Reduced sphingosine kinase 1 activity in dengue virus type-2 infected cells can be mediated by the 3′ untranslated region of dengue virus type-2 RNA

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Sphingosine kinase 1 (SphK1) is a lipid kinase with important roles including regulation of cell survival. We have previously shown reduced SphK1 activity in cells with an established dengue virus type-2 (DENV-2) infection. In this study, we examined the effect of alterations in SphK1 activity on DENV-2 replication and cell death and determined the mechanisms of the reduction in SphK1 activity. Chemical inhibition or overexpression of SphK1 after established DENV-2 infection had no effect on infectious DENV-2 production, although inhibition of SphK1 resulted in enhanced DENV-2-induced cell death. Reduced SphK1 activity was observed in multiple cell types, regardless of the ability of DENV-2 infection to be cytopathic, and was mediated by a post-translational mechanism. Unlike bovine viral diarrhea virus, where SphK1 activity is decreased by the NS3 protein, SphK1 activity was not affected by DENV-2 NS3 but, instead, was reduced by expression of the terminal 396 bases of the 3′ UTR of DENV-2 RNA. We have previously shown that eukaryotic elongation factor 1A (eEF1A) is a direct activator of SphK1 and here DENV-2 RNA co-localized and co-precipitated with eEF1A from infected cells. We propose that the reduction in SphK1 activity late in DENV-2-infected cells is a consequence of DENV-2 out-competing SphK1 for eEF1A binding and hijacking cellular eEF1A for its own replication strategy, rather than a specific host or virus-induced change in SphK1 to modulate viral replication. Nonetheless, reduced SphK1 activity may have important consequences for survival or death of the infected cell.

INTRODUCTION

Dengue virus (DENV) is a flavivirus of the Flaviviridae family that is responsible for a significant mosquito-borne infection in humans leading to mild fever (dengue fever) or the more severe dengue haemorrhagic fever and dengue shock syndrome, more recently termed dengue or severe dengue (Clyde et al., 2006; Martina et al., 2009; Simmons et al., 2012). The more severe forms of disease are characterized by increased vascular leakage that is proposed to be induced by pathogenic host responses, including elevated release of inflammatory mediators (Halstead, 2007; Rothman, 2011; Simmons et al., 2012). One important inflammatory and vasoactive factor is tumour necrosis factor alpha (TNF-α), which is released at high levels from DENV-infected cells (Carr et al., 2003; Chen & Wang, 2002) and associated with DENV-induced haemorrhage in human studies (Hober et al., 1993) and mouse models of infection (Chen et al., 2007; Shresta et al., 2006; Yen et al., 2008). We have previously shown altered TNF-α responses, with induction of cell death and inability to activate pro-survival nuclear factor kappa B (NFκB)-driven signals in DENV-2-infected cells (Wati et al., 2007, 2011). The timing of these altered TNF-α responses coincides with reduced activity of sphingosine kinase 1 (SphK1) in these cells (Wati et al., 2011). SphK1 is an important mediator of TNF-α-stimulated NFκB responses (Alvarez et al., 2010; Li & Lin, 2008; Xia et al., 2002) via the generation of the bioactive lipid mediator, sphingosine-1-phosphate (SIP) (Leclercq & Pitson, 2006; Pitson, 2011). SIP is a pro-survival and immunomodulatory factor that can act as an intracellular signalling molecule or be secreted from the cell and act extracellularly via a family of
five S1P-specific G-protein coupled receptors (S1P₁–₅) (Hannun & Obeid, 2008; Maceyka et al., 2009; Takabe et al., 2008). The activity of SphK1 is tightly regulated within cells by mechanisms including phosphorylation/dephosphorylation (Barr et al., 2008; Pitman & Pitson, 2010; Pitman et al., 2011; Pitson et al., 2003), plasma membrane localization (Jarman et al., 2010; Wattenberg et al., 2006) and interaction with cellular activators such as eukaryotic elongation factor 1A (eEF1A) (Leclercq et al., 2008, 2011). This latter interaction results in increased cellular SphK1 activity (Leclercq et al., 2008, 2011).

