with BHA concentrations less than 400 μM (EC_{50}=150 μM; CC_{50}=420 μM; therapeutic index=2.8), demonstrating that BHA-mediated changes in replicon signal at concentrations less that 400 μM were not directly caused by BHA toxicity. We therefore used 200 μM BHA for the remaining flavivirus cell culture experiments because that concentration showed about 90% reduction in viral replication while not negatively affecting cell viability compared to controls.

Antioxidants affect capped positive strand RNA synthesis

To further investigate the effect of antioxidants on flavivirus RNA replication, we examined how BHA treatment influences accumulation of KUNV negative and positive strand RNAs during infection. BHK cells were infected with KUNV and treated with 200 μM or mock treated with DMSO for 48 h. RNA was extracted from infected cells and positive and negative strand RNA levels were quantified using a KUNV RNA strand-specific qRT-PCR protocol (Ambrose and Mackenzie, 2013). In accordance with our previous findings that viral replication was diminished upon BHA treatment, we found that the overall levels of both positive and negative strands of KUNV RNA were lower upon BHA treatment. Average positive strand RNA values were 6.6×10^5 and 8×10^4 PFU equivalents/ml for DMSO and BHA treated samples, respectively. Average negative strand RNA values were 2.9×10^4 and 1.3×10^3 PFU equivalents/ml for DMSO and BHA treated samples, respectively. We observed that the positive-to-negative strand RNA ratio was approximately 24:1 in DMSO treated samples, whereas treating cells with 200 μM BHA consistently reduced the ratio to approximately 5:1 (Fig. 2A). These data indicated that BHA treatment significantly reduced the abundance of viral positive strand RNA as compared to negative strand RNA.

Positive strand flavivirus RNAs possess a 5′ RNA cap structure that protects the RNA from 5′ exonuclease degradation and directs protein translation. Because we observed a reduction in the proportion of positive strand RNAs during BHA treatment, we tested if BHA treatment could affect the formation of the viral 5′ RNA cap and increase the accumulation of uncapped viral RNAs during infection. BHK cells were infected with KUNV and treated

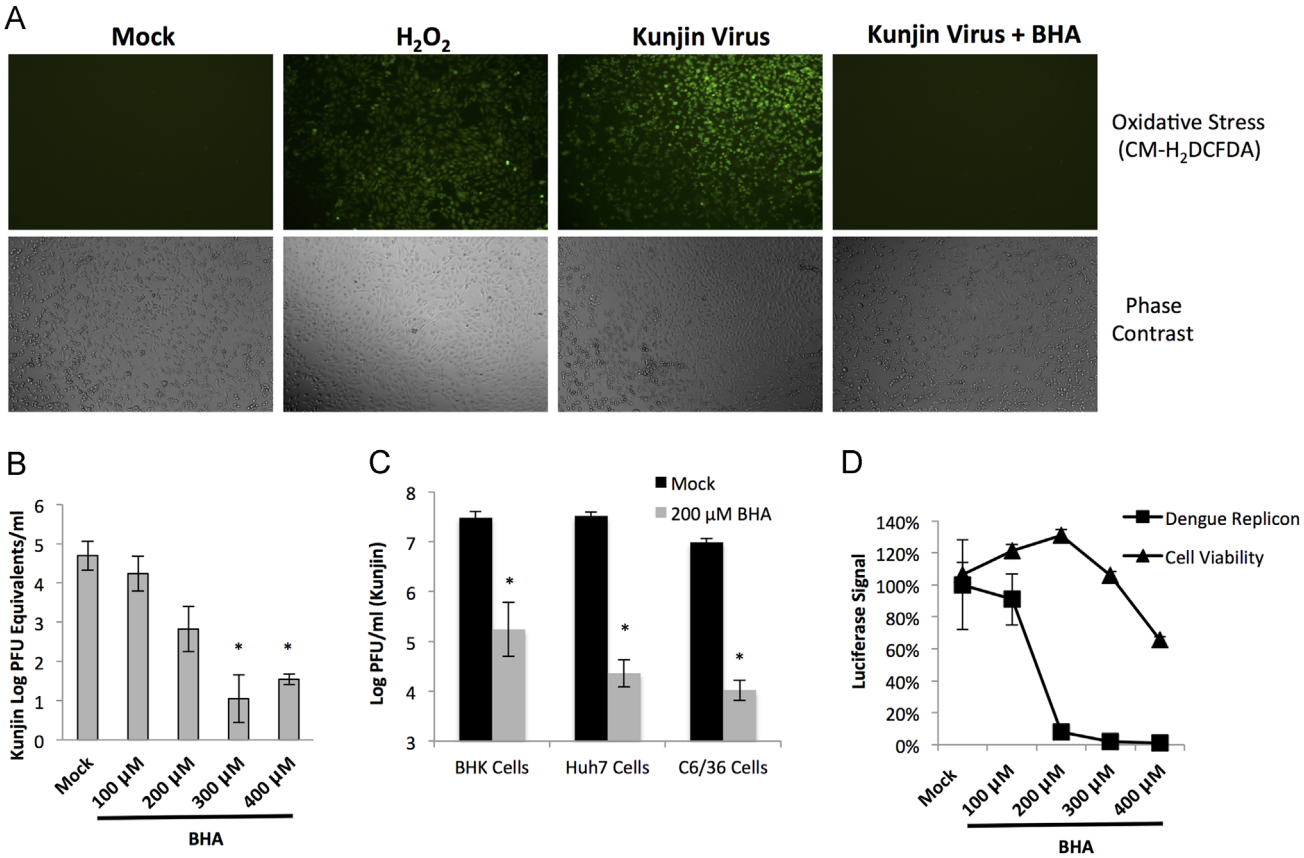

Fig. 1. Antioxidants reduce flavivirus RNA replication. (A) The antioxidant BHA blocks Kunjin virus (KUNV) induced oxidative stress. BHK cells were treated or infected as shown for 24 h, then incubated with the fluorescent oxidation sensor CM-H_2DCFDA (green) and imaged. (B) BHA reduces KUNV replication. BHK cells were infected with KUNV (MOI = 0.01) and treated with the indicated concentration of BHA. Supernatants were collected at 48 h, RNA extracted, and qRT-PCR analysis performed to determine PFU equivalents. 100 PFU/ml (2 logs) is the limit of detection in these assays. C_q values were converted to PFU equivalents using a standard curve. A pairwise t test with Bonferroni correction showed that 300 and 400 μM BHA treatments were significantly different than DMSO with $p < 0.01$. ($n = 3$) (C) BHA reduces KUNV replication in different cell species. Huh7, BHK, or C6/36 cells were infected with KUNV (MOI = 0.01) at mock treated or treated with 200 μM BHA for 72 h. Viral particles were quantified by KUNV plaque assay. Two-way ANOVA demonstrated a significant reduction in viral titer upon treatment with BHA with $p < 0.0001$ in each cell type. ($n = 3$) (D) Antiviral effect of BHA on DENV-2 replicon replication. BHK cells containing a stable DENV-2 replicon expressing *Renilla* luciferase were treated with the indicated concentration of BHA. *Renilla* luciferase and CellTiter-Glo signals were measured at 72 h post BHA addition, and the signal was reported as a percentage of the untreated sample. ($n = 3$).

