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Foot-and-mouth disease virus genome replication is unaffected by inhibition of type III phosphatidylinositol-4-kinases

Eleni-Anna Loundras, Morgan R. Herod, Mark Harris and Nicola J. Stonehouse

School of Molecular and Cellular Biology, Faculty of Biological Sciences and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds LS2 9JT, UK

Correspondence
Nicola J. Stonehouse
n.j.stonehouse@leeds.ac.uk

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Foot-and-mouth disease virus (FMDV) causes economically damaging infections of cloven-hooved animals, with outbreaks resulting in large financial losses to the agricultural industry. Due to the highly contagious nature of FMDV, research with infectious virus is restricted to a limited number of key facilities worldwide. FMDV sub-genomic replicons are therefore important tools for the study of viral translation and genome replication. The type III phosphatidylinositol-4-kinases (PI4Ks) are a family of enzymes that plays a key role in the production of replication complexes (viral factories) of a number of positive-sense RNA viruses and represents a potential target for novel pan-viral therapeutics. Here, we investigated whether type III PI4Ks also play a role in the FMDV life cycle, using a combination of FMDV sub-genomic replicons and bicistronic internal ribosome entry site (IRES)-containing reporter plasmids. We demonstrated that replication of the FMDV replicon was unaffected by inhibitors of either PI4KIIIα or PI4KIIIβ. However, PIK93, an inhibitor previously demonstrated to target PI4KIIIβ, did inhibit IRES-mediated protein translation. Consistent with this, cells transfected with FMDV replicons did not exhibit elevated levels of phosphatidylinositol-4-phosphate lipids. These results are therefore supportive of the hypothesis that FMDV genome replication does not require type III PI4K activity and does not activate these kinases.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) is the causative agent of foot-and-mouth disease: a highly contagious, systemic, vesicular infection of domestic and wild cloven-hooved animals. Major outbreaks in recent years, including the 2001 outbreak in the UK, resulted in economic losses estimated at £8 billion (Thompson *et al.*, 2002) and the culling of millions of livestock to prevent the further spread of the virus. FMDV replicates rapidly, and therefore can transmit and spread readily, in addition to being able to develop an asymptomatic carrier state (as reviewed by Alexandersen *et al.*, 2002). Although vaccines are available, effectiveness in disease control is hampered by the seven serotypes (and many sub-types) of FMDV, together with the inability to distinguish with certainty animals that are vaccinated from those having recovered from infection (Grubman & Baxt, 2004).

FMDV is a picornavirus in the genus *Aphthovirus*. It has a positive-sense ssRNA genome that, upon infection, is translated into a single polyprotein which is co- and post-translationally processed. The genome is flanked by 5′ and 3′ UTRs and is polyadenylated. The 5′ UTR contains a number of structural regions: a long-hairpin ‘S-fragment’ structure, a poly-C tract, a region containing pseudoknots, the *cis*-acting replicative element (*cre)* and the internal ribosome entry site (IRES), involved in 7-methyl-guanosine ‘cap’-independent translation (Belsham & Brangwyn, 1990; Martínez-Salas *et al.*, 2015). The coding region is separated into four regions: L^{Pro}, P1, P2 and P3. L^{Pro} encodes a protease that has been shown to shut off host-cell translation by cleaving the eIF4G subunit of the host translation initiation machinery (Devaney *et al.*, 1988; Kirchweger *et al.*, 1994). P1 encodes the structural capsid proteins. P2 and P3 encode non-structural proteins including 2C, three 3B proteins (that encode for the 3 VPg proteins that act as primers for replication), the 3C protease and the RNA-dependent RNA polymerase $3D^{Pol}$. This enzyme (in complex with other non-structural proteins) is involved in replication of the viral genome (via negative-sense intermediates).

Because of the highly infectious nature of FMDV, handling is restricted to a small number of facilities worldwide. Sub-genomic replicons were developed to allow for the study of viral replication in laboratories at lower containment level (McInerney *et al.*, 2000). The FMDV replicon constructs

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employed here (pGFP-pac-WT) have the structural proteins replaced by a GFP reporter and a puromycin acetyltransferase resistance gene cassette. Replication-deficient constructs have a GNN mutation in the active site of $3D^{pol}$ (pGFP-pac-GNN). Levels of GFP expression over time can be measured using an IncuCyte Dual Colour ZOOM FLR (Forrest *et al.*, 2014; Herod *et al.*, 2015; Tulloch *et al.*, 2014). Replicons have also been used to study the replication of a number of other viruses. Here, we have also used hepatitis C virus (HCV) sub-genomic replicons pSGR-Luc-GFP-JFH-1 (Jones *et al.*, 2007) and SGR-feo-JFH-1 (Wyles *et al.*, 2009), together with a Coxsackievirus B3 (CVB3) sub-genomic replicon, pRib-Fluc-CB3/T7 (Lanke *et al.*, 2009).