Important roles of SphK1 and S1P in viral infections are beginning to emerge (Carr et al., 2013). Studies have shown that SphK1 activity is enhanced in respiratory syncytial virus (RSV) (Monick et al., 2004) and human cytomegalovirus (HCMV)-infected cells (Machesky et al., 2008). RSV infection activates Akt and extracellular signal-related kinase (ERK), which leads to increased SphK1 activity, and this early RSV-induced pro-survival stimulus prevents premature virus-induced cell death (Monick et al., 2004). Similarly, activation of SphK1 is also proposed to prevent viral-induced cell death and allow persistence of HCMV (Machesky et al., 2008). Additionally, influenza virus infection was enhanced by overexpression of SphK1, but reduced when S1P lyase (SPL), the enzyme that degrades S1P, was overexpressed (Seo et al., 2010). Together, these data suggest an important role for enhanced host-cell SphK1 and S1P in viral infection and persistence. In contrast, SphK1 is inhibited in bovine viral diarrhea virus (BVDV) infected cells via direct binding and inhibition by the BVDV NS3 protein, with this inhibition necessary for BVDV-induced cytopathic effect (CPE) (Yamane et al., 2009). Notably, BVDV is a pestivirus of the Flaviviridae family and related to the flavivirus DENV, where we have reported a similar reduction in SphK1 activity late in infection (Wati et al., 2011).

In this study, we have investigated the biological effects and molecular mechanisms mediating the later reduced SphK1 activity in DENV-2-infected cells. Although changes in SphK1 activity had no effect on DENV-2 replication, lowering SphK1 activity enhanced DENV-2-induced cell death or cell proliferation. The reduction in SphK1 activity late in DENV-2-infected cells occurs via a post-translational effect that can be mediated by the DENV-2 3′ UTR. The DENV-2 3′ UTR is known to interact with the host-cell factor eEF1A, and we propose that the 3′ UTR sequesters this constitutive SphK1-activating protein away from SphK1, thereby reducing cellular SphK1 activity. Thus, this study provides essential understanding of how DENV-2 co-opts the host cell and can influence cellular factors, such as SphK1, that can have important implications for altered function of infected cells.

RESULTS

Alterations in SphK1 activity do not affect DENV-2 infection

To examine the biological effect that changes in SphK1 activity late in infection (the time where we previously observed a reduction in SphK1 activity) have on DENV infection, we performed DENV-2 infection in cells with overexpression or chemical reduction in SphK1 activity. HEK-293 cells with doxycycline (DOX)-inducible expression of SphK1, a catalytically inactive version of this enzyme, SphK1<sup>G82D</sup>, or vector control were utilized to provide controllable level and timing of SphK1 induction (Loveridge et al., 2010; Pham et al., 2008). DOX was added immediately post-DENV-2 infection and at 24 h post-infection (p.i.) cell lysates were collected for quantification of SphK1 activity. DOX treatment induced a substantial increase in SphK1 activity in both mock- and DENV-infected cells (Fig. 1a), although, like the effect of DENV-2 infection on endogenous SphK1, SphK1 activity tended to be lower in DOX-treated DENV-2-infected cells. Despite this, no difference in infectious DENV-2 production was observed between cells with or without elevated SphK1 activity (Fig. 1b). A visual CPE was induced by DENV-2 infection at 48–72 h p.i., as previously described (Wati et al., 2011), which presented as rounding up of cells and detachment from the plate representing cell death. This CPE was not prevented by overexpression of SphK1 (data not shown). We also assessed the effect of expression of the catalytically inactive SphK1<sup>G82D</sup>, that we have previously shown acts as a dominant negative version of SphK1 which prevents SphK1 activation (Pitson et al., 2000). Again, although there was substantial induction of SphK1<sup>G82D</sup> protein (Fig. 1c), there was no significant effect on infectious DENV-2 production (Fig. 1d).

Conversely, the reduction in SphK1 activity seen normally in DENV-2-infected cells may be required to facilitate infection and thus we assessed if a further chemical reduction in SphK1 activity late in infection could enhance DENV-2 infection. HEK-293 cells were DENV-2 infected and then treated with the SphK1 inhibitor (SKi) (Loveridge et al., 2010). At 30 h p.i., cells treated with SKi had significantly lower SphK1 activity that was maintained up to 48 h p.i. (Fig. 2a). SKi treatment, however, had no effect on production of infectious DENV-2 (Fig. 2b). Similarly, DENV-2 infection followed by SKi treatment of monocyte-derived macrophages (MDMs) did not affect production of infectious DENV-2 at 48 h p.i., which represents the peak of viraemia in this infection model (Fig. 2c).