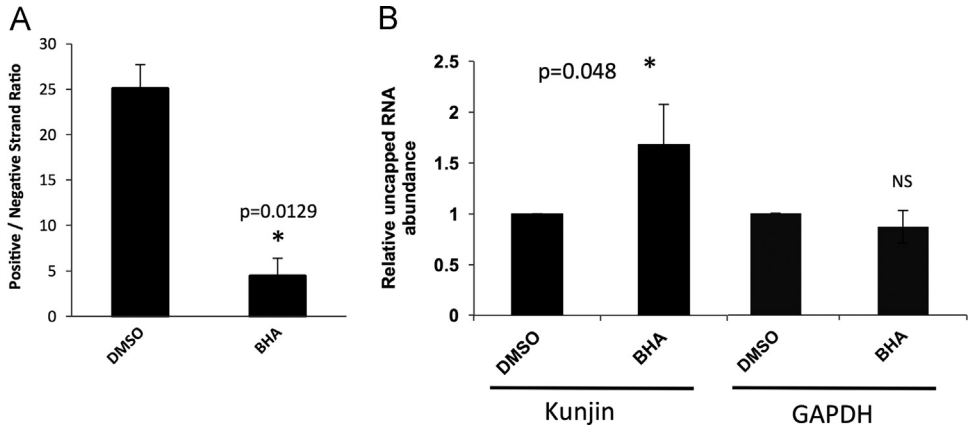

Fig. 2. BHA reduces positive strand RNA synthesis and capping. (A) Treatment of cells with BHA alters the ratio of positive to negative strand RNAs in infected cells. BHK cells were infected with KUNV (MOI = 0.1) and treated with 200 μM BHA for 48 h. Total RNA was extracted and strand specific qRT-PCR was performed. The ratio of average positive and negative strand C_q values for each sample are shown and average C_q values are in the text. An unpaired t test with equal variance yielded $p = 0.0129$, demonstrating a significant reduction in positive strand KUNV RNA in BHA as compared to DMSO. ($n = 3$) (B) Treatment with BHA increased the abundance of uncapped viral RNA. BHK cells were infected with KUNV as above and treated with DMSO or BHA and total RNA extracted at 48 h post infection. Capped and uncapped RNAs were fractionated by immunoprecipitation from total RNA samples, and KUNV and cellular *GAPDH* RNAs were detected from the uncapped fraction by qRT-PCR. An unpaired t test with equal variance yielded $p = 0.048$ for uncapped KUNV RNA but insignificant differences for GAPDH, demonstrating a significant increase in uncapped KUNV RNA but not GAPDH RNA with BHA treatment. ($n = 3$).

with DMSO or 200 μM BHA. At 48 h post infection, total RNA was extracted from cells and KUNV RNA or cellular *GAPDH* RNA was quantified by qRT-PCR. Capped RNAs were immunoprecipitated from extracted total RNA using a cap-specific antibody as previously described (Moon et al., 2012) and the relative abundance of KUNV and *GAPDH* RNAs in the immunoprecipitated fraction was determined by qRT-PCR. We observed an approximately 60% increase in the relative amount of uncapped KUNV RNA in the presence of BHA, whereas BHA treatment did not affect the proportion of capped *GAPDH* RNAs (Fig. 2B). These data indicate that blocking intracellular oxidation specifically reduces the amount of capped viral RNAs in cells but does not affect cellular RNA capping.

Oxidation upregulates NS5 guanylyltransferase activity

The finding that antioxidant treatment reduced the amount of capped viral RNA during infection suggested that oxidation may play a role in the formation of the viral RNA cap during viral RNA replication. The flavivirus NS5 protein possesses a guanylyltransferase function in its N-terminal capping enzyme domain that forms the base RNA cap structure on flavivirus genomes (Stahla-Beek et al., 2012), so we investigated if increasing oxidation could affect the guanylyltransferase function of the NS5 capping enzyme. Protein guanylation (formation of a covalent protein: GMP adduct) is considered a rate-limiting step in the guanylyltransferase reaction and is used as a measure of guanylyltransferase activity (Ahola and Kääriäinen, 1995; Guarino et al., 1998; Issur et al., 2009). Guanylation reactions with recombinant DENV-2 NS5 capping enzyme (Geiss et al., 2011) were performed with two different oxidizing agents, hydrogen peroxide (H_2O_2) and diamide. We observed that increasing concentrations of either oxidizing agent resulted in increased levels of protein guanylation (Fig. 3A). Calf intestinal phosphatase (CIP) was used as a negative control and did not incorporate GMP label even at high concentrations of oxidizing agent.

A possible mechanism for oxidative activation of guanylyltransferase activity is altering GTP binding by the capping enzyme. To test this possibility, we assessed the effect of reducing or oxidizing agents on the ability of DENV-2 capping enzyme to bind to a fluorescently labeled GTP substrate in fluorescence polarization assays as previously described (Geiss et al., 2009). We observed that TCEP and H_2O_2 did not significantly reduce GTP binding by the capping enzyme (Fig. 3C). Therefore, modulating the oxidative conditions during protein guanylation affects the enzymatic activity of the capping enzyme but does not significantly affect the ability of the enzyme to bind GTP.

Oxidation induces disulfide-bond dimerization

One consequence of protein oxidation can be the formation of disulfide bonds within and between proteins. We resolved DENV-2 capping enzyme proteins incubated with increasing concentrations of diamide on SDS-PAGE gels (after heat denaturation) with or without β-mercaptoethanol (β-ME) to monitor disulfide bond-mediated dimerization. Samples treated with β-ME resolved as a single band, whereas samples that were not treated with β-ME showed dimerization at higher diamide concentrations (Fig. 4A). Increases in guanylation activity correlated with dimerization of the NS5 protein, suggesting that disulfide bonding may influence oxidative activation of guanylyltransferase activity.