Replication of a number of positive-sense RNA viruses has been shown to occur at cytoplasmic membrane-associated sites (den Boon & Ahlquist, 2010). The formation of such membrane compartments is thought to allow for the creation of a kinetically-favourable environment in which viruses can replicate rapidly and effectively, and to protect the replication machinery from the hostile environment of the host cell. Viruses have been shown to subvert membranes of the endoplasmic reticulum (ER), Golgi and *trans*-Golgi network (TGN) in order to form these complexes. Membranes rich in phosphatidylinositol-4-phosphate (PI4P) lipids have been shown to be required for the replication of multiple members of the families *Picornaviridae* and *Flaviviridae*, including poliovirus (PV), enterovirus 71, CVB3, encephalomyocarditis virus (EMCV) and HCV (Altan-Bonnet & Balla, 2012; den Boon & Ahlquist, 2010; Dorobantu *et al.*, 2015a). Phosphorylation of phosphatidylinositol (PI) lipids on the 4-carbon of the inositol ring to generate PI4P is stimulated by the upregulation and selective recruitment of phosphatidylinositol-4-kinase (PI4K). There are two types of well-defined families of PI4Ks, type II (PI4KIIα and PI4KIIβ) and type III (PI4KIIIα and PI4KIIIβ). Previous studies on PV and CVB3 have identified PI4KIIIβ as the host enzyme upregulated in viral replication factories (Arita *et al.*, 2011; Belov *et al.*, 2007; Hsu *et al.*, 2010; Lanke *et al.*, 2009). Depletion of PI4KIIIβ activity within infected cells by RNA silencing or the use of kinase inhibitors, such as PIK93 (Knight *et al.*, 2006), appeared to block PV and CVB3 viral RNA synthesis and virus replication. In contrast, HCV replication is generally accepted to be dependent on PI4KIIIα activity (Berger *et al.*, 2011; Bishé *et al.*, 2012; Reiss *et al.*, 2011), although there is some evidence for a dependence on PI4KIIIβ (Arita *et al.*, 2011; Borawski *et al.*, 2009; Zhang *et al.*, 2012).

The PI4K family represent a possible pan-viral therapeutic target, however, involvement in FMDV replication has yet to be clearly defined. Using bicistronic reporter constructs and sub-genomic replicons, we have compared the effects of type III PI4K inhibitors on FMDV with those on other positive-sense RNA viruses. Using this approach, we have separated effects on RNA translation from direct effects on genome replication and show that FMDV replication appears not to be dependent on this pathway.

RESULTS

The small molecule inhibitor PIK93 inhibits IRES-mediated translation

Many positive-sense RNA viruses have been documented to replicate in membrane-associated complexes (den Boon & Ahlquist, 2010), and there have been several reports demonstrating effects of inhibitors of membrane lipid modification, such as PIK93, on viral replication, as described above. However, in many cases, it has been difficult to separate effects on translation of input viral RNA from those affecting genome replication. To elucidate effects of PIK93 on host and viral translation, we exploited the use of bicistronic vectors containing renilla luciferase (Rluc) and firefly luciferase (Fluc) reporter genes under the control of either cap- or IRES-dependent translation mechanisms, respectively (Licursi *et al.*, 2011) (Fig. 1a). We have employed a similar approach previously to demonstrate the absence of any non-specific effects on translation with RNA aptamers selected to FMDV $3D^{pol}$ (Forrest *et al.*, 2014).

Five different constructs were used: a control (pRF) with no IRES structure, resulting in minimal Fluc expression, and constructs in which Fluc translation was controlled by the IRES from FMDV, HCV, human rhinovirus (HRV) or EMCV. The HRV IRES is type I, EMCV and FMDV are type II and the HCV IRES is classed as a type III (Belsham, 2009; Martínez-Salas *et al.*, 2015; Tsukiyama-Kohara *et al.*, 1992). For all constructs there was a modest, non-significant inhibition of cap-mediated translation in the presence of an increasing concentration of PIK93 (0–5 μM) (Fig. 1b). However, the effects of PIK93 on IRES-mediated translation (Fig. 1c) were more profound. There was a mean decrease in luciferase signal ranging from 44.2±13.5 % in constructs treated with 1 μM to 73.3±5.0 % in those treated with 5 μM PIK93. Treatment with concentrations higher than 1 μM of PIK93 significantly reduced FMDV, HCV and EMCV IRES-driven luciferase expression. However, with the HRV IRES, a significant reduction in luciferase expression was only observed at 5 μM PIK93. This effect of PIK93 was not due to cytotoxicity; MTT assays did not reveal any reduction in cell viability in the presence of PIK93 at any of the concentrations used in the translation assay (Fig. 1d).