Notably, the visual CPE induced by DENV-2 infection of HEK-293 cells at 48–72 h p.i. was further exacerbated by treatment with SKi, and this was quantified by vital dye staining. HEK-293 cells were DENV-2 infected and treated with SKi. Cell lysates were collected from mock- and DENV-infected cells and SphK1 activity assayed from 24–72 h p.i. DMSO treatment of cells did not affect SphK1 activity in mock-infected cells (data not shown), but we observed the DENV-2-induced reduction in SphK1 activity, as described previously (Wati et al., 2011) (Fig. 3a). SKi treatment of mock- or DENV-2-infected cells reduced SphK1 activity, with a significantly greater reduction in SphK1 activity in DENV-2 compared...
Fig. 1. Increased SphK1 activity does not affect DENV-2 infection. HEK-293 cells with DOX-inducible SphK1, SphK1G82D or vector control were mock or DENV-2 infected then treated with DOX at 1 ng ml$^{-1}$. (a) At 24 h p.i. cells were lysed and SphK1 activity assayed. (b) At the indicated time point p.i. supernatant was collected and analysed for infectious virus release. Results represent cumulative virus titre, mean ± SEM (n=4). Results in (a) and (b) are from the same experiment. (c) At 24 h p.i. cells were lysed and SphK1 protein detected by Western blot with normalization against actin. (d) At 48 h p.i. supernatant was collected and analysed for infectious virus release. Results represent cumulative virus titre, mean ± SEM (n=3). Results shown in (c) and (d) are from the same experiment.

Based on the data above, SphK1 itself shows no ability, in the context of physiologically relevant levels of SphK1 protein, to regulate DENV-2 infection, but reduced levels can enhance the DENV-2-induced CPE in HEK-293 cells. Furthermore, reduced SphK1 activity has been linked to induction of a CPE in BVDV (Yamane et al., 2009) and RSV infections (Monick et al., 2004). To examine if this is also the case in DENV-2 infection, SphK1 activity was assessed in cells that show visual cell death and CPE upon DENV-2 infection (BHK-21, HEK-293 and C6/36) and where virus infection is non-cytopathic (K562, primary MDMs). Cells were mock or DENV-2 infected and, prior to the onset of visual CPE in BHK-21, HEK-293 and C6/36 cells or at high-level infectious virus production (K562,
MDM), cells were lysed and SphK1 activity was quantified. Results show a significant reduction in SphK1 activity in all cell types analysed, irrespective of the ability of DENV-2 to subsequently induce a CPE (Fig. 4), indicating that the DENV-2-induced reduction in SphK1 activity occurs in many different cell types, but does not necessarily lead to DENV-2-induced cell death.

**Reduced SphK1 activity in DENV-2-infected cells is mediated by post-translational mechanisms**

We next determined the mechanism by which SphK1 activity is reduced in DENV-2-infected cells. We used techniques for analysis of gross changes in HEK-293 cells, where the majority of cells are infected (Western blot and
total RNA analysis), and techniques to look at individual cells in MDMs, where only approximately 10% of the cells become DENV infected (immunostaining and microscopy). This has the advantages of direct comparison of DENV-negative and -positive cells from the same cell population and also potentially demonstrating any effects in uninfected bystander cells. Western blots of cell lysates were taken at 30 h p.i., the time point in HEK-293 cells at which reduced SphK1 activity becomes significant but prior to the onset of CPE. Results showed comparable levels of total SphK1 protein from DENV-2 and mock-infected HEK-293 cells (Fig. 5a). Similarly, immunostaining for SphK1 did not differ between mock- and DENV-2-infected primary MDMs (Fig. 5b) or between DENV-2 antigen-negative and -positive cells of the DENV-infected population (Fig. 5b). The validity of our ability to detect differences in SphK1 protein levels using Western blot or immunostaining has been demonstrated (Fig. S1, available in JGV Online). We next assessed the effect of DENV-2 infection on SphK1 steady-state mRNA levels. Total RNA was extracted from HEK-293 cells at 30 h p.i. and SphK1 mRNA levels were examined by quantitative reverse transcriptase-PCR (qRT-PCR). Results showed no difference in SphK1 mRNA levels in mock- or DENV-2-infected cells (Fig. 5c). We have recently reported the presence of trans-acting SphK1-inhibitory or -activating factors in lysates from cells (Chan & Pitson, 2013) and we assessed the presence of SphK1-inhibitory or -enhancing activity in DENV-2-infected compared with mock-infected cell lysates using an in vitro activity assay with recombinant SphK1. Notably, the activity of recombinant human SphK1
(rSphK1) was selectively enhanced by incubation with lysates from mock- but not DENV-2-infected cells (Fig. 5d). Importantly, the contribution of SphK1 activity in the lysates was insignificant (less than 1%) compared to the activity of rSphK1 in this system. Thus, these results suggest post-translational effects on SphK1 either via the presence of SphK1-inhibitory factors or loss of SphK1-activating factors in the lysates from DENV-2-infected cells.