We next examined the effect of protein concentration on NS5 guanylyltransferase activity. Disulfide bond-mediated dimerization would result in increased local concentration of the NS5 protein, and if protein:protein interactions between NS5 monomers alter enzymatic activity, we would anticipate that NS5 would be less active at lower molar concentrations and more active at higher molar concentrations. To examine the effect of concentration on NS5 guanylyltransferase activity we incubated 30 pmol of recombinant wild-type DENV-2 NS5 capping enzyme in 100 μl (0.3 μM), 30 μl (1 μM), and 10 μl (3 μM) guanylation reactions for 4 h at 37 °C. At the end of the reaction the volumes of each sample were adjusted to 100 μl, the proteins were subjected to heat denaturation with β-ME and resolved on SDS-PAGE gels. We observed that the guanylation activity at lower concentrations (0.3 μM and 1 μM) were significantly reduced as compared to our standard 3 μM concentration (Fig. 4B), indicating that the guanylation reaction is affected by protein concentration and supporting the hypothesis that protein:protein interactions enhance the guanylation reaction.

To determine if particular cysteine residues may be involved in the observed oxidative activation, we mutated each of the five cysteine residues in recombinant DENV-2 NS5 capping enzyme to serine residues and tested each mutant's ability to be activated by oxidation. Cys82Ser showed a small reduction of guanylation activity in the absence of oxidation, whereas each of the other mutants showed similar levels of activity as the wild-type control (Fig. 4C). Treatment of the mutant proteins with H_2O_2 showed normal oxidative activation with Cys82Ser, Cys91Ser, Cys145Ser, and Cys179Ser but significantly reduced activation with Cys140Ser,

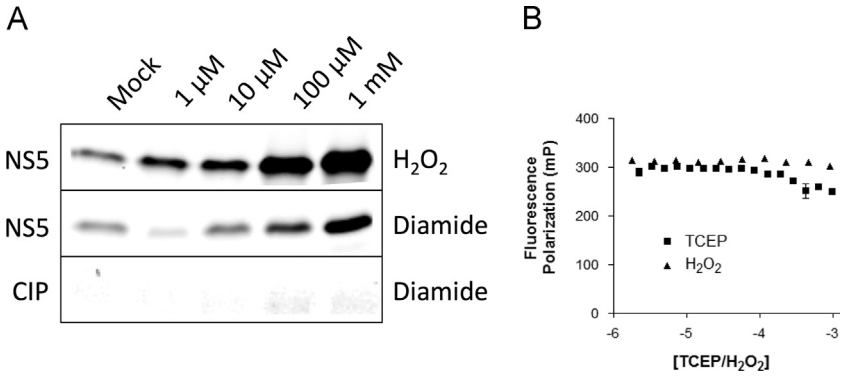

Fig. 3. Oxidizing agents increase capping enzyme guanylation activity. (A) DENV-2 NS5 capping enzyme guanylation is enhanced with oxidizing agents. Recombinant DENV-2 capping enzyme was incubated with 1 μM GTP-ATTO 680 and the indicated concentration of H_2O_2 or diamide for 4 h at 37 °C, then resolved on a 12% polyacrylamide gel. Protein guanylation activity was measured by ATTO-680 signal tracking with protein. CIP was used as a negative control in these experiments. Representative gels are shown. ($n=3$) (B) Oxidizing and reducing agents do not significantly affect DENV-2 capping enzyme GTP binding. 500 nM DENV-2 capping enzyme was incubated with 10 nM GTP-Bodipy, then the indicated concentrations of TCEP or H_2O_2 were added and the reaction was incubated at 22 °C for 1 h. Fluorescence polarization signal was detected and plotted against concentration of H_2O_2 or TCEP. ($n=3$).

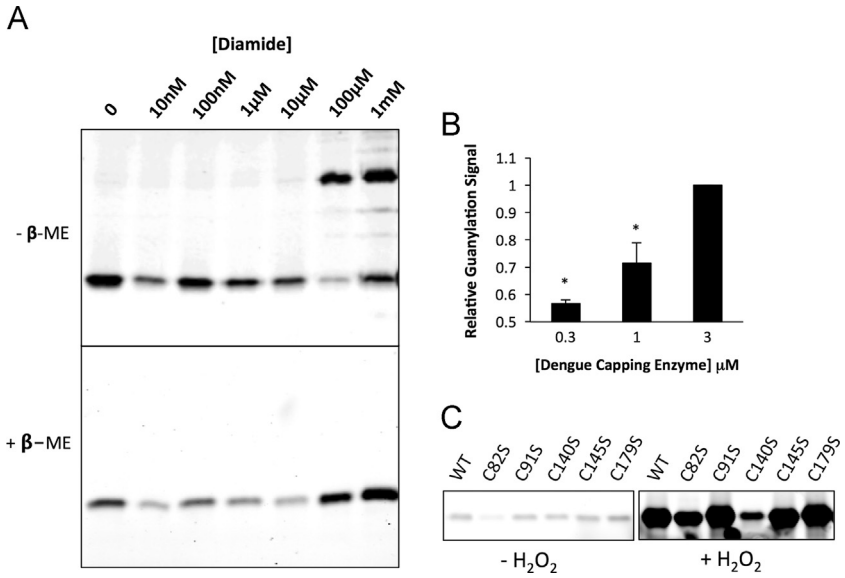

Fig. 4. Oxidation induces NS5 dimerization via disulfide bond formation. (A) Oxidation induces NS5 dimerization. Recombinant DENV-2 capping enzyme was incubated with 1 μM GTP-ATTO 680 and the indicated concentration of H_2O_2 or diamide for 4 h at 37 °C. Samples were boiled in Laemmli buffer with or without 2.5% β-mercaptoethanol and resolved on 12% polyacrylamide gels. Protein guanylation activity was measured by GMP-ATTO 680 signal tracking with protein. A representative gel is shown. (B) The NS5 guanylation reaction is concentration dependent. 30 pMol of dengue NS5 capping enzyme was incubated with 1 μM GTP-ATTO 680 in 100 μl, 30 μl, and 10 μl volumes for 4 h at 37 °C. At the end of the incubation sample volumes were all increased to 100 μl, Laemmli buffer added, and proteins boiled and resolved on 12% SDS-PAGE gels. GMP-ATTO 680 guanylation signals for each concentration were normalized for protein loading and the 3 μM dengue NS5 guanylation signal was set to 1. One-way ANOVA analysis yielded a significant different between the relative guanylation signal at 0.3 and 1 μM with $p=0.0041$ as compared to 3 μM concentration. ($n=3$) (C) Mutation of cysteine residues alters NS5 oxidative activation. 3 μM of mutant DENV-2 NS5 capping enzyme proteins were incubated with 1 μM GTP-ATTO 680 in the presence or absence of 1 mM H_2O_2 for 4 h at 37 °C. Reactions were resolved on 12% SDS-PAGE gels and GMP-ATTO 680 signal detected. A representative gel is shown. ($n=3$).

indicating that disulfide bonding with Cys140 may be partially involved in oxidative activation of NS5 guanylyltransferase activity.

