Cell Host & Microbe
Article

Cholesterol Manipulation by West Nile Virus Perturbs the Cellular Immune Response

Jason M. Mackenzie,[1,4,*] Alexander A. Khromykh,[1,3] and Robert G. Parton[2,3]
[1]School of Molecular and Microbial Sciences
[2]Institute for Molecular Bioscience and Centre for Microscopy and Microanalysis
University of Queensland, Brisbane, Queensland 4072, Australia
[3]These authors contributed equally to this work.
[4]Present address: Department of Microbiology, La Trobe University, Victoria 3086, Australia.
*Correspondence: j.mackenzie@uq.edu.au
DOI 10.1016/j.chom.2007.09.003

SUMMARY

Complex membrane structures induced by West Nile virus (WNV), an enveloped RNA virus, are required for efficient viral replication. How these membranes are induced and how they facilitate the viral life cycle are unknown. We show that WNV modulates host cell cholesterol homeostasis by upregulating cholesterol biosynthesis and redistributing cholesterol to viral replication membranes. Manipulating cholesterol levels and altering concentrations of cellular geranylgeranylated proteins had a deleterious effect on virus replication. Depletion of the key cholesterol-synthesizing enzyme 3-hydroxy-methyglutaryl-CoA reductase drastically hampered virus replication. Significantly, virus-induced redistribution of cellular cholesterol downregulated the interferon-stimulated Jak-STAT antiviral signaling response to infection. This defect could be partially restored by exogenous addition of cholesterol, which increased the ability of infected cells to respond to interferon. We propose that, by manipulating cellular cholesterol, WNV utilizes the cellular response to cholesterol deficiency and dependence of antiviral signaling pathways on cholesterol-rich microdomains to facilitate viral replication and survival.

INTRODUCTION

The *Flaviviridae* are a family of highly pathogenic, small, enveloped RNA viruses that cause a range of infections in man, for example, yellow fever, encephalitis, dengue (by species of the *Flavivirus* genus), and hepatitis C (by hepatitis C virus [HCV], a species within the hepacivirus genus). Hundreds of thousands of deaths associated with flavivirus infections occur each year, many of which are unpreventable with current vaccine and therapeutic strategies. The recent emergence and spread of West Nile virus New York 99 (WNV_{NY99}) strains within North America has also presented a considerable concern, as 24,734 cases with 920 fatalities have been reported since 1999. The prevalence of HCV is also increasing, and liver cirrhosis and carcinoma, pathological outcomes of HCV infection, are likewise on the increase.

Like all viruses, flaviviruses exploit the cellular machinery to facilitate propagation of infectious progeny virions. An Australian strain of WNV virus, Kunjin (WNV_{KUN}) represents one of the best studied models of intracellular changes induced by flavivirus infections. During the infection process highly complex membrane structures are induced, which act as platforms for viral replication (Mackenzie et al., 1999; Westaway et al., 2002). How these membranes facilitate viral RNA replication and virion assembly and the virus-cell interactions involved in inducing these specialized membrane subdomains are currently unknown (Mackenzie, 2005). In addition to utilizing the cellular machinery for replication, viruses must also disable and/or circumvent the cellular immune response. A key mediator of our host defense against pathogens is the interferon response. During viral infection this response is triggered by recognition of viral products by pattern recognition receptors (Akira et al., 2006; Basler and Garcia-Sastre, 2007; van Boxel-Dezaire et al., 2006). This recognition leads to cascade of signaling events including the activation of transcription factors involved in induction of expression of IFN-a/β. Secreted IFN-α/β then binds to specific cell surface receptors to initiate signaling cascade via the Jak-STAT pathway, culminating in the production of a wide range of antiviral products (Akira et al., 2006; Garcia-Sastre and Biron, 2006). Like many viruses, flaviviruses can combat IFN-α/β signaling, primarily by interfering with the activation of Jak-STAT pathway (Guo et al., 2005; Jones et al., 2005; Lin et al., 2004; Liu et al., 2005; Munoz-Jordan et al., 2003). A number of flavivirus proteins have been implicated in inhibition of IFN signaling, although different flaviviruses appear to utilize different viral proteins for this purpose (Best et al., 2005; Lin et al., 2006; Liu et al., 2005; Munoz-Jordan et al., 2003, 2005). However, why different viral proteins from different flaviviruses are required to perform the same function and what the mechanism of this inhibition is are not clear.

In vertebrate cells cholesterol homeostasis is tightly regulated in a feedback mechanism via transcriptional factors that sense intracellular cholesterol levels (Adams et al., 2003; Brown et al., 2002; Brown and Goldstein, 1997; Goldstein et al., 2006; Horton et al., 2002; Nohturfft et al., 2000; Radhakrishnan et al., 2007; Sun et al., 2007). These transcription factors are termed sterol-regulatory element-binding proteins (SREBPs) that associate tightly with the sterol-sensing protein SREBP cleavage-activating protein (SCAP) within the ER membrane, via an additional interaction with the ER resident protein Insig. SCAP has an additional role as an escort protein that mediates transport of the SREBP-SCAP complex to the Golgi apparatus where SREBP is proteolytically cleaved by two resident Golgi proteases (S1P and S2P) to release the transcriptionally active form of SREBP from the membrane. The released form of SREBP is transported to the nucleus and activates transcription of target genes required for cholesterol biosynthesis, including HMGCR and fatty acid synthase. When cholesterol levels are high, SCAP binds to cholesterol in the ER, promoting an association with Insig, retaining the complex within the ER, thus reducing the synthesis of cholesterol. Conversely, when cholesterol levels are low, binding of SCAP to Insig is disrupted, and cholesterol metabolism is initiated. As the virus-induced membranes show similarities to membranes induced upon perturbation of cholesterol-synthesizing enzymes (Orci et al., 1984), we speculated that changes in cholesterol could underlie the cellular changes occurring upon viral infection.

RESULTS

WNV Infection Leads to a Redistribution of Cholesterol and Cholesterol-Synthesizing Proteins to the Sites of Virus Replication

We first examined the intracellular distribution of cholesterol in control and WNV_{KUN}-infected cells using filipin as a marker of unesterified cholesterol. Filipin stained the plasma membrane and internal organelles of mock-infected cells (Figures 1Ac and 1Ad), but this distribution was markedly different in WNV_{KUN}-infected cells (Figure 1Ab). Infection of cells with WNV_{KUN} led to a more confined perinuclear localization of filipin-bound cholesterol and a dramatic loss of cholesterol staining at the plasma membrane (indicated by arrowheads in Figures 1Ac and 1Ad). Perinuclear foci detected with filipin also coincided with those stained with virus-specific anti-dsRNA antibodies (compare Figures 1Aa and 1Ab). Thus, WNV_{KUN} appears to coordinate a redistribution of cellular cholesterol to the sites of virus replication.

We next examined the possible role of the key cholesterol-synthesizing enzyme, 3-hydroxy-methyglutaryl-CoA reductase (HMGCR), in WNV_{KUN} replication. HMGCR is required for the synthesis of mevalonate, a precursor for isoprenoids and cholesterol, and is upregulated in response to cholesterol depletion or degraded in response to high sterol levels (Goldstein and Brown, 1990). Additionally, overexpression of HMGCR during compactin treatment resulted in the proliferation and induction of reticular membranes visually similar to those observed during flavivirus infection (Anderson et al., 1983; Orci et al., 1984). Expression of HMGCR was induced 3- and 5-fold after 24 hr of infection of Vero cells with either WNV_{NY99} or WNV_{KUN}, respectively (Figure 1B). In addition, immunofluorescence (IF) analysis indicated that a significant pool of HMGCR was confined to the large cytoplasmic foci recognized by antibodies directed against the WNV_{KUN} nonstructural protein NS1 in both WNV_{NY99}- and WNV_{KUN}-infected cells (Figure 1C). This was further confirmed by immunolabeling of thawed cryosections from WNV_{KUN}-infected cells where anti-HMGCR labeling was associated with the virus-induced convoluted-membrane (CM) structures (Figure 1D, quantitation analysis in Figure S2 in the Supplemental Data available with this article online). These observations suggest a role for HMGCR and cholesterol in establishing membrane platforms required for efficient WNV replication. The localization of HMGCR within proliferated reticulum membranes in infected cells also implicates a possible role for HMGCR in the biogenesis of these membrane platforms, potentially via the virus-induced relocation of cellular cholesterol pools in concert with the WNV_{KUN} NS4A protein, previously shown to proliferate internal membranes (Roosendaal et al., 2006).