**DENV-2-induced reduction in SphK1 activity can be mediated by the DENV-2 3′ UTR that interacts with the SphK1 activator, eEF1A**

Since the NS3 protease/helicase from the related Flaviviridae BVDV has been previously reported to bind and inhibit SphK1 activity (Yamane et al., 2009), we next assessed if expression of the NS3 protease/helicase from DENV-2 could similarly inhibit SphK1 activity. HEK-293 cells were transfected with vectors encoding DENV NS3-GFP fusion protein or control GFP, and at 24 h post-transfection expression was confirmed by visualization of GFP (58±4% and 63±5% of cells GFP positive, respectively), cells were harvested, lysed and assayed for SphK1 activity. This time point post-transfection was chosen since it represented good transgene expression without the later observed CPE, possibly due to a high-level GFP expression which has the potential to influence SphK1 activity. This time point post-transfection was significant (less than 1%) compared to the activity of rSphK1 in this system. Thus, these results suggest post-translational effects on SphK1 either via the presence of SphK1-inhibitory factors or loss of SphK1-activating factors in the lysates from DENV-2-infected cells.

**It is well established that the flavivirus 3′ UTR can bind via the 3′ UTR stem–loop (SL) structure to eEF1A (Blackwell & Brinton, 1997; Davis et al., 2007). eEF1A is a highly expressed cellular protein that we have previously shown also binds and enhances the catalytic activity of SphK1 and acts as a constitutive endogenous activator of this enzyme (Leclercq et al., 2008).** We thus assessed the ability of the DENV-2 3′ UTR to inhibit eEF1A-stimulated SphK1 activity. The terminal 396 bases of the DENV-2 3′ UTR was cloned downstream of GFP to create a construct that would express GFP mRNA as a 3′ UTR RNA fusion and also produce GFP as a visual marker of transfection. HEK-293 cells were transfected with GFP control or GFP 3′ UTR RNA-expressing constructs. Transfection and expression of GFP was confirmed by fluorescence microscopy (63±5% and 70±4% of cells GFP positive, respectively). The presence of DENV-2 3′ UTR RNA was also quantified by qRT-PCR. Results demonstrated comparable levels of 3′ UTR RNA in transfected cells to those seen in DENV-2-infected HEK-293 cells (10±7, 2.8±0.6 and 5.6±1.8 ng/10⁶ cells for transfected GFP 3′ UTR and DENV-2 HEK-293 cells at 24 and 48 h p.i., respectively). Lysis of transfected cells and analysis of SphK1 activity showed a significant reduction in SphK1 activity in GFP 3′ UTR, compared with GFP expressing cells (Fig. 6b). We rationalized that a reduction in SphK1 activity mediated by the 3′ UTR should be specifically observed late in viral infection when levels of positive and negative strand DENV-2 RNA increase and higher levels of a small flavivirus RNA containing the 3′ UTR are present (Funk et al., 2010). We thus analysed the time course of SphK1 activity during DENV-2 infection. As predicted, a significant DENV-2-induced reduction in SphK1 activity was specifically observed late in infection (Fig. 7a), which coincided with high levels of infectious virus release (Fig. 7b) and levels of 3′ UTR RNA (Fig. 7c).

**eEF1A has been shown to co-localize with DENV NS3/NS5 in potential replication complexes and is required for DENV replication (Davis et al., 2007).** We next investigated changes in eEF1A in DENV-2-infected cells at the time we observed reduced SphK1 activity. Western blot analysis of cell lysates taken at 30 h p.i. from DENV-2- and mock-infected HEK-293 showed no difference in the levels of eEF1A protein (Fig. 8a). Furthermore, DENV-2- or mock-infected 293-T cells were fixed and immunostained for eEF1A. The staining for eEF1A is diffuse in mock-infected cells and of similar intensity in DENV-2-infected cells (Fig. 8b). A more punctate eEF1A staining was observed in DENV-2-infected cells with regions of co-localization of eEF1A with dsRNA (Fig. 8b). The physical association of eEF1A and DENV-2 RNA was then assessed. Lysates from DENV-2-infected cells were incubated with or without an eEF1A antibody, precipitated with protein A-Sepharose and co-precipitation of DENV-2 RNA assessed by qRT-PCR. Results showed co-precipitation of DENV-2 RNA with an eEF1A antibody, confirming the interaction of these two factors (Fig. 8c), and extending prior studies showing an association of eEF1A with the NS3 and NS5 proteins of DENV and West ...