This result brings into question whether some of the documented effects of PIK93 on replication could be the result of suppression of input viral RNA translation. In order to clarify this, we compared the effects of PIK93 on the replication of WT and replication-deficient FMDV replicons with those on the replication of CVB3, previously shown to be dependent on PI4K activity (Lanke *et al.*, 2009). Because of the different reporters used, replication was monitored by luciferase assay or by GFP expression.

The development of the FMDV GFP-pac replicon and optimization of the replication assay has been described previously (Forrest *et al.*, 2014). Replication was detected by measuring the levels of GFP expression over time using an IncuCyte Dual Colour ZOOM FLR (Tulloch *et al.*, 2014),

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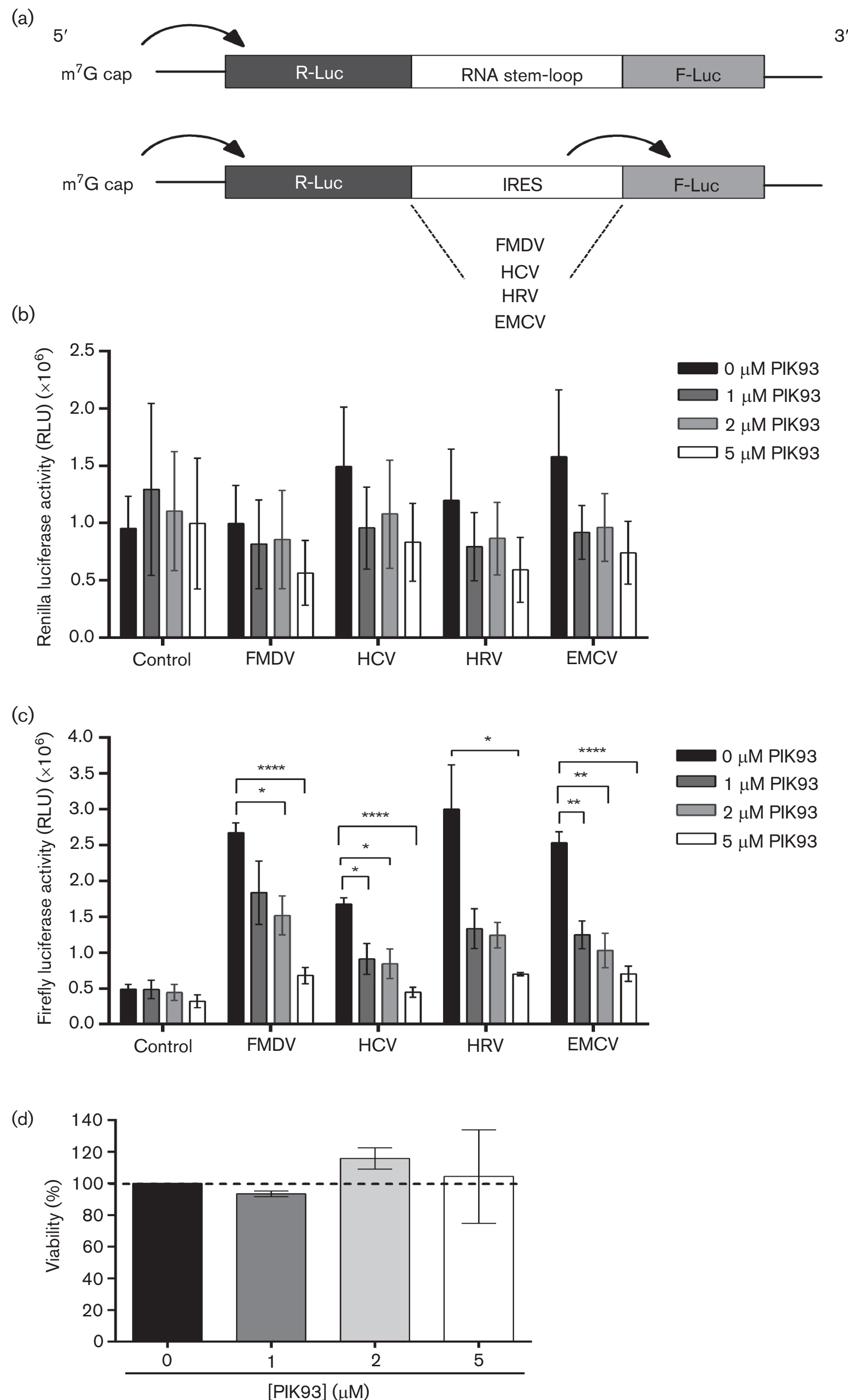