Changes in Cholesterol Biosynthesis and/or Trafficking Affect WNV RNA Replication

To determine whether the observed redistribution of cholesterol (Figure 1A) was essential for WNV replication, we used a WNV_{KUN} replicon encoding the reporter protein β-galactosidase in conjunction with treatments that affect cholesterol biosynthesis (see Table S1). We have shown previously that β-galactosidase expression in this system correlates well with WNV_{KUN} replicon RNA replication efficiency (Liu et al., 2002). To dissect the role of HMGCR during WNV infection, we utilized treatments that directly modulate HMGCR activity (namely the HMGCR inhibitor lovastatin [Lov]) and that affect the level of cellular cholesterol (methyl-β-cyclodextran [M-β-CD]) or the processing of cholesterol transcription factors (25-hydroxycholesterol [25-HC]; Wang et al., 1994). In addition, we increased cholesterol levels by the addition of exogenous cholesterol. Addition of Lov, 25-HC, or cholesterol itself to the culture medium had deleterious effects on replicon RNA replication (48%, 30%, and 59% decreases, respectively; see Figure 2A). Interestingly, a greater synergistic effect was observed when Lov and cholesterol were added together. In this case replicon replication was decreased by 89%. All of these observed effects were also reflected in decreases in virus replication (Figures 3A and 3B), the production of infectious virus (Figure 3C), and the formation of the virus-induced membrane structures when assessed on WNV_{KUN}-infected cells (Figure 3D). The observed effects of the cholesterol-modulating compounds appeared specific for WNV_{KUN} replication, as similar treatments of cells transfected with a Semliki Forest virus (SFV) replicon expressing the β-galactosidase gene showed

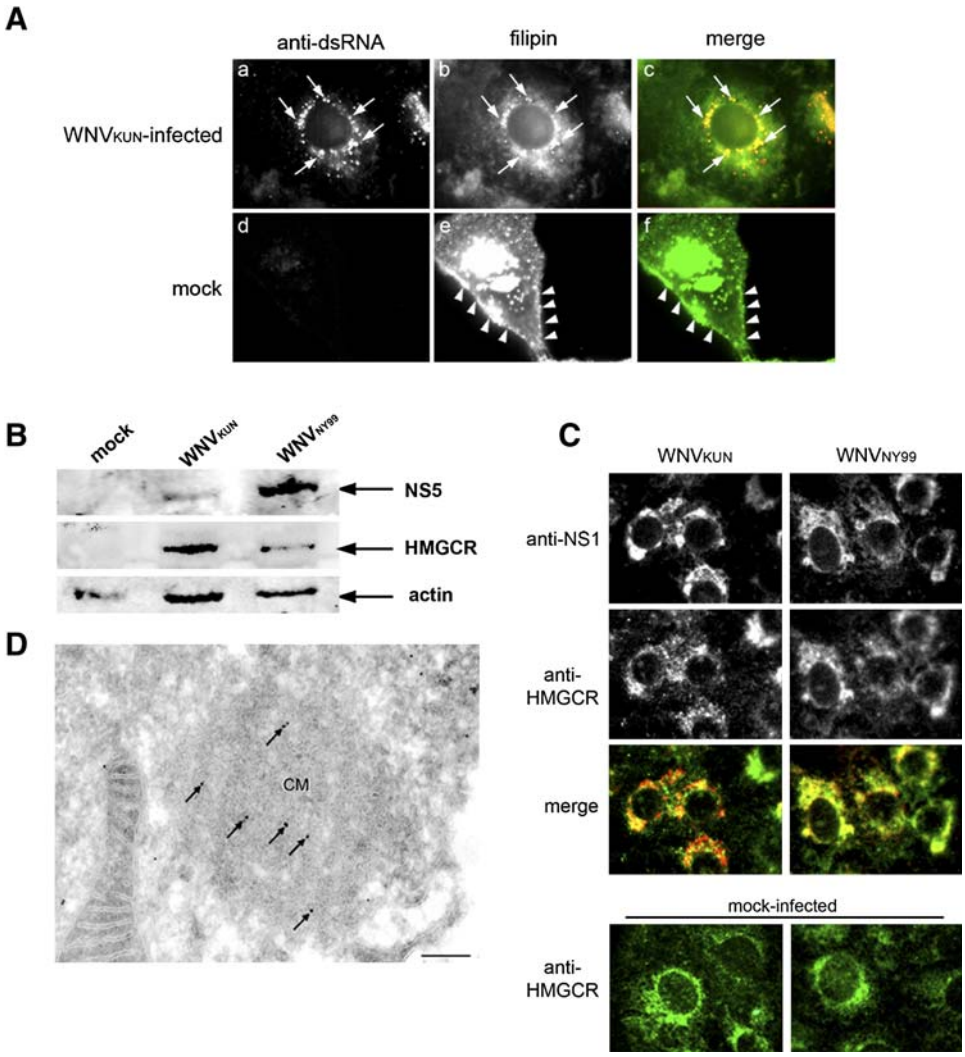

Figure 1. Infection of Mammalian Cells with WNV Redistributes Intracellular Cholesterol and HMGCR to the Sites of Virus Replication

Vero cells infected with WNV_{KUN} for 24 hr were stained with the cholesterol-binding compound filipin (Aa–Ac) and viewed by fluorescence microscopy. A drastic decrease in the amount of cholesterol at the plasma membrane is observed when compared to uninfected cells (arrowheads in [Ae] and [Af]), with an associated increase of cholesterol staining in the perinuclear area of the infected cells (arrows in [Aa]–[Ab]) with a majority colocalizing with virus replication sites immunostained with antibodies to anti-dsRNA (Aa). For ease of visualization, the filipin staining was imported into the green channel during processing in Adobe Photoshop software, and a yellow hue in (Ac) indicates overlap of the anti-dsRNA and filipin staining. (B) Western blot analysis of lysates prepared from mock-, WNV_{KUN}-, or WNV_{NY99}-infected cells indicated that HMGCR protein expression was upregulated during virus infection. Subcellular localization studies revealed that a majority of the HMGCR protein accumulated within virus-induced membrane structures, as assessed by IF analysis (C) with rabbit antibodies specific to HMGCR visualized with anti-rabbit Oregon green and mouse antibodies to WNV NS1 visualized with anti-mouse Texas red. Colocalization is observed as a yellow hue in the right-hand panels. Additionally, immunolabeling of thawed cryosections from WNV_{KUN}-infected cells with antibodies specific to HMGCR (D) clearly indicated accumulation within the virus-induced CM structures. Abbreviations in (D): CM, convoluted membranes; VP, vesicle packets. Scale bars, 200 nm.

different effects on SFV replication (Figure S1A). For example, treatment of SFV-transfected cells with cholesterol had no effect on SFV replication, whereas treatment with Lov increased SFV replication by ~100% (Figure S1A). These observations demonstrate specific inhibitory effects of the cholesterol-modulating drugs on WNV replication. Additionally, only minor cytotoxic effects were observed when transfected or mock-infected cells were incubated with the cholesterol compounds (Figures S1B and S1C). Taken together, these results suggest that viral infection causes redistribution of intracellular cholesterol and an associated upregulation of HMGCR and imply that WNV replication is dependent on HMGCR activity. The corresponding influence of HMGCR on virus replication appears to be tightly linked to efficient viral replication and induction of virus-induced membrane platforms. We have previously shown that efficient WNV_{KUN} is intimately linked with membrane induction (Mackenzie et al., 2001).