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**Fig. 6.** Inhibition of SphK1 activity is mediated by the DENV-2 3′ UTR. HEK-293 cells were transfected to express (a) GFP control or GFP-NS3 or (b) GFP control or GFP-3′ UTR. At 24 h post-transfection cells were lysed and SphK1 activity assayed; SphK1 activity is normalized per microgram of total protein and expressed relative to GFP-transfected control. Results represent mean±SEM (n=5), *P<0.05, Students t-test.
Nile virus (Davis et al., 2007) and RNA of West Nile virus (Blackwell & Brinton, 1997).

**DISCUSSION**

SphK1 is a lipid kinase recognized as a central and important mediator of pro-survival and cell-death signals (Leclercq & Pitson, 2006; Pitson, 2011), but few studies have assessed SphK1 activity in virally infected cells (Carr et al., 2013). SphK1 activity is increased following HCMV infection.

**Fig. 7.** The reduction in SphK1 activity correlates with high levels of the DENV-2 3’ UTR RNA. HEK-293 cells were DENV-2 or mock infected. Prior to collection of samples, cells were incubated overnight in 0.1% FCS containing media, and then samples collected at the indicated time points p.i. (a) SphK1 activity was quantified in cell lysates; SphK1 activity is normalized per µg of total protein and expressed relative to mock-infected control. (b) Infectious virus production was analysed in culture supernatant by plaque assay. Results represent mean ± SEM from one of three independent experiments. *P<0.05, Students t-test. (c) RNA was extracted from DENV-2-infected HEK-293 cells and 3’ UTR RNA was quantified by qRT-PCR and normalized for cellular RPLPO.

**Fig. 8.** eEF1A protein is unaltered in DENV-2-infected cells, localizes to dsRNA and binds to DENV-2 RNA. HEK-293 cells were mock or DENV-2 infected and at 30 h p.i. cells (a) were lysed and 30 µg of lysate analysed by Western blot for eEF1A and actin, and (b) fixed and immunostained for dsRNA (mouse anti-dsRNA, English and Scientific Consulting, J2, 1/200) and eEF1A (rabbit anti-eEF1A, Cell Signalling, 1/25 dilution) with detection of complexes with Alexa 488 and 647 conjugated antibodies, respectively, and visualization by confocal microscopy. Nuclei were visualized by Hoechst staining. (c) At 30 h p.i. cells were lysed and incubated with eEF1A or control antibody. Immunoglobulin complexes were precipitated with protein A-Sepharose, washed and analysed for DENV-2 RNA by qRT-PCR. Results are expressed as copy number precipitated per equivalent millilitre of cell lysate (n=6, from three independent precipitations, P<0.05).
and RSV infection (Machesky et al., 2008; Monick et al., 2004), but reduced late in infection with the Flaviviridae, DENV-2 (Wati et al., 2011) and BVDV (Yamane et al., 2009). Interestingly, SphK2 gene expression is reduced in cells expressing proteins of hepatitis C virus (HCV), a member of the Flaviviridae (Vandermeeren et al., 2008). The study herein has focused on the biological effect and mechanisms of the observed reduction in SphK1 activity late in infection in DENV-2 infected cells.

Our results show that overexpression or further inhibition of SphK1 activity after DENV-2 infection has no effect on DENV-2 infection and suggests that once an infection is established, the SphK1 enzyme itself does not regulate DENV-2 replication and virus production. This contrasts with BVDV infection in which BVDV RNA production was increased following SKI treatment of infected cells (Yamane et al., 2009). Importantly, the functional changes that alterations in SphK1 activity may impart to the infected cell may be significant, particularly in relation to cell death. Virus-mediated inhibition of SphK1 activity during BVDV infection is related to the ability of different BVDV isolates to induce CPE in vitro, with SphK1 overexpression blocking virus-induced CPE (Yamane et al., 2009). Chemical inhibition of SphK1 activity also increased RSV-induced cell death (Monick et al., 2004). Conversely, overexpression of SPL, the S1P degrading enzyme, inhibited influenza virus-induced CPE (Seo et al., 2010).