Fig. 1. (a) Schematic of bicistronic reporter constructs. (b, c) Levels of luciferase expression in BHK-21 cells transfected with bicistronic luciferase reporter constructs. Transfected cells were pre-treated for 2 h with PIK93 at the concentrations indicated. Levels of luciferase expression were measured at 48 h post-transfection. The effect of PIK93 on (b) cap-mediated translation and (c) IRES-mediated translation is shown. The control (pRF) contains the firefly reporter under the control of a non-IRES structure and the renilla reporter under the control of cap-mediated translation. (d) MTT assay of PIK93 on BHK-21 cells shows no cytotoxicity at the concentrations used for functional assays. Data show mean values with SEM (n=3); statistical analysis was performed using a two-tailed unpaired t-test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). RLU, Relative light units.

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with maximum fluorescence values being observed at 8–10 h post-transfection. At this time point up to 80 % of cells transfected with the GFP-pac-WT replicon RNA expressed high levels of GFP, and therefore replication could be equally assessed by either number of GFP-positive cells or total GFP fluorescence (data not shown). Use of the replication-deficient mutant replicon (GFP-pac-GNN) resulted in a markedly lower GFP signal (i.e. as a result of input translation only). Levels of GFP expression in GFP-pac-GNN replicon-transfected cells were similar to those with a second replication-deficient replicon, GFP-pac-Δ3D (Tulloch *et al.*, 2014), with a large deletion in the $3D^{pol}$ gene (data not shown).

The addition of PIK93 at 2 h pre-transfection resulted in a dose-dependent reduction in GFP expression in cells transfected with GFP-pac-WT or -GNN replicon RNA (Fig. 2). Reduction of GFP expression in cells transfected with GFP-pac-WT ranged from 30.9±6.7 % to 50.0±3.9 % after treatment with 1 and 5 μM PIK93, respectively, when compared with untreated control cells (Fig. 2a, b). In GFP-pac-GNN transfected cells there was a 45.5±2.3 % to 69.8±5.2 % reduction (Fig. 2c, d). The reduction in GFP expression in cells transfected with GFP-pac-GNN in the presence of 5 μM PIK93 (69.8±5.2 %) was similar to the decrease observed in FMDV IRES-mediated translation with the same concentration of PIK93 (74.1±5.4 %).

We then assayed the effects of PIK93 on WT and replication-deficient CVB3 replicons (Fig. 3). As expected, and in contrast to the results with FMDV replicons (Fig. 2b), there was a more pronounced dose-dependent effect of PIK93 on CVB3 replication. At 5 μM, PIK93 treatment resulted in a 98.7±0.6 % decrease in luciferase expression in cells transfected with WT Rib-Fluc-CB3/T7 RNA, compared with untreated controls (Fig. 3b). However, cells transfected with replication-deficient Rib-Fluc-CB3/T7-3A RNA and treated with 5 μM PIK93 only, exhibited a 38.9 ±13.7 % decrease in luciferase expression (Fig. 3c), much less than that seen with FMDV (Fig. 2d). It should be noted that the genetic backgrounds of the FMDV and CVB3 constructs are different; however, our data were consistent with the hypothesis that FMDV genome replication did not require PI4K activity, and that the effect of PIK93 was an indirect effect of inhibition of protein translation.

FMDV replication does not require PI4KIIIα or β activity

PIK93 was originally developed as an inhibitor of PI3K (IC_{50} PI3K p110α: 39 nM), but was shown to have selective activity against PI4KIIIβ (IC_{50} PI4KIIIα: 1.1 μM, PI4KIIIβ: 19 nM) (Knight *et al.*, 2006). Given that some positive-strand RNA viruses have been shown to require PI4KIIIα for genome replication (e.g. HCV), it was thus formally possible that the lack of effect of PIK93 could be explained if FMDV genome replication exhibited a requirement for PI4KIIIα but not PI4KIIIβ.

We therefore proceeded to directly test if the lack of sensitivity to PIK93 could be explained by a requirement for PI4KIIIα in FMDV genome replication. To achieve this we took advantage of two inhibitors developed by AstraZeneca with complementary selectivities for PI4KIIIα and PI4KIIIβ (Raubo *et al.*, 2015; Waring *et al.*, 2014). CMPD (7) exhibits selective inhibition of PI4KIIIα (IC_{50} PI4KIIIα: 7 nM, PI4KIIIβ: 1.8 μM), whereas CMPD (3) exhibits a similar selectivity to PIK93 (IC_{50} PI4KIIIα: 7.3 μM, PI4KIIIβ: 15 nM). As a positive control for inhibition of PI4KIIIα we utilized Huh7.5 cells transiently expressing an HCV subgenomic replicon (SGR-Luc-GFP-JFH1), derived from the JFH-1 infectious clone and containing an insertion of GFP into domain III of NS5A (Jones *et al.*, 2007). This allowed HCV genome replication to be assayed using the IncuCyte system, as described for FMDV above.