Methionine 219 is involved in oxidative activation of NS5 guanylyltransferase activity

Methionine residues can be oxidized to methionine sulfoxide or methionine sulfone, so we examined how oxidation of a conserved methionine residue (Met219) affected oxidative activation of NS5 guanylyltransferase activity. We first determined if oxidants increased oxidation of NS5 Met219 in vitro. We treated purified wildtype DENV-2 NS5 capping enzyme with H_2O_2 and assessed the proportion of methionine sulfoxide and methionine sulfone produced by Orbitrap mass spectrometry. We observed that treatment with H_2O_2 increased the amount of both methionine sulfoxide and sulfone species present on purified protein (Fig. 5A), indicating that oxidation could induce the formation of each of these species. We then tested the effects of Met219 oxidation on guanylyltransferase activation using mutational analysis. Met219 was mutated to Ile (mimicking non-oxidized methionine), Gln (mimicking hemi-oxidized methionine sulfoxide), or Glu (mimicking fully oxidized methionine sulfone) as previously described (Drazic et al., 2013). Each mutant displayed approximately wild-type levels of guanylyltransferase activity in the absence of H_2O_2, but treatment with H_2O_2 resulted in attenuated activation with Met219Ile and hyperactive activation with Met219Glu (Fig. 5B). Met219Gln showed similar activation as wild-type control, indicating that modulation of the oxidation state of Met219 contributes to the oxidation activation of the NS5 guanylyltransferase enzyme.

To examine what effect M219 mutants had on the structure of the NS5 capping enzyme, we performed thermal scanning (Lavinder et al., 2009) with each mutant to determine protein stability (Fig. 5C). Wild-type NS5 capping enzyme displayed a melting temperature of 44 °C, whereas the Met219Ile and Met219Glu mutants had temperatures of 34 °C and 35 °C, respectively, suggesting that the folding of the mutant proteins were both relatively destabilized by the mutations. The Met219Gln mutant had an intermediate melting temperature of 39 °C, indicating that while the M219Gln mutant was less stable than wild-type it was more stable than the Ile and Glu mutants. Therefore, mutations at Met219 that mimic non-oxidized or methionine sulfone forms result in increased protein instability.

To test if the oxidation status of Met219 is important for viral replication we used a firefly luciferase expressing West Nile virus replicon (Pierson et al., 2005, 2006) to determine how modulating methionine oxidation status affects viral replication. BHK cells were transfected with the wild-type replicon expression plasmid, Met219Ile, Met219Gln, Met219Glu or a replication defective polymerase active site GVD mutant (Khromykh et al., 1998). Met219Ile and Met291Glu mutants were unable to replicate whereas the Met219Gln mutant showed about 30% of wildtype replication (Fig. 5D). These results demonstrate that Met219 is essential for viral replication and that mutations that mimic unoxidized or fully oxidized methionine completely block replication, whereas a mutation that mimics partially oxidized methionine is somewhat tolerated. These results suggest that a dynamic balance between unoxidized and oxidized Met219 may be necessary for viral replication, and locking Met219 into an unoxidized or fully oxidized form would be detrimental to viral replication.

Oxidation influences alphavirus replication

Oxidative stress appears to play a role in flavivirus RNA replication, but can a similar effect also be occurring among other positive sense RNA viruses? To address this question we determined if blocking oxidative stress with BHA affected the replication of Sindbis virus (a prototypical alphavirus). Treatment of BHK cells infected with a luciferase-expressing Sindbis virus (SINV) with increasing amounts of BHA resulted in a significant reduction of luciferase signal indicative of repressed SINV propagation (Fig. 6A). To ensure that this effect was not a result of a non-specific inhibition of luciferase activity by BHA, we also infected