In our study, the reduction in SphK1 activity in different DENV-2-infected cell types is not linked to the ability of DENV-2 to induce a CPE and, further, upregulating SphK1 did not counteract the DENV-2 induced CPE. This suggests that additional cell-death pathways independent or downstream of SphK1 actions are important in eliciting DENV-2-induced cell killing. In contrast, SKI treatment has a greater effect on cellular SphK1 activity and cell-death/proliferation rate in DENV-2- compared with mock-infected cells, and suggests that during DENV-2 infection the further reduction in SphK1 activity can tip the balance towards cell death, potentially in response to additional cell-death triggers. For example, we have previously reported that TNF-α responses are altered in DENV-2-infected cells, with reduced ability of TNF-α to degrade IκB-α and activate NFκB and induction of cell death (Wati et al., 2011). These responses are coincident with the DENV-2-induced reduction in SphK1 activity, which may be a significant contributor to these virus-induced changes in TNF-α responses.

Interestingly, changes in SphK1 activity can influence the levels of the substrates/substrate precursors for SphK1, such as sphingosine, ceramide, sphingomyelin and other components of the sphingolipid biosynthetic pathway. Indeed, changes in the lipidome during virus infections are a new area of active interest. Studies have shown increased levels of ceramide and sphingomyelin in HCV-infected cells (Diamond et al., 2010). Sphingolipids have also been suggested to promote HCV replication, potentially by direct binding of NS5B to sphingomyelin in detergent resistant membranes (Hirata et al., 2012) and SphK1 gene expression is reportedly increased in HCV-susceptible Huh7.5.1 cells compared to HCV-resistant Huh7 cell lines (MacPherson et al., 2011). Similarly, increased levels of ceramide and sphingomyelin have been observed in DENV-infected mosquito cells (Perera et al., 2012). Our observed changes in SphK1 activity in DENV-infected cells may be an important contributor to these changes in cellular sphingolipid profiles. Although not directly related to sphingolipid biosynthesis, DENV infection has been demonstrated to alter lipid droplets (Heaton & Randall, 2011) and stimulate fatty acid synthetase enzyme activity (Heaton et al., 2010), highlighting the importance of lipid remodelling during DENV infection, with these changes being a clear area of future interest.

In this study, we have defined a novel mechanism by which DENV-2 can influence cellular enzymic functions. We observed unchanged SphK1 mRNA and protein levels and the ability of lysates from mock- but not DENV-2-infected cells to activate rSphK1 in vitro, indicating a post-translational mechanism of DENV-2-inhibition of SphK1 activity. Similarly, in BVDV-infected cells (Yamane et al., 2009) the reduction in SphK1 activity is not associated with a change in SphK1m RNA. This is in contrast to RSV and HCMV infections, where the virus-induced increase in SphK1 activity is accompanied by an increase in SphK1 mRNA. In BVDV infection, inhibition of SphK1 activity is mediated by direct binding of BVDV NS3 protease/helicase to bovine SphK1 (Yamane et al., 2009). In contrast, we show that expression of DENV-2-NS3 in mammalian cells did not affect SphK1 activity, consistent with findings seen with HCV NS3, a hepacivirus of the Flaviviridae family (Yamane et al., 2009). SphK1 activity, however, was reduced by expression of the 3′ UTR region of DENV-2 RNA. Consistent with this, 3′ UTR RNA levels are high later in DENV-2 infection, the time frame over which we observe reduced SphK1 activity in DENV-2-infected cells. eEF1A is known to bind to the WNV 3′ UTR SL region and promote viral replication (Blackwell & Brinton, 1997) and to co-localize with DENV NS3/NS5 (Davis et al., 2007). We have shown that eEF1A also binds to SphK1 and enhances the catalytic activity of this enzyme (Leclercq et al., 2008). We have extended these studies to show that eEF1A protein levels are not altered in DENV-2-infected cells, but that eEF1A co-localizes to sites of DENV-2 dsRNA and co-precipitates with DENV-2 RNA. We thus propose that the high levels of 3′ UTR RNA in DENV-2-infected cells compete with SphK1 for binding to eEF1A, resulting in less eEF1A being available to stimulate SphK1. We have further investigated the 3′ UTR, eEF1A and SphK1 interaction using recombinant proteins and in vitro transcribed 3′ UTR RNA. Results support the ability of the DENV-2 3′ UTR to prevent eEF1A-stimulated SphK1 activity (Fig. S2). Our ongoing research aims to further define this interaction using phosphorylated eEF1A (Blackwell & Brinton, 1997) and 3′ UTR mutants that lack the ability to bind eEF1A (Davis et al., 2007).
Interestingly, although our data suggest the potential for a competitive interaction between the 3′ UTR and SphK1 for binding eEF1A, we also show that inducing changes in SphK1 activity after an established infection had no effect on infectious DENV-2 production. Similarly, overexpression of SphK1 did not affect BVDV infection, although, in contrast to our observations in DENV-infected cells, a reduction in SphK1 activity by SKI treatment enhanced BVDV RNA replication (Yamane et al., 2009). Furthermore, in our study, overexpression of a dominant negative mutant, SphK1G82D, which inhibits the activation of endogenous SphK1, still did not significantly affect infectious DENV-2 production. The specific details of the biochemical competition between SphK1 and the 3′ UTR RNA for eEF1A remain to be investigated, but factors such as the cellular colocalization of DENV-2 RNA, eEF1A and SphK1, the high levels of DENV-2 RNA and the relative affinity of DENV-2 RNA for eEF1A compared with SphK1, may all favour the 3′ UTR/eEF1A interaction in an infected cell. It has been previously shown that DENV capsid (C) protein interacts with and degrades host cell Sec3 protein, also an eEF1A-binding protein (Bhuwanankantham et al., 2010). Disruption of the SEC3–eEF1A interaction by DENV-C provides an active mechanism to release cellular eEF1A and thus promote DENV-2 replication. Our studies provide a contrasting example whereby DENV-2 RNA may sequester eEF1A at the detriment of the host-cell eEF1A-interacting protein, SphK1, and together these findings highlight the importance of cellular eEF1A to DENV replication. Recently, eEF1A has been identified as a host-cell component of the HIV reverse transcription complex (Warren et al., 2012), suggesting broader roles for eEF1A in viral replication, as recently reviewed by Li et al. (2013).