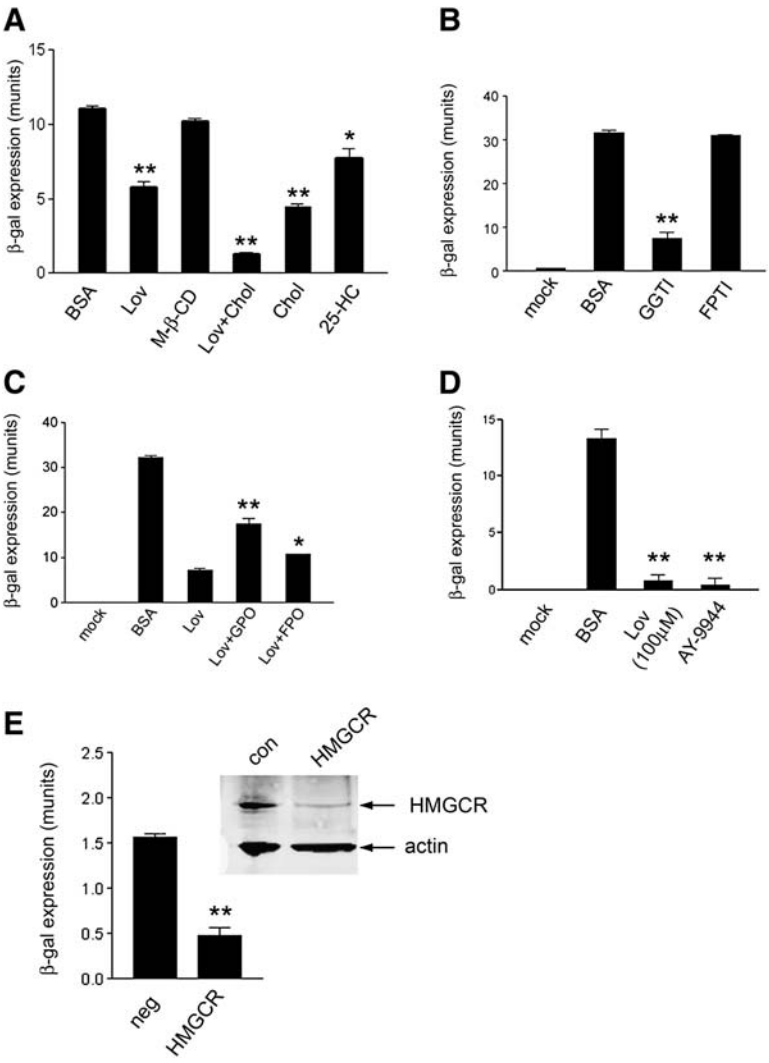

Figure 2. WNV_{KUN} Replication Is Dependent on HMGCR Activity, Implicating a Role for Both Cholesterol and Geranylgeranylated Host Proteins during Replication

(A) Quantification of the observed effects of lovastatin, M-β-CD, cholesterol, 25-hydroxycholesterol, and a combination of lovastatin and cholesterol on WNV_{KUN} replicon RNA replication. Addition of lovastatin (**$p < 0.0001$, Student's t test), cholesterol (**$p < 0.0001$, Student's t test), and 25-hydroxycholesterol (*$p < 0.002$, Student's t test) all efficiently reduced replication of the replicon RNA, whereas the combination of lovastatin and cholesterol (**$p < 0.0001$, Student's t test) had a greater synergistic effect on the replicon replication.

(B and C) Quantification of the effect of geranylgeranylation inhibitors on WNV RNA replication. The geranylgeranylation inhibitor GGTI had a marked effect on replicon replication (**$p < 0.0001$, Student's t test), whereas the farnesylation inhibitor FPTI had no effect on replication (not significant). The role for geranylgeranylation was further substantiated, as addition of geranyl-phosphate could partially rescue the observed replicon replication inhibition by lovastatin (**$p < 0.0001$, Student's t test), whereas the addition of farnesyl-phosphate was less effective (*$p < 0.01$, Student's t test).

(D) AY-9944 treatment dramatically impaired WNV_{KUN} RNA replication (**$p < 0.0001$, Student's t test), establishing a strict requirement for both cholesterol and geranylgeranylation during efficient replication.

(E) RNAi-mediated knockdown of the HMGCR gene efficiently reduced WNV_{KUN} replicon replication (**$p < 0.0001$, Student's t test). The western blot indicates that expression of the HMGCR protein was effectively reduced by the RNAi treatment but not with a control (con) RNAi.

The role for HMGCR during viral membrane formation is exemplified by the profound effects of cholesterol and Lov addition, whereby the induction of viral membranes is hampered (Figure 3D) and correspondingly reduces virus replication. This is in contrast to the recent report indicating that HCV RNA was retained within membranous structures in the presence of cholesterol inhibitors (Sagan et al., 2006). WNV replication complex is derived from *trans*-Golgi membranes, and the HCV replication complex is derived from ER membranes; thus, the effects of cholesterol-modulating drugs on the composition of these two different cellular membranes modified by virus infection may be different. Exogenous addition of cholesterol was previously observed to lead to dissolution of HMGCR-induced membrane proliferation (Anderson et al., 1983), correlating with our observations presented here.

An additional effect of HMGCR upregulation is to increase synthesis of prenylated (geranylgeranylated and farnesylated) proteins. Previous investigations have reported that the observed deleterious effects of Lov on HCV replicon replication (Sagan et al., 2006; Ye et al., 2003) involved geranylgeranylation of the host protein FBL2 (Wang et al., 2005). To investigate whether WNV replication was dependent on prenylation, we assessed WNV_{KUN} replicon replication in the presence of inhibitors of protein geranylgeranylation and farnesylation. WNV_{KUN} replicon RNA replication was unaffected in the presence of an inhibitor of farnesylation (FPTI) but was significantly impaired in the presence of the geranylgeranylation inhibitor GGTI (decreased to approximately 24% of untreated controls; Figure 2B). Inhibition of WNV_{KUN} replicon replication imposed to some extent by the prevention of prenylation modification induced by Lov treatment (Figures 2A and 2C) could be partially restored by the addition of geranyl-pyrophosphate (Lov + GPO; to approximately 60% of untreated levels) and to some extent by the addition of farnesyl-pyrophosphate (Lov + FPO; 34% of untreated levels) (Figure 2C). As HMGCR-mediated modification of prenylated proteins occurs before the

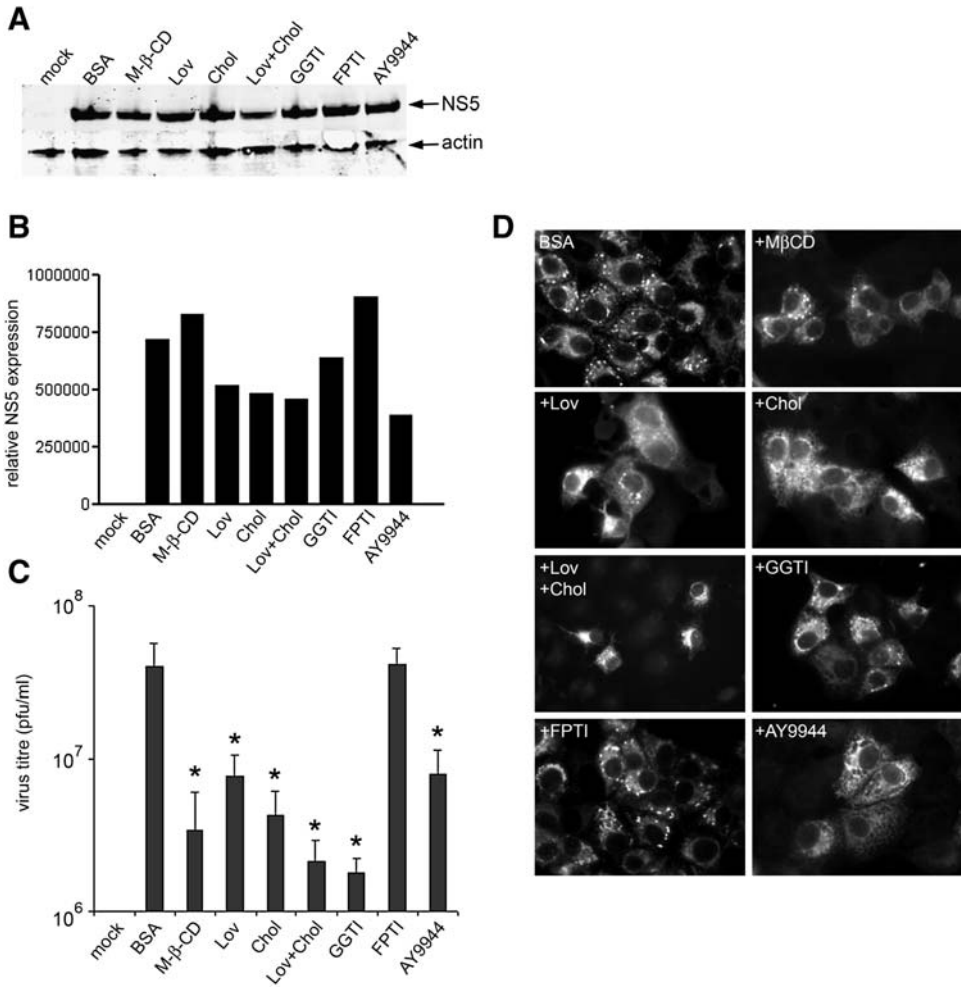

Figure 3. Modulation of HMGCR Activity and Cholesterol Biosynthesis Has Deleterious Effects on WNV_{KUN} Virus Production

(A) WNV_{KUN}-infected Vero cells were treated with the appropriate drugs for 22.5 hr, at which point the infected lysates were harvested for protein analysis by western blotting for the WNV_{KUN} protein NS5, and the host protein actin was used as a loading control.