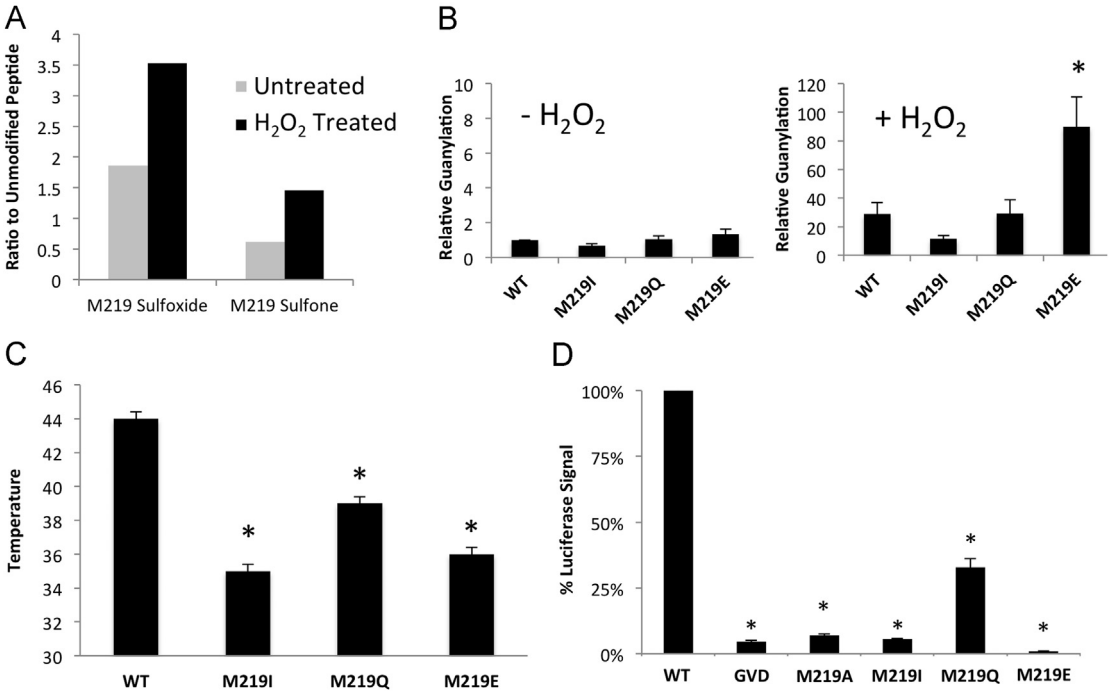

Fig. 5. Met219 influences oxidative activation of NS5 guanylyltransferase function. (A) Oxidation of DENV-2 capping enzyme increases proportion of methionine sulfoxide and sulfone species. Wild-type DENV-2 capping enzyme was mock treated or treated with 1 mM H_2O_2 for 1 h at 37 °C and resolved on SDS-PAGE gel. Proteins were trypsin digested, extracted from gels, and subjected to Orbitrap mass spectrometry. The proportion of Met219 sulfoxide and sulfone species for each sample was determined and compared to unmodified Met219. A representative experiment showing the proportion of Met219 sulfoxide and sulfone species for each sample compared to unmodified Met219 is shown. (B) Mutation of oxidation sensitive residues alters response to oxidizing agents. 3 μM of wild-type or mutant NS5 proteins were incubated with 1 mM H_2O_2 for 4 h, then resolved on SDS-PAGE gel and GMP-ATTO 680 detected. Guanylation signal was normalized to Commassie blue staining. A one-way ANOVA yielded significant difference with p=0.00693 and a pairwise *t* test with a Bonferroni comparison yielded significant difference between WT M219E with $p=0.00347$. ($n=3$) (C) Mutants mimicking Met219 methionine sulfoxide and sulfone have altered thermal stability. 30 μM of wild-type or mutant NS5 proteins were denatured in the presence of Krypton Infrared Protein stain, and melting temperature was determined by maximum d(RFU)/dT value. A one-way ANOVA yielded significant difference between samples with p=0.026 and a pairwise t test with a bonferroni comparison yielded significant difference between WT and each mutant with $p<0.001$. ($n=3$) (D) Oxidation mimicking mutations of NS5 Met219 block replicon replication. Mutant West Nile virus replicon plasmids that express firefly luciferase as a function of viral positive strand RNA abundance were transfected into BHK cells, and luciferase activity was determined 24 h later on a Victor X5 platereader. A pairwise t test with Bonferroni correction yielded a significant difference for each mutant compared to the wild type with $p<0.001$ in each case. Additionally, each mutant was significantly different than M219Q with $p<0.001$. ($n=3$).

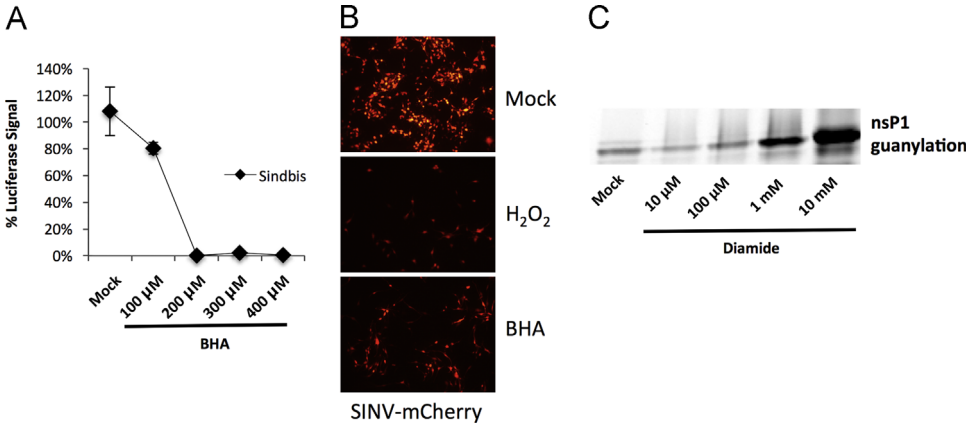

Fig. 6. Alphavirus replication and nsP1 guanylyltransferase activity is sensitive to oxidation. (A) Antioxidant treatment reduces alphavirus replication. BHK cells treated with the indicated concentrations of BHA were infected with firefly luciferase expressing Sindbis virus for 24 h. Firefly luciferase activity was detected in infected cell lysates on a Victor X5 platereader as a measure of viral propagation ($n=3$). (B) Oxidants and antioxidants agents alter Sindbis virus mediated mCherry expression. BHK cells were treated with DMSO, 200 μM BHA, or 200 μM H_2O_2 and infected with mCherry expressing Sindbis virus. At 24 h post infection fluorescent images were collected. (C) Alphavirus nsP1 guanylyltransferase is activated by oxidant treatment. 3 μM recombinant VEEV nsP1 protein was incubated with 1 μM GTP-ATTO 680 in the presence of the indicated concentration of diamide for 4 h at 37 °C. Samples were boiled in Laemmli buffer with β-mercaptoethanol and resolved on 12% polyacrylamide gels. A representative gel is shown. ($n=3$).

BHK cells with an mCherry-expressing SINV treated with either the oxidant H_2O_2 or the reducing agent BHA and observed a significant reduction of mCherry fluorescence in each case (Fig. 6B). These data suggest that alphavirus replication is also sensitive to oxidative stress conditions similar to flaviviruses and that a sensitive oxidation balance is required for efficient viral replication. We then examined if the activity of the alphavirus RNA capping enzyme nsP1 is enhanced under oxidizing conditions in a similar manner as the flavivirus NS5 protein. We expressed and purified an N-terminal His-tagged nsP1 protein from the related

alphavirus Venezuelan Equine Encephalitis (VEE) virus. Incubation of the VEE nsP1 protein with GTP results in the formation of the guanylated intermediate as seen with NS5, demonstrating that VEE nsP1 has guanylyltransferase activity (Fig. 6C). To determine if the guanylyltransferase activity of nsP1 could be affected by oxidation, we monitored nsP1 activity in the presence of increasing levels of the oxidant diamide. Increasing diamide concentrations resulted in increased nsP1 guanylation activity, indicating that VEE nsP1 is sensitive to oxidative conditions and that alphavirus RNA capping may also be responsive to oxidative stress during infection.