In conclusion, we have demonstrated that changes in SphK1 activity after infection do not affect DENV-2, but the reduced SphK1 activity in DENV-2-infected cells can enhance DENV-2-induced cell death. The reduction in SphK1 activity in DENV-2-infected cells can be mediated by the 3′ UTR RNA and we propose that high levels of 3′ UTR RNA accumulate late in infection and competitively bind the SphK1 activator eEF1A, resulting in less eEF1A-activated SphK1. These results provide a new and novel role for the DENV-2 3′ UTR in affecting SphK1 activity that has the potential to influence important cell-death, cell signalling and lipid changes in infected cells.

**METHODS**

**Cells and virus.** Monocytes were generated by adherence of peripheral blood mononuclear cells isolated from blood from healthy donors taken with consent at the Australian Red Cross Blood Service and used in accordance with approval from the Southern Health Human Ethics Committee. Monocytes were cultured to differentiate donors taken with consent at the Australian Red Cross Blood Service peripheral blood mononuclear cells isolated from blood from healthy donors. Monocytes were generated by adherence of

**DNA constructs and transfection of cells.** The terminal 396 bases of the 3′ UTR was PCR amplified using primers 5′-CTATGCT-TTTTGGCTGGCTGCCTGTC-3′ and 5′-GGATCCCTGATCGCACTGTTG-3′, digested with HindIII/BamHI and cloned into pBluescript KS(−) to generate in vitro transcripts (pBS-WT-3′ UTR) and pEGFP-C1 for expression of RNA in mammalian cells (pEGFP-WT-3′ UTR). The DENV-2 NS3 expression construct (pEPI-GFP-NS3) was generated and kindly donated by Dr Stephen Rawlinson and Professor David Jans (Monash University, Australia).

HEK-293 cells were transfected with 1 μg of GFP control vector, GFP-NS3 or GFP-3′ UTR using PolyFect (Qiagen) as per manufacturer’s instructions. Transfected cells were incubated overnight in 0.1% FCS then at 24 h p.i., GFP expression was visualized by fluorescent microscopy and cells were lysed and analysed for SphK1 activity, as below. For 3′ UTR-expressing constructs, RNA was also extracted and analysed by RT-PCR for 3′ UTR RNA, as described below.

**RNA extraction and RT-PCR.** At 30 h p.i. mock- and DENV-infected cells were lysed and total RNA extracted (TRIzol, Life Technologies). To quantify SphK1 mRNA, 1 μg of total RNA was subjected to qRT-PCR for SphK1 and results were normalized against 18S rRNA. Quantification of 3′ UTR RNA in DENV-infected or transfected cell lysates was performed by RT-PCR with primers as described for 3′ UTR cloning. Results were normalized against cellular ribosomal protein large PO (RPLPO) as described previously (Helbig et al., 2005). Reactions were performed using Mu-MLV Reverse transcriptase and Bio-Rad IQ Sybergreen mix. PCR was performed in a real-time format using a Rotorgene 6000 (Corbett Research). In vitro transcribed 3′ UTR RNA was used as standards in RT-PCRs and was generated by linearization of pBS-3′ UTR constructs with HindIII and in vitro transcription with T3 polymerase. Transcribed RNA was DNase treated, purified and quantified (Nanodrop 1000 spectrophotometer, Thermo Scientific).