(B) The relative expression of the NS5 protein was quantified by normalizing its expression based on the expression of the host protein actin.

(C) Tissue culture fluid was harvested from treated and WNV-infected cells to assess the production of infectious virus particles upon treatment with the appropriate drugs. Statistical analysis: *$p < 0.05$, Student's t test.

(D) Formation of viral membrane platforms associated with viral replication is regulated by HMGCR activity. WNVKUN-infected cells were treated with the appropriate concentration of drugs for 22.5 hr and subsequently assessed for virus-induced membrane formation by IF. Subcellular localization of WNVKUN NS3 was identified by immunostaining with rabbit mono-specific antibodies and visualized with rabbit-specific IgG conjugated to Oregon green.

synthesis of cholesterol, we sought to address whether the observed Lov-induced defects were only due to the reduction in available geranylgeranylated proteins or also due to the production of cholesterol itself. For these analyses we took advantage of a drug, AY-9944, that specifically blocks the host enzyme 7-dehydrocholesterol Δ7-reductase responsible for the final conversion step of 7-dehydrocholesterol to cholesterol (Kolf-Clauw et al., 1996). Addition of AY-9944 dramatically decreased the replication efficiency of the WNV_{KUN} replicon (Figure 2D) to levels equivalent to that observed with GGTI. In addition, RNAi knockdown of the HMGCR gene resulted in a significant reduction in WNV_{KUN} RNA replication (Figure 2E). These results suggest that the replication of WNV_{KUN} shows a strict requirement for both intracellular cholesterol and geranylgeranylated host proteins that depends on the expression and activity of HMGCR.

WNV Infection Severely Compromises Formation of Cholesterol-Rich Microdomains and Their Function in Activation of Interferon Signaling

In view of the redistribution of cholesterol from the plasma membrane in response to WNV infection (Figure 1), we speculated that cellular cholesterol-dependent processes may be affected, including the formation of cholesterol-rich microdomains (or lipid rafts) at the plasma membrane.

We hypothesized that such modulation may regulate the interferon (IFN)-mediated response to WNV_{KUN} infection. We first tested whether the formation of cholesterol-dependent surface microdomains was affected upon viral infection. As an indicator of surface cholesterol disruption, we assayed association of caveolin-1 (Cav1) with detergent-resistant membranes (DRMs). Cav1 was concentrated in DRMs in mock-infected cells (Figure 4A) but was absent in DRMs isolated from WNV_{KUN}-infected cells. Instead, Cav1 was primarily associated with heavy fractions containing the WNV_{KUN} protein NS5 (Figure 4A). These observations are consistent with disruption of cholesterol-dependent lipid raft domains.

We next examined whether the observed disruption of cholesterol-rich microdomains was associated with the lack of Stat protein phosphorylation observed during flavivirus infection (Best et al., 2005; Lin et al., 2004, 2006; Liu et al., 2005; Munoz-Jordan et al., 2003). Localization of Stat proteins 3, 5, and 6 within cholesterol-rich microdomains has been documented (Maziere et al., 2001; Sehgal et al., 2002; Shah et al., 2002), but the association of IFN-stimulated Stat-1 and Stat-2 proteins has not been so well described (Marchetti et al., 2006; Sehgal, 2003). Thus, we utilized three independent assays to assess the role of cholesterol in facilitating effective intracellular signaling via the interferon receptor. Depletion or binding of cholesterol at the plasma membrane with M-β-CD or filipin severely hampered efficient phosphorylation and nuclear translocation of Stat-1 protein in response to IFN-α stimulation and impaired transcription of a transfected reporter plasmid encoding the firefly luciferase gene under control of an interferon-stimulated responsive element (ISRE; King and Goodbourn, 1994) (Figures 4B–4D). In all cases this impairment could be partially rescued with the exogenous addition of cholesterol (Figures 4D and 4E), strongly implying a direct involvement of cholesterol in these processes.

In view of these results and the dramatic effect of the virus on cholesterol distribution (Figure 1), we tested the possibility that virus-mediated inhibition of interferon signaling was due, in part, to an effect on surface cholesterol levels. If so, we would predict that inhibition of signaling pathways during virus infection should be rescued by cholesterol addition. As shown in Figure 5, using two independent assays we could show that Stat-1 phosphorylation and translocation to the nucleus was inhibited in WNV_{KUN}-infected cells in the presence of IFN-α, and the majority of Stat-1 was not phosphorylated and remained within the cytoplasm (Figures 5B and 5D). However, addition of cholesterol prior to, and during, IFN stimulation resulted in substantial rescue of Stat-1 phosphorylation (Figure 5D) and a striking 3-fold increase in nuclear accumulation of Stat-1 protein (arrows in Figure 5B; quantitation in Figure 5C). This can be attributed to the replenishment of cholesterol at the plasma membrane in WNV_{KUN}-infected cells during incubation with exogenous cholesterol (Figure 5E). We could also show that the replenishment of cholesterol-rich microdomains and the dissolution of the WNV membranous replication complex in infected cells upon incubation with exogenous cholesterol (Figure 5E and Figure 3, respectively) have accordingly increased the ability of infected cells to respond to the antiviral effects of IFN-α (Figure 6). In the presence of both Chol and IFN, WNV RNA replication was significantly impaired by restored IFN-mediated signaling and by the negative regulation of cholesterol biosynthesis.

These results provide compelling evidence to support the hypothesis that the observed redistribution of cholesterol causes disruption of cholesterol-rich microdomain function and disables the ability of cells to respond to IFN-mediated stimulation upon WNV_{KUN} infection.

DISCUSSION

Our results show that WNV directly manipulates the host cell pathway involved in cholesterol biosynthesis in order to facilitate both virus replication and viral evasion from the cellular antiviral response. Our observations indicate that WNV manipulates the intracellular distribution of cholesterol from the plasma membrane to membranous sites of virus replication. We hypothesize that upon replication WNV_{KUN} redistributes intracellular cholesterol pools (presumably from the plasma membrane) to the sites of virus replication. This relocation of intracellular cholesterol (away from sites normally harboring cholesterol sensory molecules, such as the SREBP-Insig complex) triggers the cell's natural response to reduced cholesterol levels. The associated upregulated expression of HMGCR leads to an increase in cholesterol and geranylgeranylated protein biosynthesis and proliferation of intracellular membranes. The expression of HMGCR is tightly regulated at the transcriptional level via the nuclear import of the posttranslationally cleaved SREBP-2 protein. The proteolysis of SREBP-2 occurs in the Golgi apparatus, and transport of SREBP-2 only occurs when sterol levels are low via dissociation of the SREBP complex from the ER-retained Insig molecule. Therefore, our observations that HMGCR is upregulated in infected cells strongly suggest that cholesterol homeostasis and distribution have been affected. Overexpression of HMGCR is known to induce the proliferation of intracellular membranes (Orci et al., 1984) similar to that observed during WNV_{KUN} infection, and we speculate that HMGCR may play a synergistic role with WNV_{KUN} NS4A (Roosendaal et al., 2006) in the induction of viral-induced membranes. We envision that the increased membrane platform aids the virus by providing a larger surface area for viral protein production and virion formation. However, the replenishment of cholesterol appears to direct cholesterol to the "correct" intracellular location where the increased presence of sterol induces Insig binding to SREBP, thereby facilitating its retention within the ER (Goldstein and Brown, 1990; Sun et al., 2005; Yang et al., 2002). The retention of SREBP-Insig within the ER is a negative regulator of HMGCR synthesis and thus mevalonate biosynthesis. This accordingly has a detrimental effect on virus replication through the reduced levels of cholesterol and geranylgeranylated proteins and reduced levels of HMGCR contributing to