We first determined whether either compound exhibited any cytotoxicity in BHK-21 cells (for FMDV experiments) or Huh7.5 (for HCV). As shown in Fig. 4a, b the compounds were tolerated up to 10 μM by both cell types, although at 20 μM both exhibited significant cytotoxicity. We therefore tested the effects of the two compounds on both FMDV (Fig. 4c) and HCV (Fig. 4d) replication at 0.5 and 10 μM. As shown in Fig. 4c FMDV replication was only modestly reduced (~20 %) by the higher concentration of both compounds. Reassuringly, whereas CMPD (7) (selective for PI4KIIIα) inhibited HCV replication even at 0.5 μM (Fig. 4d), CMPD (3) (selective for PI4KIIIβ) had no effect. We deduced that FMDV genome replication is not dependent on either PI4KIIIα or PI4KIIIβ.

FMDV replication does not result in upregulation of PI4P lipids

It has previously been described (Reiss *et al.*, 2011; Ross-Thriepland *et al.*, 2015; Zhang *et al.*, 2012) that HCV utilizes the PI4K pathway to assist in the formation of membranous intracellular replication factories, termed the 'membranous web', and consequently the abundance of PI4P lipids is upregulated during HCV RNA replication. We predicted that, because we have no evidence that FMDV replication is dependent on PI4K activity, cells harbouring FMDV replicons would not exhibit a similar upregulation of PI4P lipids. To test this, we compared the levels of PI4P lipids in cells harbouring either HCV- or FMDV- derived replicons. As shown in Fig. 5a, b, Huh7.5 human hepatoma cells harbouring an HCV replicon showed high levels of PI4P lipids as judged by immunofluorescence analysis using antibodies specific for PI4P (Ross-Thriepland *et al.*, 2015) that was lost following treatment with PIK93 and CMPD (7) (selective for PI4KIIIα), consistent with the replication data shown in Fig. 4d.

In contrast, BHK-21 cells transfected with FMDV GFP-pac RNA did not exhibit an increase in PI4P staining compared with untransfected cells (Fig. 5c, d). Furthermore, there were no significant differences in the levels of PI4P staining in BHK-21 cells after treatment with any of the inhibitors

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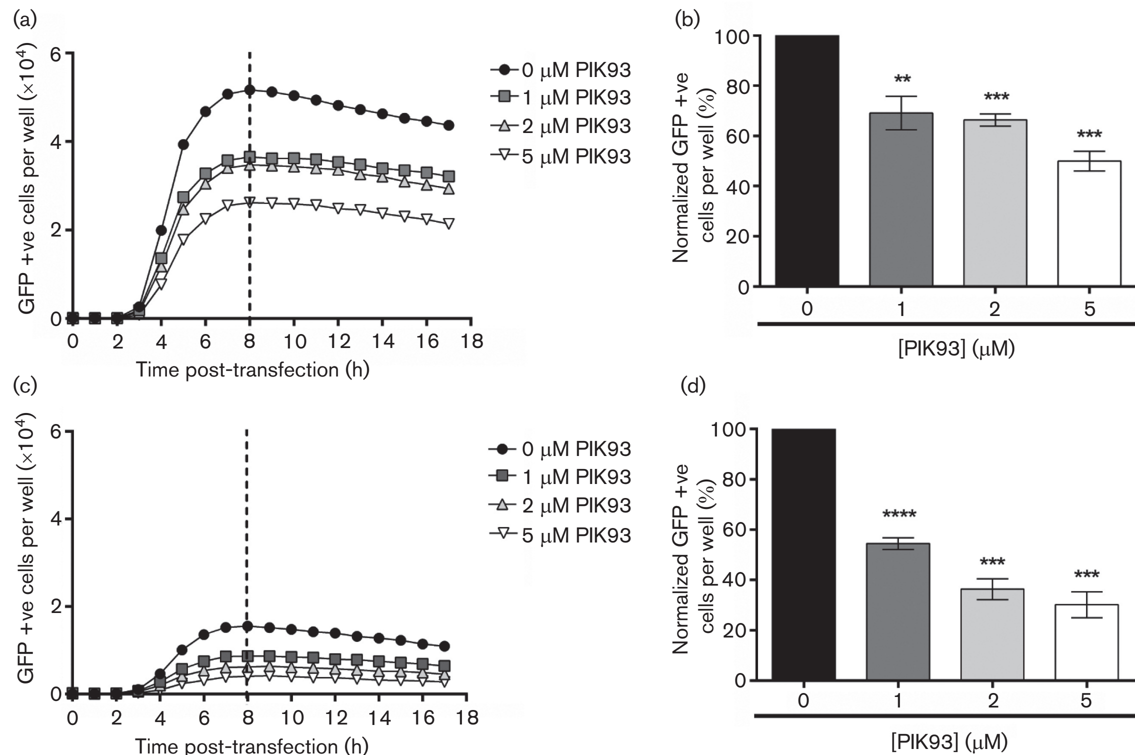