**SphK1 activity assays.** Cells were lysed in 100 mM Tris pH 7.4, 10 mM MgCl2, 1 mM Na2VO4, 10 mM NaF, 10% (v/v) glycerol, 100 mM β-glycerophosphate, 0.05% (v/v) Triton X-100 and protease inhibitors (Roche, Complete mini). Lysates were sonicated and total protein concentration quantified (Bio-Rad protein assay). Protein content was normalized and SphK1 activity determined by [γ32P]ATP transfer to d-cycloheximide under conditions of 0.3% (v/v) Triton X-100 where SphK2 activity is inhibited, as described previously (Pitman et al., 2012). SphK1 activity was also analysed in vitro using 0.5 μg of recombinant human SphK1 (rSphK1), generated in E. coli, as described previously (Leclercq et al., 2008; Pitson et al., 2002).

**Immunoprecipitation of eEF1A.** HeLa were DENV-2-infected, as above, and at 30 h p.i. cells were lysed 50 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, pH 7.4 with protease inhibitors (Roche, Complete mini) for 1 h at 4 °C. Lysates were

**HEK-293 cells containing DOX-inducible expression of WT SphK1, a dominant negative mutant of SphK1 (SphK1G82D) (Pitson et al., 2000) and control vector-only cells were generated as previously described (Pham et al., 2008; Pitson et al., 2000).**

**DENV-2 infection and plaque assay.** Cells were infected at an m.o.i. of 1 (HEK-293) or 3 (MDM) or as indicated for 90 min at 37 °C, as described previously (Pryor et al., 2001). In some experiments cells were treated with 5 μM of the SphK1 inhibitor (SKI) 1-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole or DMSO vehicle control. At the indicated time points post-infection (p.i.) supernatants were collected clarified by centrifugation and snap frozen at −80 °C. Plaque assays were performed in Vero cells and infectious virus quantified as p.f.u. mℓ−1.
clarified by centrifugation (10,000 g, 1 min, 4 °C) and incubated with 1 µg of eEF1A control antibody (mouse anti-eEF1A, clone CBP-KK1, #05-235, Millipore). Complexes were captured with protein A-Sepharose, washed six times [50 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, 0.1% (v/v) Triton X-100, pH 7.4] and pellets were resuspended in water. Co-precipitated DENV-2 RNA was analysed by real-time RT-PCR, using primers DENV3.2 and DENV5.1 targeted against the DENV-2 C region, as previously described (Wati et al., 2007).

**Immunostaining and confocal microscopy.** Cells were grown on gelatin-coated glass coverslips, fixed in 1% (v/v) formaldehyde and stored at −20 °C. Slides were washed in PBS, permeabilized with 0.05% (v/v) IGEPAL and blocked in 4% (v/v) human serum. Cells were stained using mouse anti-DENV-2 (serotypes 1–4, Santa Cruz Biotechnology, 1/100 dilution) and rabbit anti-SphK1 (Cell Signalling, #3297, 1/200) or mouse anti-dsRNA (English and Heaton, 2011). Both virus and tumor necrosis factor alpha are critical for endothelial cell monolayers. J Med Virol 69, 521–528.


**Western blot analysis.** Total protein (30 µg) was subjected to SDS-PAGE and Western blot. Filters were probed for SphK1 (Cell Signalling, #3297, 1/1000) and eEF1A (Cell Signalling, #3251, 1/1000) with detection of proteins with goat anti-rabbit and goat anti-mouse IgG-HRP conjugate, respectively. Bound complexes were visualized by chemiluminescence and images captured (LAS 4000, Fuji Imaging Systems). Filters were reprobed for actin (Chemicon International, MAB1501R, 1/1000), with detection as above.

**Quantification of cell viability.** HEK-293 cells were cultured in 96-well plates, DENV-2 infected and treated ± SKI. At the indicated time point p.i., supernatant was removed and cells fixed with 0.2 % (w/v) crystal violet in 20% (v/v) methanol for 15 min at room temperature. Stain was removed and cells washed in water. Bound stain was solubilized in 1% (w/v) SDS for 60 min and absorbance at 520 nm measured.

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Attenuation of leakiness in doxycycline-inducible expression via incorporation of 3′ A-UTR.