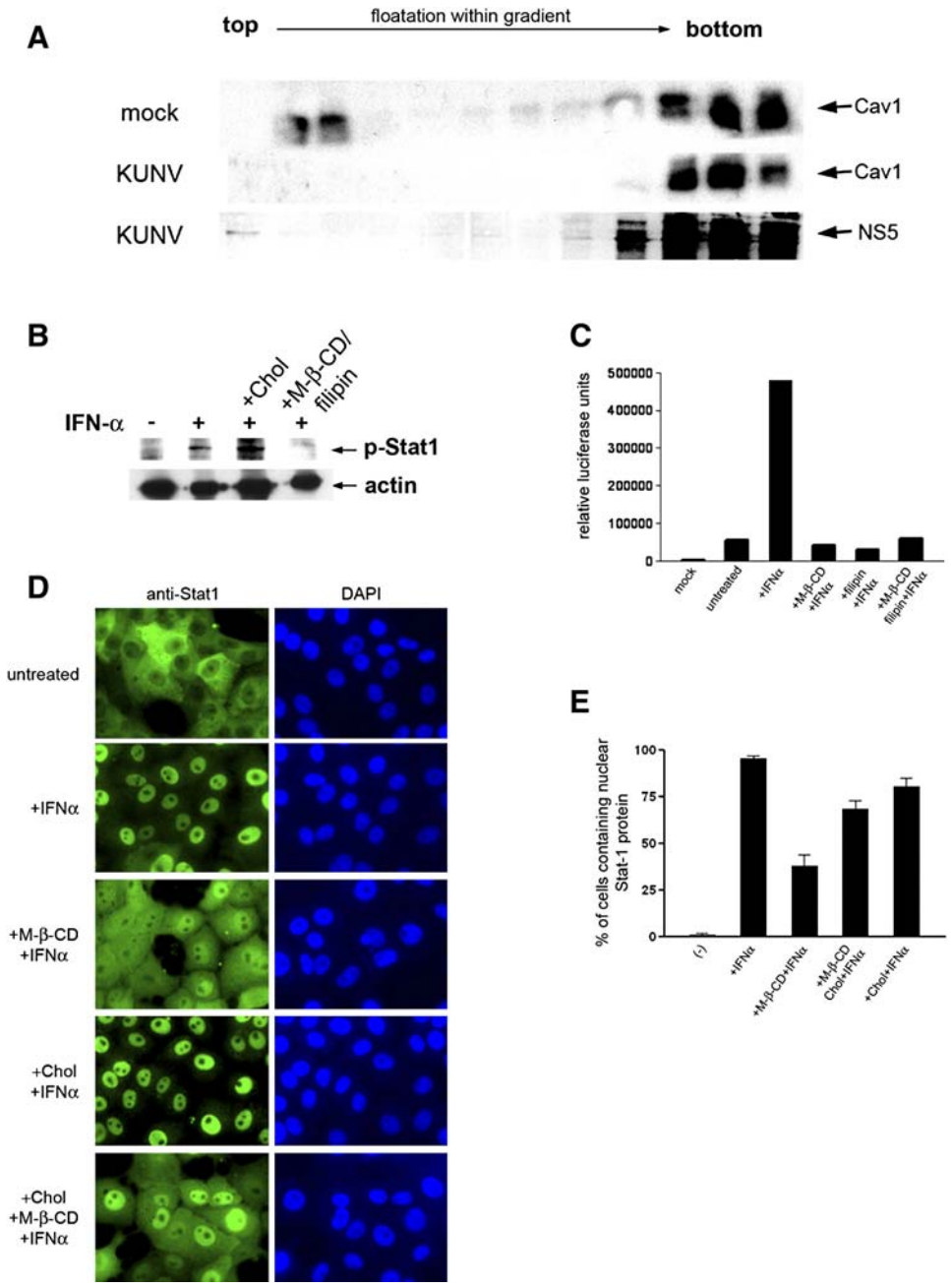

Figure 4. Redistribution of Intracellular Cholesterol in WNV_{KUN}-Infected Cells Leads to a Loss of Lipid Raft Function

(A) Western blot and sucrose flotation analysis of caveolin-1 within detergent resistant membranes isolated from mock- and WNV_{KUN}-infected cells reveals that the ability of caveolin-1 to float within the gradient from infected cells is impaired.

(B) Western blotting for phosphorylated-Stat-1 protein after treatment of cells with cholesterol or M-β-CD and filipin and subsequent stimulation with interferon-α reveals a dependency on cholesterol-rich microdomains for efficient IFN signaling.

(C) Activation of interferon-stimulated regulatory element (ISRE)-driven transcription is shown as induction of luciferase expression in IFNα-stimulated cells treated or untreated with the cholesterol depleting agents M-β-CD and filipin.

(D) Nuclear translocation of Stat-1 protein was significantly impaired in the presence of M-β-CD, as assessed by IF analysis, but could be rescued by cotreatment with cholesterol.

(E) These results were quantified after triplicate experiments in both Vero and A549 cells.

membrane induction. In considering this model it is important to highlight that the natural vector of flavivirus infection, the mosquito, is a cholesterol auxotroph and cannot synthesize cholesterol. However, mosquito survival is dependent on cholesterol, and this dietary need is acquired through the blood meal. Insects still contain all

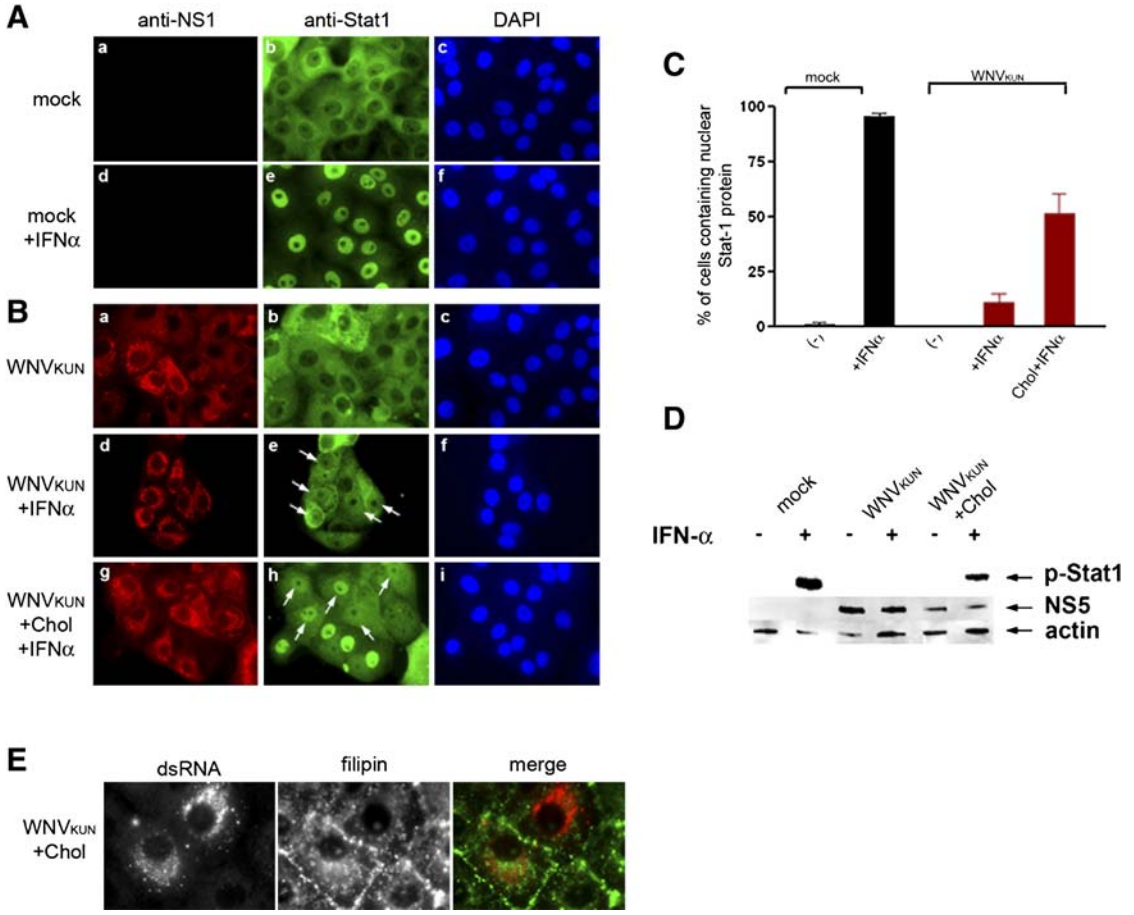

Figure 5. Addition of Cholesterol Can Rescue Stat-1 Nuclear Translocation and Phosphorylation in Response to IFN-α in WNV_{KUN}-Infected Cells