Fig. 2. Levels of GFP expression as a measure of replication in BHK-21 cells transfected with (a, b) GFP-pac-WT and (c, d) GFP-pac-GNN replicon RNAs. Transfected cells were pre-treated for 2 h with PIK93 at the concentrations indicated, and levels of GFP expression were compared against untreated controls. Data were collected by imaging GFP fluorescence hourly in transfected cells using an IncuCyte Dual Colour ZOOM FLR. Levels of GFP expression 8 h post-transfection are shown in (b) and (d), and indicated by a dotted line in (a) and (c). Mean values with SEM (n=3) are shown; statistical analysis was performed using a two-tailed unpaired t-test (**P<0.01, ***P<0.001, ****P<0.0001). +ve, Positive.

tested. We propose that FMDV does not require type III PI4K activity for genome replication and consequently does not upregulate activity of these kinases.

DISCUSSION

Phosphatidylinositol lipids (and phosphorylated derivatives) are key membrane components and have been demonstrated to be involved in entry of a number of viruses, including FMDV (Vázquez-Calvo *et al.*, 2012). Furthermore, the PI4K family of enzymes has been shown to be involved in the genome replication of many positive-sense RNA viruses, primarily in the formation of intracellular membranous compartments by generating PI4P lipids. These compartments are proposed to house the viral replication factories to protect the viral RNA from degradation and recognition by the host cell innate immune response. It has also been observed that some viruses, such as HCV, upregulate PI4K expression and activity. PIK93 is a PI4KIIIβ small molecule inhibitor, but has been shown to have an additional inhibitory effect on PI4KIIIα and PI3K (Knight *et al.*, 2006; Rutaganira *et al.*, 2016). It has been demonstrated previously that the treatment of cells with PIK93 downregulated the generation of PI4P lipids by inhibiting the activity of PI4KIIIα and β. Furthermore, studies with HCV, CVB3 and PV have shown that treating infected cells with PIK93 reduced virus genome replication (Altan-Bonnet & Balla, 2012). Here, we have demonstrated that IRES-mediated translation is sensitive to PIK93. There appears to be an additional effect on genome replication for CVB3, but not for FMDV.

Consistent with these observations, we found no evidence (by immunofluorescence microscopy staining for PI4P lipids) that FMDV replication leads to an accumulation of these lipids. However, in cells electroporated with HCV replicon RNA levels of PI4P were stimulated, as expected. These data suggest that the PI4K pathway is not the primary pathway involved with FMDV genome replication.

Membrane reorganization during HCV infection has been well defined, and it has been reported that PI4KIIIα plays an essential role in the formation of intracellular replication factories required for replication. PI3K has also been implicated in HCV replication by recruiting membranous