Discussion

In this study we examined how oxidative stress induced during flavivirus and alphavirus infection affects viral RNA replication. We present data indicating that antioxidant treatment can significantly reduce viral RNA replication and that antioxidant treatment preferentially reduces positive strand RNA synthesis during infection. We show that antioxidant treatment can alter the amount of capped positive strand viral RNAs whereas cellular mRNAs are unaffected, indicating that antioxidant treatment specifically interferes with viral RNA cap formation. Biochemical analysis of the effect of oxidation on the flavivirus NS5 capping enzyme demonstrated that oxidation significantly increases the guanylyltransferase activity of NS5, and our data indicate that the increase in activity may be at least partially attributable to disulfide-bond mediated protein dimerization and oxidation of a conserved methionine residue within the NS5 capping enzyme. Finally, we found that replication of alphaviruses also appears to be sensitive to oxidation and that the alphavirus RNA capping enzyme activity is enhanced by oxidation.

Taken as a whole, these data indicate that flaviviruses and alphaviruses may use oxidative stress produced during infection to help regulate their RNA replication. This raises the question of why oxidative stress would be useful to viruses as means to help regulate viral RNA replication. Our hypothesis is that using oxidative conditions to control RNA capping and positive strand RNA synthesis provides a mechanism to temporally control the RNA replication cycle. During flavivirus infection, negative strand RNA synthesis occurs early after entry of the viral genome into cells and is not thought to occur within membranous replication compartments. The cytoplasmic environment would be reducing at this point and based on our data RNA capping would be minimal. It has previously been reported that treatment of cells and in vitro RNA replication assays with the oxidant SNAP to produce nitric oxide reduces negative strand DENV-2 RNA replication (Charnsilpa et al., 2005; Takhampunya et al., 2006), indicating that negative strand synthesis is more active in a reducing environment than an oxidizing environment. Therefore, limiting oxidative stress with antioxidants will likely block positive strand RNA synthesis and bias replication towards negative strand synthesis. These effects would manifest as changes in the ratio of RNA species produced during infection. We observed that treatment of infected cells with BHA reduced the levels of both positive and negative strand RNAs, but positive strand RNA accumulation was affected to a greater extent than negative strand accumulation. As viral RNA replication is cyclic and newly synthesized positive strand RNAs serve as templates for synthesis of the next round of negative strand RNAs, the change in proportions of positive strand capped RNAs suggests that blocking oxidative stress interferes with positive strand RNA synthesis and RNA capping, which corresponds well with results of our in vitro assays.

Many positive strand RNA viruses (e.g. flaviviruses, alphaviruses, caliciviruses, coronaviruses, etc.) replicate their positive strand capped genomes in induced compartments derived from cellular membranes with differential environmental conditions on either side, including the ER, mitochondria, plasma membrane, and endosomes. The prevailing rationale for forming these membranous replication compartments is protection of their dsRNA intermediates from cellular antiviral defense mechanisms such as Toll-like receptors and RNAi. Formation of these replication compartments would also provide a unique microenvironment that the virus could modulate during the course of infection. Once the replication compartments are formed, reactive oxygen species could permeate the replication compartment from the ER lumen and modify viral replication enzymes (either by forming disulfide bonds or inducing methionine oxidation) and/or lipids within the replication compartment. Therefore, in our current model, a reducing environment early in infection biases towards negative strand RNA synthesis whereas increasing cytoplasmic oxidation later in infection biases replication towards positive strand synthesis.

There are several possibilities for how oxidation affects viral RNA replication. Oxidative activation of the NS5 guanylyltransferase is reminiscent of an oxidative switch, where redox sensitive cytoplasmic proteins are activated by ROS-mediated oxidation of amino acid residues. Recent examples of oxidative activation of enzyme include methionine oxidation of the *Escherichia coli* HypT transcription factor (Drazic et al., 2013) and activation of the ataxia-telangiectasia mutated (ATM) protein kinase, which is activated upon oxidative stress by ROS-induced disulfide-bonded dimers (Guo et al., 2010). The reduction in oxidative activation observed with the NS5 Cys140 mutant suggests that flaviviruses may use cysteine oxidation and disulfide-bond mediated dimerization as a mechanism to activate their enzymes. The molecular architecture of the flavivirus RNA replication complex within the membranous replication compartments is not yet known, but a potential consequence of disulfide bond formation within or between replication proteins could be the stabilization of the RNA replication complex as a whole. Although our data support the hypothesis that disulfide bond formation is likely contributing to enhanced viral RNA capping rates, the Cys140S mutant DENV-2 NS5 protein showed reduced but not complete oxidative activation as compared to wildtype NS5 protein, indicating that other mechanisms may be involved in the process. Oxidation of NS5 Met219 appears to enhance guanylation activity as demonstrated by decreased in vitro activity with the Met219Ile mutant and increased activity with the Met219Glu mutation. Mutating M219 to either Ile or Glu completely disrupts replication, whereas mutating M219 to Gln partially restores replication, suggesting that the Ile mutation biases towards negative strand synthesis while the Glu mutation biases towards positive strand synthesis. These data correlate well with the cyclic nature of viral RNA replication, as a defect in one part of RNA replication will adversely affect the other parts. If the Met219Ile mutant is strongly biased towards negative strand synthesis, positive strand synthesis would not be able to occur. Met219Glu biasing towards positive strand synthesis may inhibit negative strand synthesis. In either case the RNA replication cycle is aborted. Met219Gln, which shows similar oxidative activation as wild-type protein, may be able to switch between negative and positive strand synthesis more effectively than the Ile and Glu mutants and allow the replication cycle proceed, albeit less efficiently than the wild-type protein. These data suggest that modulating the oxidation status of Met219 may significantly contribute to the flavivirus RNA replication cycle. Methionine sulfone has a similar structure to a phosphorylated residue, and it is possible that Met219 sulfone (and Met219Glu) act like a phosphorylation event that alters protein conformation and alters NS5 enzymatic function. Using this logic Met219Ile would be analogous to an un-modified residue and Met219Gln would be analogous to a partially phosphorylated residue. Therefore, methionine oxidation could act as a redox sensitive switch that alters NS5

enzymatic activity as a function of oxidation status. We are currently pursuing experiments to ascertain the oxidation status of Met219 and other residues during infection by mass spectrometry to better understand the kinetics of the process.