Nuclear translocation of Stat-1 occurred efficiently in uninfected cells in response to IFN-α stimulation but was impaired under similar conditions in WNV_{KUN}-infected cells (A and B). The translocation defect could be partially rescued upon incubation of WNV_{KUN}-infected cells with cholesterol and IFN-α (compare arrows in [Be] and [Bh]) as quantified in (C) after triplicate experiments in Vero and A549 cells. The ability of cholesterol to rescue interferon-mediated signaling was also reflected in the phosphorylation status of Stat-1 upon IFN-α treatment (D). (E) Exogenous addition of cholesterol to WNV_{KUN}-infected cells restores cholesterol concentration at the plasma membrane. Filipin staining at the plasma membrane is now clearly evident in the infected cells during incubation with cholesterol.

of the enzymes require to metabolize cholesterol and also encode an HMGCR protein for the production for prenylated proteins (Belles et al., 2005). Thus, it is intriguing to consider these traits between the vector hosts (mosquito) and the dead-end host (humans). Comparisons between mammalian and insect cells will be part of our ongoing studies in this area.

We propose that the redistribution of cholesterol from the plasma membrane has an additional, equally important effect, which is crucial for virus survival. WNV infection causes disruption of cholesterol-dependent surface domains, and this inactivates the IFN-regulated Jak/Stat signaling pathway, as demonstrated by the rescue of signaling by cholesterol addition to infected cells. Thus, the redistribution of intracellular cholesterol to sites of WNV RNA replication appears to play a key regulatory role in the dissociation of lipid raft formation and function, culminating in the loss of ability of infected cells to respond to IFN and to restrict viral replication. This is exemplified by the increased ability of infected cells to activate IFN signaling upon incubation with exogenous cholesterol (Figure 6). In this situation plasma membrane concentrations of cholesterol have been restored (Figure 5E), enabling the recruitment and activation of the IFN receptor and associated signaling molecules. This is also accentuated by the reduction in virus replication presumably due to the dissolution of the membranous replication complex (Figure 3). These observations have fundamental importance for understanding virus-cell interactions with potential therapeutic implications. WNV has exploited the complex machinery involved in cellular cholesterol regulation to serve its own replication needs, while utilizing the same cholesterol-based mechanism to inactivate surface signaling domains. Compounds that target cholesterol biosynthesis and/or HMGCR activity may provide candidate antiviral treatments to combat

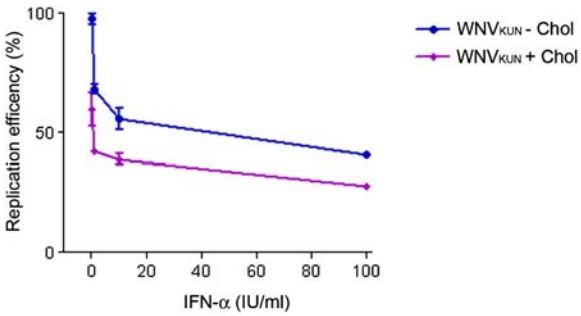

Figure 6. Addition of Cholesterol Enhances the Ability of IFN to Restrict WNV_{KUN} Replication

Quantification of the observed effects of increasing concentrations of IFN-α on WNV_{KUN} RNA replication in A549 cells, in the absence or presence of cholesterol, was assessed after two independent experiments performed in triplicate. A significant shift in the ability of IFN-α to restrict WNV_{KUN} replication was observed in the presence of cholesterol (*$p < 0.05$, Student's t test).

this pathogenic family of viruses. Cotreatment of WNV-infected patients with Lov and cholesterol could provide a safe and low-cost application for future clinical trials.

EXPERIMENTAL PROCEDURES

Viruses and Cells

Vero cells were grown and maintained in DMEM medium (GIBCO BRL) supplemented with 5% fetal calf serum and penicillin/streptomycin. Cells were infected with WNV_{KUN} strain MRM61C or WNV_{NY99} at a multiplicity of infection (moi) of 5, as previously described (Westaway et al., 1997), and infected cells were maintained in DMEM medium containing 0.1% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO).

Reagents

Antibodies

WNV-specific anti-NS1 (clone 4G4; Macdonald et al., 2005) monoclonal antibodies and guinea pig anti-dsRNA antibodies have been described previously (Magliano et al., 1998). Mouse monoclonal anti-NS5 (clone 5H1.1) antibodies were generously provided by Dr. Roy Hall (university of Queensland, Brisbane, Australia). Rabbit anti-HMGCR antibodies were generously provided by Dr. Peter Edwards (UCLA, CA). Anti-Caveolin-1, anti-phosphorylated-Stat-1 antibody (clone 550428), and rabbit anti-actin antibodies were purchased from PharMingen (San Diego, CA) and Sigma Aldrich (St. Louis, MO) respectively. Anti-rabbit- and anti-mouse-specific IgG Oregon green, Texas red, and horseradish peroxidase were purchased from Molecular Probes (Invitrogen, Leiden, The Netherlands).

Chemicals

Filipin compound, Lov, methyl-β-cyclodextran, 25-hydroxycholesetrol, cyclodextran complexed cholesterol, geranylgeranylation inhibitor GGTI-298, farnesylation inhibitor B2559 B581 (FPTI), AY-9944, and DAPI were all purchased from Sigma Aldrich (St. Louis, MO).

IF Analysis

Vero cell monolayers on coverslips were infected with WNV at approximately 3–5 moi and incubated at 37°C for 24 hr. The cells were subsequently washed with PBS and fixed with 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO) for 10 min at 20°C and permeabilized with 0.1% Triton X-100 in 4% paraformaldehyde for an additional 10 min at 20°C; the cells were washed with PBS, and aldehyde groups were quenched with 0.2 M glycine in PBS and additionally washed twice with PBS before incubation with antibodies. Primary and secondary antibodies were incubated within blocking buffer (PBS containing 1% BSA) and washed with PBS containing 0.1% BSA between incubation steps. After a final wash with PBS, the coverslips were drained and mounted onto glass slides with a quick-dry mounting medium (United Biosciences, Brisbane, Australia) before visualization on a Zeiss Axiophot 2 IF microscope. Images were collected using a Zeiss AxioCam MRm digital camera and Axiovision AC software before processing for publication using Adobe Photoshop software.

Western Blotting

WNV_{KUN}-infected or uninfected cells were lysed in 1× SDS-PAGE loading buffer, and proteins were separated on 10% polyacrylamide-SDS gels. Then proteins were subsequently transferred to an Hybond-ECL nitrocellulose membrane (Amersham Biosciences Corp., Piscataway, NJ) using the BioRad wet-transfer blotting module. The membrane was subsequently blocked with 5% Skim Milk Powder (Diploma) in PBS containing 0.1% Tween-20 at room temperature. Incubation with the appropriate antibodies was performed in blocking buffer on a rotating wheel overnight at 4°C. The bound antibodies were subsequently visualized with species-specific IgG conjugated to horseradish peroxidase and the ECL Plus chemiluminescence kit (Amersham Biosciences Corp.) by exposure to X-ray film or by detection and visualization using species-specific Alexa Fluor 680 (Molecular Probes) or IRDye 800CW (Rockland Incorporated)-conjugated IgG antibodies and the LI-COR Odyssey scanner. The resulting images were digitally scanned and processed in Adobe Photoshop for publication.