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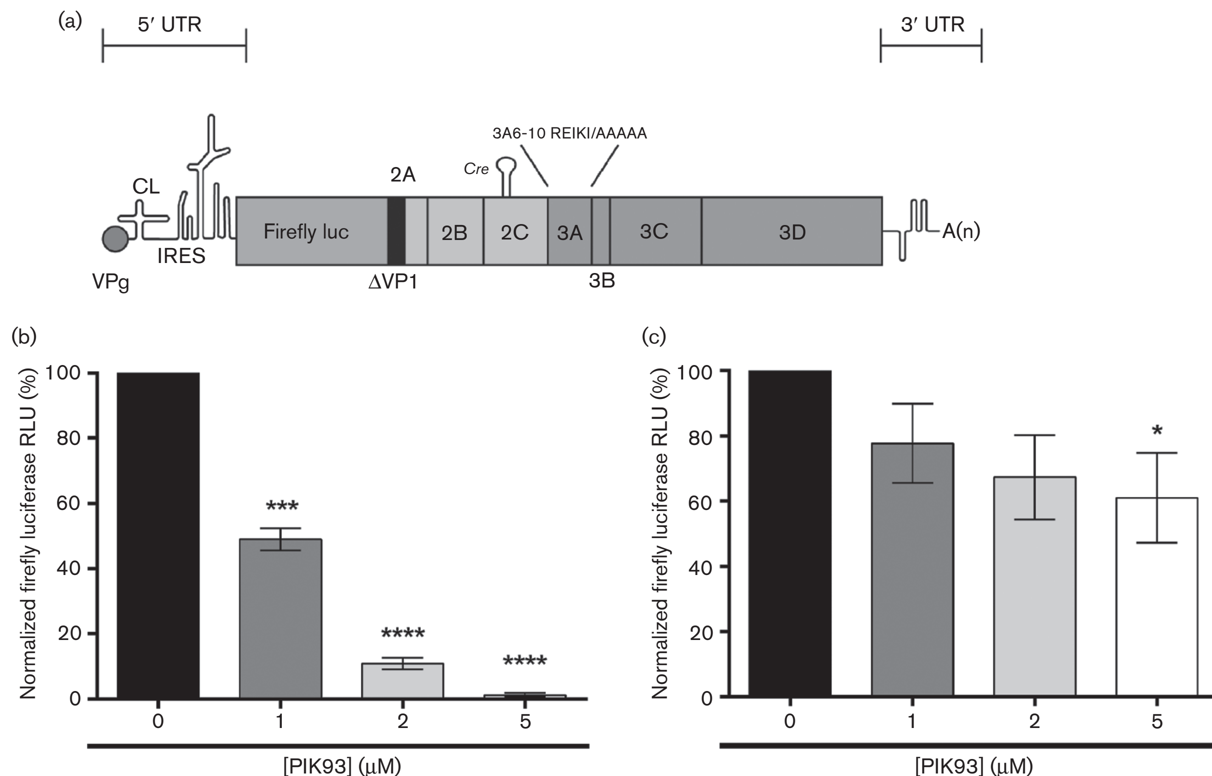

Fig. 3. (a) Schematic diagram of the CVB3 replicon indicating location of the 5′ and 3′ UTR flanking the genes encoding replication proteins. The firefly luciferase reporter gene replaces the structural capsid proteins. Levels of luciferase expression in HeLa cells transfected with (b) Rib-Fluc-CB3/T7 or (c) Rib-Fluc-CB3/T7-3A CVB3 replicon RNAs are shown. Transfected cells were pre-treated for 2 h with PIK93 at the concentrations indicated and levels of luciferase expression were compared against an untreated control. Levels of luciferase expression were measured at 8 h post-transfection. Data show mean values with SEM (n=3); statistical analysis was performed using a two-tailed unpaired t-test (*$P<0.05$, ***$P<0.001$, ****$P<0.0001$). RLU, Relative light units.

compartments from the endocytic pathway. It has been demonstrated that FMDV induces the formation of autophagosomes from the endocytic pathway to facilitate cell entry, but this does not appear to be involved in viral replication. Autophagosome formation is induced in a PI3K-independent manner (Berryman *et al.*, 2012), whereas inhibition of both PI3K and PI4KIIIα significantly inhibits HCV replication in cells (Berger *et al.*, 2009; Gosert *et al.*, 2003). There is evidence from the literature that reorganization of cellular membranes during FMDV infection is different to that seen during infection with other picornaviruses. FMDV infection results in a dramatic condensation and relocalization of intracellular organelles to one side of the cytoplasm in the perinuclear region (Monaghan, 2004) and is unaffected by brefeldin A, a fungal metabolite that disrupts retrograde Golgi–ER transport. It has been shown that brefeldin A interacts with Arf1/GBF1, and interference with GBF1 affects the recruitment of PI4KIIIβ and subsequent PI4P-lipid upregulation. Interestingly, treatment of cells with brefeldin A has been shown to enhance FMDV infection (Midgley *et al.*, 2013). Recent studies have shown that PV, CVB3 and HRV are able to recruit PI4KIIIβ in an Arf1/GBF1-independent manner, highlighting the complexity of the mechanisms by which picornaviruses recruit intracellular membranes (Dorobantu *et al.*, 2015b, 2014; Midgley *et al.*, 2013; O'Donnell *et al.*, 2001). Overall, these studies support the results shown here suggesting that type III PI4Ks are not involved in FMDV replication. It is interesting to note that, during the revision of this manuscript, a further paper has been published that supports our conclusions (Berryman *et al.*, 2016). We therefore propose that FMDV must subvert an alternative cellular pathway to effect the membrane reorganization required to support virus replication.