While we have focused on the NS5 guanylyltransferase function with this study, there are other potential redox switches in NS5 that could influence enzymatic activity. The flavivirus NS5 polymerase contains a conserved cysteine pair at Cys449 and Cys451 with a very similar structure to the CxxC redox switch found in thioredoxin (Andersen et al., 1997). Additionally, a disulfide bond is observed between conserved residues Cys733 and Cys852 in the WNV RNA dependent RNA polymerase (RdRp) crystal structure near the GDD active site loop (Malet et al., 2007), suggesting that disulfide bonding could take place within the NS5 RdRP protein and affect enzymatic activity. The effects of these potential disulfide bonds within NS5 on viral RNA replication have not yet been assessed. We also speculate that part of the effect on viral RNA replication observed with BHA treatment is due to changes in disulfide bonding within NS1, which is a luminal ER protein that contains several disulfide bonds and is known to be involved in RNA replication (Blitvich et al., 2001; Youn et al., 2013). However, our data demonstrates that oxidation directly affects the function of the NS5 RNA capping enzyme, so the effect of oxidation on viral replication appears to be complex and proteins other than just NS1 appear to be sensitive to oxidative conditions. We are currently examining how antioxidant treatment affects NS1 localization and disulfide bond formation to better define the effect of NS1 oxidation status on viral RNA replication.

To our knowledge, there have been no studies on the effects of oxidation within or between any of the cytoplasmic nonstructural viral proteins primarily because it has been assumed that the viral replication proteins would be in a reducing environment and not susceptible to disulfide bond formation or methionine oxidation. Experiments to examine if disulfide bonds are formed within and between flavivirus and alphavirus nonstructural proteins during replication will be critical for further definition of the effects of oxidation on flavivirus RNA replication. Methionine oxidation during viral infection has also not been examined and represents a new line of inquiry into how changing intracellular conditions affect viral RNA replication. There are, however, several technical hurdles in assessing the status of methionine oxidation in proteins isolated from the cell. Simple lysis of the cell or disruption of the endoplasmic reticulum can lead to methionine residues becoming non-specifically oxidized during purification, and methionine sulfoxide is routinely observed during mass spectrometry analysis due to oxidation during ionization (Morand et al., 1993), complicating the identification of intracellular oxidation events. Additionally, only a small fraction of total NS5 produced during infection is thought to be found in positive strand RNA replication complexes, and the remaining NS5 protein is present in the cytoplasm interacting with cellular proteins (Fischl and Bartenschlager, 2011; Khadka et al., 2011) or is in some cases translocated to the nucleus (Pryor et al., 2007). Late in infection viral polyprotein translation is ramped up to increase the amount of structural proteins available for virion formation, and the nonstructural proteins are produced in excess. Therefore the challenge becomes purifying replication complex-associated NS5 in a manner that maintains natural disulfide bonds or methionine oxidation. We are currently in the process of developing protocols to determine the oxidation status of flavivirus nonstructural proteins during infection.

How do flaviviruses induce oxidative stress during infection? There is currently no definitive data on this topic, but several tantalizing possibilities exist. Japanese Encephalitis virus infection down-regulates thioredoxin expression (Yang et al., 2010), which would increase cytoplasmic oxidation and interfere with homeostatic redox balance during infection. Dengue virus infection can induce autophagy, which is used to support virus-induced membrane remodeling (McLean et al., 2011). Autophagy can induce a release of ROS, although this may not be a common mechanism, as WNV does not appear to utilize autophagy during replication (Vandergaast and Fredericksen, 2012). An interesting candidate for inducing oxidative stress is NS2A. NS2A is a transmembrane ER protein and is involved in RNA replication and evasion of the interferon response (Jones et al., 2005; Muñoz-Jordan et al., 2003). NS2A is partially responsible for the cytopathic effect produced during viral infection (Rossi et al., 2007). Furthermore, NS2A can induce interferon-independent apoptosis (Melian et al., 2013), and oxidative stress is well known to induce apoptosis. Two cell-adapted mutants in the NS2A protein, D73H and M108K, increase cellular viability and reduce RNA replication during infection (Rossi et al., 2007). A recent topology study of NS2A localizes D73H and M108K to transmembrane regions of NS2A (Xie et al., 2013), which raises the possibility that NS2A possesses channel or pore-like activity that allows ROS into the cytoplasmic side of the ER membrane. Indeed, NS2A possesses pore-like activity when expressed in *E. coli* (Chang et al., 1999). These results support the hypothesis that NS2A could alter the cellular redox balance late in infection and support a switch from negative strand to positive strand RNA synthesis.

Conclusion

Virus-controlled use of oxidative, pH, or ionic potential differences on either side of a replication membrane would provide an elegant mechanism for viruses to regulate their genomic replication. Many RNA viruses replicate their RNA on modified intracellular membranes with different environmental conditions on either side, and our findings with the flavivirus and alphavirus families may have broad implications for many different types of positive strand RNA viruses. Further analysis of how RNA viruses use oxidation and other environmental conditions to control RNA replication will help increase our understanding of how these pathogens usurp cellular resources to control the replication of their genomes and provide novel points of intervention that may be exploited for antiviral or vaccine development.

Materials and methods

Cells, chemicals, and viral assays

Baby Hamster Kidney (BHK) cells and *Aedes albopictus* C6/36 cells were obtained from the American Type Culture Collection (ATCC). Human Huh7 cells were generously provided by Dr. Keril Blight. BHK and Huh7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Atlas Biologicals), 5% Pen/Strep, 5% Sodium Pyruvate and 5% L-Glutamine, and C6/36 cells were maintained in L-15 medium (Life Technologies) in 10% FBS, 5% Pen/Strep, 5% Sodium Pyruvate and 5% L-Glutamine. West Nile virus (subtype Kunjin) and luciferase expressing Sindbis virus (Steel et al., 2011) were used for viral infection studies. Butylated hydroxyanisole (BHA) and DMSO were obtained from Sigma-Aldrich (St. Louis, MO); an equal volume of DMSO was used as a control in experiments with BHA. KUNV RNA was collected from culture media and extracted using Trizol LS (Invitrogen, La Jolla, CA). KUNV plaque assays and real-time quantitative reverse transcriptase PCR assays (qRT-PCR) were performed as described (Stahla-Beek et al., 2012). Strand-specific qRT-PCR was performed using the protocol described by Youn et al. (2013). Dengue virus type 2 replicon assays were performed using persistent DENV-2 replicon cells expressing *Renilla* luciferase (Whitby et al., 2005).