Electron Microscopy

Methods for resin embedding, cryofixation, preparation of cryosections, and immunolabeling have been described elsewhere (Mackenzie et al., 1996a, 1996b; Westaway et al., 1997). Briefly, WNV-infected cells were fixed with 4% paraformaldehyde/0.1% glutaraldehyde (ProSciTech, Kirwan, Australia) in PBS on ice for 60 min and subsequently embedded within a 10% gelatin block before postfixing with 1% paraformaldehyde in PBS. The sample blocks were infused with a mixture of sucrose and polyvinylpyrrolidone and mounted onto cryostubs (Leica) for cryosectioning. Ultrathin cryosections (~55 nm) were cut with a Diatome Cryo-P diamond knife and retrieved from the cryochamber with a droplet of 14:1 2.3 M sucrose/2% methylcellulose. The recovered sections were subsequently immunolabeled with the appropriate antibodies and protein-A gold conjugated to 10 nm gold and contrasted with 1.8 M methylcellulose containing 0.4% uranyl acetate. The sections were then viewed on JOEL 1010 transmission electron microscope, and images were captured on a MegaView III side-mounted CCD camera (Soft Imaging Systems, USA) and processed for publication in Adobe Photoshop.

Chemical Treatments and Reporter Assay

Vero cells in quadruplicate in 96-well plates were infected with virus-like particles encapsulating WNV_{KUN} replicon RNA encoding for the β-galactosidase (β-gal) gene (Harvey et al., 2004; Liu et al., 2002) at an moi of 5. After the initial infection period of 90 min at 37°C, the inoculum was removed and replaced with DMEM containing 0.1% BSA and the corresponding chemicals to the following final concentrations: Lov, 10 or 100 μM; M-β-CD, 8 μg/ml in media; CD-cholesterol, 10 μg/ml; 25-hydroxycholesterol, 10 μg/ml; AY-9944, 1 μM; GGTI-298, 10 μM; FPTI, 10 μM; geranyl- and farnesyl-phosphate; 10 μM. The infected cells were then harvested at 48 hr p.i., and detection and quantitation of replicon replication were performed by β-Gal assay of lysed cells using a β-galactosidase enzyme assay kit (Promega, Madison, WI) essentially as described by the manufacturer. Effects on IFN signaling were assessed as above except Vero cells were only transfected with an IFN-α/β-responsive (ISRE) luciferase reporter plasmid p(9-27)4thΔ (−39)Lucter (King and Goodbourn, 1994). Thirty-six hours after transfection, cells were incubated with filipin and/or CD-cholesterol for 60 min before stimulation with 1000 U of IFN-αA (I-4276; Sigma) per ml for an additional 14 hr before lysis within luciferase

reporter assay buffer (Promega, Madison, WI) and analysis of luciferase expression.

RNAi-Mediated Knockdown of HMGCR

For HMGCR gene silencing, Vero cells were transfected for 24 hr with 200 pmol of Stealth RNAi specific for human HMGCR purchased from Invitrogen. The transfected cells were then infected with virus-like particles encapsulating WNV_{KUN} replicon RNA encoding for the β-gal gene at a moi of 5. A cocktail of three RNAi duplexes were used for transfection using Lipofectamine 2000, and the transfections occurred every 24 hr until 48 hr postinfection. Transfected cells were subsequently harvested in Promega Reporter lysis buffer for analysis of β-galactosidase expression and for western blot analysis of HMGCR expression, as described above. A medium G-C content RNAi duplex was purchased from Invitrogen and used as a control.

Sucrose Flotation Gradients

Separation of detergent resistant membranes was performed according to Fra et al. (1994). WNV_{KUN}-infected Vero cells were lysed with TNE buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100 and protease inhibitors (PMSF, aprotinin, and leupeptin). Insoluble material was removed by centrifugation, and the resulting supernatant was equilibrated to 1.5 M sucrose and overlaid with volumes of 1.8 M and 0.15 M sucrose. The DRMs were separated by centrifugation in a SW41Ti rotor (Beckman Coulter) at 38,000 rpm for 18 hr at 4°C. One milliliter fractions were collected from the top and analyzed by SDS-PAGE and western blotting using anti-Caveolin-1 and anti-NS5 antibodies.

IFN Sensitivity Assay

A549 cells grown in 96-well plates were infected with virus-like particles encapsulating WNV_{KUN} replicon RNA encoding for the β-galactosidase (β-gal) gene (Harvey et al., 2004; Liu et al., 2002) at an moi of 5. After the initial infection period of 90 min at 37°C, the inoculum was removed and replaced with DMEM/F12 media containing 2% fetal calf serum in the presence or absence of 10 μg/ml CD-cholesterol. At 6 hr p.i., increasing amounts of IFN-α were added to the medium. The infected cells were subsequently harvested at 48 hr p.i., and detection and quantitation of replicon replication were performed by β-Gal assay of lysed cells using a β-galactosidase enzyme assay kit (Promega, Madison, WI) essentially as described by the manufacturer.

Supplemental Data

The Supplemental Data include Supplemental Results and Experimental Procedures, one supplemental table, and two supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/2/4/229/DC1/.

ACKNOWLEDGMENTS

We wish to thank Ari Helenius and Gareth Griffiths for critical review of the manuscript. We also wish to thank Peter Edwards for contribution of antibodies and Steve Goodbourn for the ISRE reporter plasmid. This work was supported by a project grants from the National Health and Medical Research Council of Australia to J.M.M. and A.A.K. and a program grant to R.G.P.

Received: February 26, 2007
Revised: July 10, 2007
Accepted: September 5, 2007
Published: October 10, 2007

REFERENCES

Adams, C.M., Goldstein, J.L., and Brown, M.S. (2003). Cholesterol-induced conformational change in SCAP enhanced by Insig proteins and mimicked by cationic amphiphiles. Proc. Natl. Acad. Sci. USA *100*, 10647–10652.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell *124*, 783–801.

Anderson, R.G., Orci, L., Brown, M.S., Garcia-Segura, L.M., and Goldstein, J.L. (1983). Ultrastructural analysis of crystalloid endoplasmic reticulum in UT-1 cells and its disappearance in response to cholesterol. J. Cell Sci. *63*, 1–20.

Basler, C.F., and Garcia-Sastre, A. (2007). Sensing RNA virus infections. Nat. Chem. Biol. *3*, 20–21.

Belles, X., Martin, D., and Piulachs, M.-D. (2005). The mevalonate pathway and the synthesis of juvenile hormone in insects. Annu. Rev. Entomol. *50*, 181–199.

Best, S.M., Morris, K.L., Shannon, J.G., Robertson, S.J., Mitzel, D.N., Park, G.S., Boer, E., Wolfinbarger, J.B., and Bloom, M.E. (2005). Inhibition of interferon-stimulated JAK-STAT signaling by a tick-borne flavivirus and identification of NS5 as an interferon antagonist. J. Virol. *79*, 12828–12839.

Brown, M.S., and Goldstein, J.L. (1997). The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell *89*, 331–340.

Brown, A.J., Sun, L., Feramisco, J.D., Brown, M.S., and Goldstein, J.L. (2002). Cholesterol addition to ER membranes alters conformation of SCAP, the SREBP escort protein that regulates cholesterol metabolism. Mol. Cell *10*, 237–245.

Fra, A., Williamson, E., Simons, K., and Parton, R. (1994). Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. J. Biol. Chem. *269*, 30745–30748.

Garcia-Sastre, A., and Biron, C.A. (2006). Type 1 interferons and the virus-host relationship: A lesson in detente. Science *312*, 879–882.

Goldstein, J.L., and Brown, M.S. (1990). Regulation of the mevalonate pathway. Nature *343*, 425–430.

Goldstein, J.L., DeBose-Boyd, R.A., and Brown, M.S. (2006). Protein sensors for membrane sterols. Cell *124*, 35–46.

Guo, J.T., Hayashi, J., and Seeger, C. (2005). West Nile virus inhibits the signal transduction pathway of alpha interferon. J. Virol. *79*, 1343–1350.

Harvey, T.J., Liu, W.J., Wang, X.J., Linedale, R., Jacobs, M., Davidson, A., Le, T.T., Anraku, I., Suhrbier, A., Shi, P.Y., and Khromykh, A.A. (2004). Tetracycline-inducible packaging cell line for production of flavivirus replicon particles. J. Virol. *78*, 531–538.

Horton, J.D., Goldstein, J.L., and Brown, M.S. (2002). SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. J. Clin. Invest. *109*, 1125–1131.

Jones, M., Davidson, A., Hibbert, L., Gruenwald, P., Schlaak, J., Ball, S., Foster, G.R., and Jacobs, M. (2005). Dengue virus inhibits alpha interferon signaling by reducing STAT2 expression. J. Virol. *79*, 5414–5420.