Renilla luciferase activity and cellular viability were determined using Viviren Live Cell *Renilla* Luciferase substrate and CellTiter-Glo Cell Viability Assay (Promega), respectively, on a Victor X5 Multi-Mode plate reader as previously described (Stahla-Beek et al., 2012; Whitby et al., 2005). *Renilla* and cell viability curves were generated using the Prism Graphpad software.

Intracellular oxidative stress was detected in BHK cells 24 h after chemical treatment or virus infection. Cells were washed with PBS and incubated with CM-H_2DCFDA according to the manufacturers recommendations (Life Technologies, Carlsbad CA). All fluorescence microscopy images were collected on a Nikon Diaphot 200 inverted fluorescence microscope.

Plasmid-launched West Nile virus replicon expressing firefly luciferase was previously described (Pierson et al., 2006). Site-directed mutagenesis of the replicon expression plasmid was performed using Quick Change mutagenesis (Stratagene, La Jolla, CA) according to the manufacturers recommendations. To test the effect of mutations of on viral RNA replication, wildtype or mutant replicon plasmids were transfected into BHK cells using Lipofectamine 2000 (Life Technologies, Carlsbad CA). 24 h post-transfection the cells were lysed with Glo Lysis buffer (Promega, Madison, WI) and 20 μl of the lysates were transferred to an opaque 96 well plate. The firefly luciferase signal was detected on a Perkin Elmer Victor X5 Multi-Mode plate reader by injecting 100 μl of firefly luciferase substrate (Promega, Madison, WI) into each wells and measuring relative light units.

Expression and purification of recombinant proteins

Expression and purification of the wild-type dengue virus type 2 NS5 protein for oxidation studies was performed as described previously (Geiss et al., 2009; Henderson et al., 2011). Small-batch purification was used to prepare mutant NS5 capping enzymes. Each cysteine in the DENV-2 NS5 was mutated to serine using Quick Change mutagenesis and verified by sequencing. Mutant and wild-type plasmids were transformed into BL21 DE3 pLysS *E. coli* cells and induced with 400 μM IPTG overnight. Bacterial pellets were collected in low imidazole buffer, disrupted by sonication, and clarified lysates collected. Clarified lysates were incubated with HIS-select HF Nickel Affinity beads (Sigma-Aldrich, St. Louis MO) at 4 °C for 30 min, then the beads were washed with low imidazole buffer on Micro Bio-spin columns (Bio-Rad, Hercules, CA) and eluted in 250 mM imidazole buffer. Proteins were buffer exchanged on Zeba Spin Desalting Columns (Pierce) into gel filtration buffer (5 mM Tris pH 7.5, 300 mM NaCl, 20% Glycerol). Protein concentrations for purified proteins were determined using A_{280} absorbance and extinction coefficients obtained from the ExPASy website. The expression clone for recombinant VEEV nsP1 protein (Trinidad Donkey Strain) was obtained from the Seattle Structural Genomics Center for Infectious Disease (SSGCID) and was expressed and purified as described above.

Thermal scanning calorimetry was performed on a BioRad CFX96 Real-Time PCR detection system using a protocol adapted from Lavinder et al. (2009). Briefly, 1 μl of 10X Krypton Infrared Protein stain (Pierce) was added to 30 μM of each protein in gel filtration buffer in a final volume of 10 μl in optically transparent qPCR tubes. Melt curve analysis was performed using the Quasar 705 channel on a BioRad CFX96 Real-Time PCR detection system, and melting temperature for each mutant was determined by maximum d(RFU)/dT value. Due to the low resolution of the instrument we acquired three replicates of the exact same temperature per sample we had to add a decimal place to the values to generate variance for statistical analysis. In order to do this we assumed maximum variance in the tenths position that would still yield the same average temperature and then performed a one-way ANOVA analysis using these values.

Mass spectrometry analysis of DENV-2 NS5 capping enzyme methionine oxidation was performed at the CSU Proteomics and Metabolomics Facility. Mock treated or H_2O_2 treated proteins were resolved on 12% SDS-PAGE gels, trypsin digested, and peptides analyzed on a Velos Orbitrap mass spectrometer in HCD mode. Quantitative analysis of Met219 oxidation status was performed using Skyline (MacLean et al., 2010).

Protein guanylation assay

Protein guanylation assays were performed as previously described (Geiss et al., 2011). Briefly, the 3 μM enzyme was incubated with 1 μM GTP-ATTO 680 (Jena Bioscience, Germany) in 5 mM Tris-base (pH 7.5), 500 nM $MgCl_2$ and 0.1% NP-40 for 120 min at 37 °C. We have observed that VEEV nsP1 (New World Alphavirus) does not require *S*-adenosylmethionine for guanylyltransferase activity whereas Old World Alphavirus nsP1 requires *S*-adenosylmethionine (Ahola and Kääriäinen, 1995; Tomar et al., 2011), so *S*-adenosylmethionine was not added to VEEV nsP1 guanylation reactions. The reactions were resolved on 12% polyacrylamide gels, and the extent of protein guanylation was quantified using an Odyssey IR Imaging system (Li-Cor, Lincoln, NE). Gels were stained with Coomassie Blue and GTP-ATTO 680 signals were normalized for protein loading.

Cap immunoprecipitation and qRT-PCR analysis

Total RNA was extracted from KUNV infected BHK-21 cells treated with BHA or DMSO at 24 h post infection using TRIzol (Invitrogen, La Jolla CA). A mouse monoclonal antibody to the m3G/m7G RNA cap structure (Synaptic Systems) was used to fractionate 5 μg of total RNA into capped and uncapped portions as previously described (Moon et al., 2012). An uncapped, 5′ tri-phosphorylated negative control RNA was prepared by in vitro transcription from linearized pGEM-4 plasmid using a MEGAscript SP6 kit (Invitrogen) followed by gel purification. Equal volumes of each RNA fraction were reverse transcribed using Improm-II reverse transcriptase (Promega) and qRT-PCR was performed with SYBR Green Supermix (BioRad) on a BioRad CFX96 Real-Time PCR detection system. The relative amount of uncapped KUNV or *GAPDH* RNA in each treatment was determined by comparing the amount of RNA in the unbound fraction to the 10% input fraction using the $\Delta\Delta Ct$ method with pGEM set as the reference gene.

Fluorescence polarization assays

GTP displacement assays were performed as described (Geiss et al., 2009). Fluorescence polarization (FP) values were determined on a Victor X5 Multi-Mode plate reader (Perkin-Elmer). Curves were generated with the Prism Graphpad software package.

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