King, P., and Goodbourn, S. (1994). The beta-interferon promoter responds to priming through multiple independent regulatory elements. J. Biol. Chem. *269*, 30609–30615.

Kolf-Clauw, M., Chevy, F., Wolf, C., Siliart, B., Citadelle, D., and Roux, C. (1996). Inhibition of 7-dehydrocholesterol reductase by the teratogen AY9944: A rat model for Smith-Lemli-Opitz syndrome. Teratology *54*, 115–125.

Lin, R.J., Chang, B.L., Yu, H.P., Liao, C.L., and Lin, Y.L. (2006). Blocking of interferon-induced Jak-Stat signaling by Japanese encephalitis virus NS5 through a protein tyrosine phosphatase-mediated mechanism. J. Virol. *80*, 5908–5918.

Liu, W.J., Sedlak, P.L., Kondratieva, N., and Khromykh, A.A. (2002). Complementation analysis of the flavivirus Kunjin NS3 and NS5 proteins defines the minimal regions essential for formation of a replication complex and shows a requirement of NS3 in cis for virus assembly. J. Virol. *76*, 10766–10775.

Lin, R.J., Liao, C.L., Lin, E., and Lin, Y.L. (2004). Blocking of the alpha interferon-induced Jak-Stat signaling pathway by Japanese encephalitis virus infection. J. Virol. *78*, 9285–9294.

Liu, W.J., Wang, X.J., Mokhonov, V.V., Shi, P.Y., Randall, R., and Khromykh, A.A. (2005). Inhibition of interferon signaling by the New York 99 strain and Kunjin subtype of West Nile virus involves blockage of STAT1 and STAT2 activation by nonstructural proteins. J. Virol. *79*, 1934–1942.

Macdonald, J., Tonry, J., Hall, R.A., Williams, B., Palacios, G., Ashok, M.S., Jabado, O., Clark, D., Tesh, R.B., Briese, T., and Lipkin, W.I. (2005). NS1 protein secretion during the acute phase of West Nile virus infection. J. Virol. *79*, 13924–13933.

Mackenzie, J. (2005). Wrapping things up about virus RNA replication. Traffic *6*, 967–977.

Mackenzie, J.M., Jones, M.K., and Young, P.R. (1996a). Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. Virology *220*, 232–240.

Mackenzie, J.M., Jones, M.K., and Young, P.R. (1996b). Improved membrane preservation of flavivirus-infected cells with cryosectioning. J. Virol. Methods *56*, 67–75.

Mackenzie, J.M., Jones, M.K., and Westaway, E.G. (1999). Markers for trans-Golgi membranes and the intermediate compartment localize to induced membranes with distinct replication functions in flavivirus-infected cells. J. Virol. *73*, 9555–9567.

Mackenzie, J.M., Khromykh, A.A., and Westaway, E.G. (2001). Stable expression of noncytopathic Kunjin replicons simulates both ultrastructural and biochemical characteristics observed during replication of Kunjin virus. Virology *279*, 161–172.

Magliano, D., Marshall, J.A., Bowden, D.S., Vardaxis, N., Meanger, J., and Lee, J.Y. (1998). Rubella virus replication complexes are virus-modified lysosomes. Virology *240*, 57–63.

Marchetti, M., Monier, M.N., Fradagrada, A., Mitchell, K., Baychelier, F., Eid, P., Johannes, L., and Lamaze, C. (2006). Stat-mediated signaling induced by type I and type II interferons (IFNs) is differentially controlled through lipid microdomain association and clathrin-dependent endocytosis of IFN receptors. Mol. Biol. Cell *17*, 2896–2909.

Maziere, C., Conte, M.-A., and Maziere, J.-C. (2001). Activation of the JAK/STAT pathway by ceramide in cultured human fibroblasts. FEBS Lett. *507*, 163–168.

Munoz-Jordan, J.L., Sanchez-Burgos, G.G., Laurent-Rolle, M., and Garcia-Sastre, A. (2003). Inhibition of interferon signaling by dengue virus. Proc. Natl. Acad. Sci. USA *100*, 14333–14338.

Munoz-Jordan, J.L., Laurent-Rolle, M., Ashour, J., Martinez-Sobrido, L., Ashok, M., Lipkin, W.I., and Garcia-Sastre, A. (2005). Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. J. Virol. *79*, 8004–8013.

Nohturfft, A., Yabe, D., Goldstein, J.L., Brown, M.S., and Espenshade, P.J. (2000). Regulated step in cholesterol feedback localized to budding of SCAP from ER membranes. Cell *102*, 315–323.

Orci, L., Brown, M.S., Goldstein, J.L., Garcia-Segura, L.M., and Anderson, R.G. (1984). Increase in membrane cholesterol: A possible trigger for degradation of HMG CoA reductase and crystalloid endoplasmic reticulum in UT-1 cells. Cell *36*, 835–845.

Radhakrishnan, A., Ikeda, Y., Kwon, H.J., Brown, M.S., and Goldstein, J.L. (2007). Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: Oxysterols block transport by binding to Insig. Proc. Natl. Acad. Sci. USA *104*, 6511–6518.

Roosendaal, J., Westaway, E.G., Khromykh, A., and Mackenzie, J.M. (2006). Regulated cleavages at the West Nile virus NS4A–2K-NS4B junctions play a major role in rearranging cytoplasmic membranes and Golgi trafficking of the NS4A protein. J. Virol. *80*, 4623–4632.

Sagan, S.M., Rouleau, Y., Leggiadro, C., Supekova, L., Schultz, P.G., Su, A.I., and Pezacki, J.P. (2006). The influence of cholesterol and lipid metabolism on host cell structure and hepatitis C virus replication. Biochem. Cell Biol. *84*, 67–79.

Sehgal, P.B. (2003). Plasma membrane rafts and chaperones in cytokine/STAT signaling. Acta Biochim. Pol. *50*, 583–594.

Sehgal, P.B., Guo, G.G., Shah, M., Kumar, V., and Patel, K. (2002). Cytokine signaling: STATS in plasma membrane rafts. J. Biol. Chem. *277*, 12067–12074.

Shah, M., Patel, K., Fried, V.A., and Sehgal, P.B. (2002). Interactions of STAT3 with Caveolin-1 and Heat Shock Protein 90 in plasma membrane raft and cytosolic complexes. Preservation of cytokine signaling during fever. J. Biol. Chem. *277*, 45662–45669.

Sun, L.-P., Li, L., Goldstein, J.L., and Brown, M.S. (2005). Insig required for sterol-mediated inhibition of Scap/SREBP binding to COPII proteins in vitro. J. Biol. Chem. *280*, 26483–26490. Published online May 16, 2005. 10.1074/jbc.M504041200.

Sun, L.P., Seemann, J., Goldstein, J.L., and Brown, M.S. (2007). Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: Insig renders sorting signal in Scap inaccessible to COPII proteins. Proc. Natl. Acad. Sci. USA *104*, 6519–6526.

van Boxel-Dezaire, A.H., Rani, M.R., and Stark, G.R. (2006). Complex modulation of cell type-specific signaling in response to type I interferons. Immunity *25*, 361–372.

Wang, X., Sato, R., Brown, M.S., Hua, X., and Goldstein, J.L. (1994). SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. Cell *77*, 53–62.

Wang, C., Gale, M., Jr., Keller, B.C., Huang, H., Brown, M.S., Goldstein, J.L., and Ye, J. (2005). Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. Mol. Cell *18*, 425–434.

Westaway, E.G., Mackenzie, J.M., Kenney, M.T., Jones, M.K., and Khromykh, A.A. (1997). Ultrastructure of Kunjin virus-infected cells: Colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus- induced membrane structures. J. Virol. *71*, 6650–6661.

Westaway, E.G., Mackenzie, J.M., and Khromykh, A.A. (2002). Replication and gene function in Kunjin virus. Curr. Top. Microbiol. Immunol. *267*, 323–351.

Yang, T., Espenshade, P.J., Wright, M.E., Yabe, D., Gong, Y., Aebersold, R., Goldstein, J.L., and Brown, M.S. (2002). Crucial step in cholesterol homeostasis: Sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell *110*, 489–500.

Ye, J., Wang, C., Sumpter, R., Jr., Brown, M.S., Goldstein, J.L., and Gale, M., Jr. (2003). Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. Proc. Natl. Acad. Sci. USA *100*, 15865–15870.