METHODS

Replicons. Development and use of the FMDV pGFP-pac-WT replicon construct has been described previously (Forrest *et al.*, 2014; Tulloch *et al.*, 2014). The replication-defective FMDV replicon, pGFP-pac-GNN, bearing a inactivating point mutation to the $3D^{pol}$ active site was generated by standard PCR mutagenesis (Herod *et al.*, 2015). An HCV bi-cistronic sub-genomic replicon (pSGR-Luc-GFP-JFH1) (Jones *et al.*,

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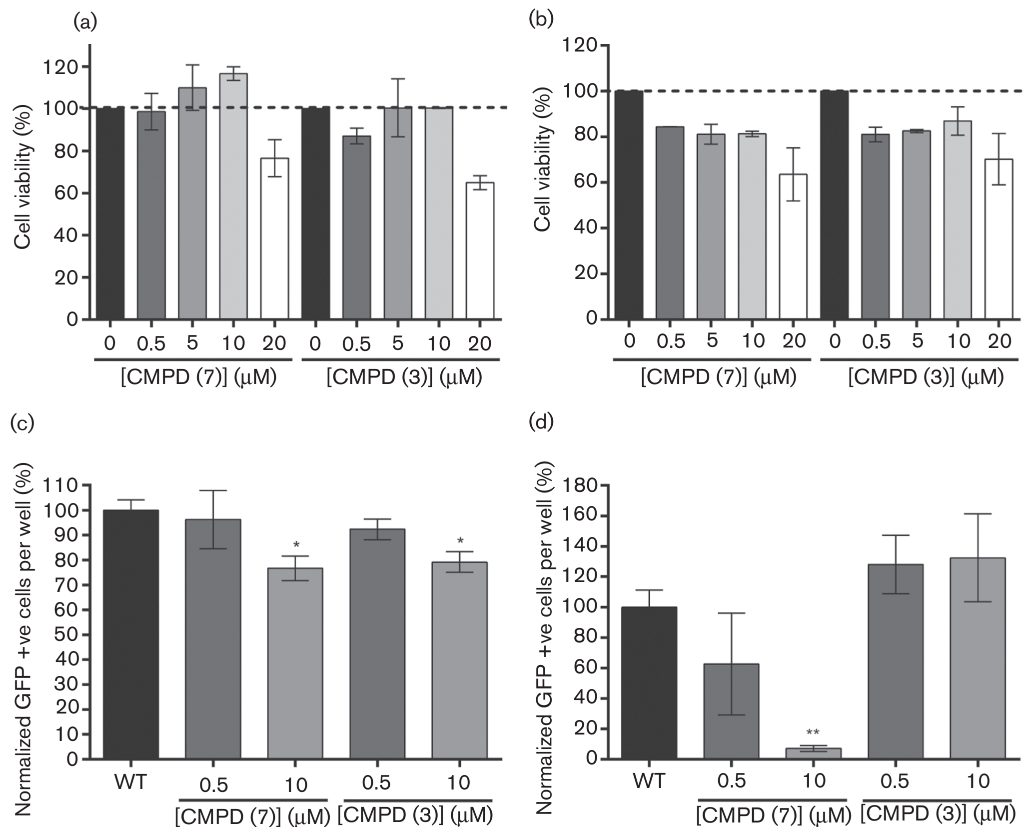

Fig. 4. MTT assay of (a) BHK-21 cells or (b) Huh7.5 cells treated with either a selective PI4KIIIα inhibitor CMPD (7) or PI4KIIIβ inhibitor CMPD (3) at the concentrations indicated. (c) GFP-pac-WT replicon RNA-transfected BHK-21 cells were treated with inhibitors as indicated and levels of GFP expression were compared against an untreated control. Levels of GFP expression were measured at 8 h post-transfection. (d) HCV SGR-Luc-GFP-JFH1 replicon RNA-electroporated Huh7.5 cells were treated with inhibitors as indicated and levels of NS5A-GFP expression were compared against an untreated control. Levels of NS5A-GFP expression were measured at 48 h post-electroporation. Data show mean values with SEM (n=3); statistical analysis was performed using a two-tailed unpaired t-test (*P<0.05, **P<0.01). +ve, Positive.

2007) was also employed, where the coding sequence for the structural proteins (Core, E1 and E2), the viroporin p7 and non-structural protein NS2 were replaced by a luciferase reporter gene under the translational control of the HCV IRES, and translation of NS3, NS4A, NS4B, NS5A and NS5B was driven by the EMCV IRES. A GFP reporter was inserted into domain III of the NS5A protein; this insertion was tolerated such that the sub-genomic replicon retained WT replicative ability, as described previously (Moradpour *et al.*, 2004). A CVB3 sub-genomic replicon, pRib-Fluc-CB3/T7, was also employed, and a replication-deficient mutant (pRib-Fluc-CB3/T7-3A) was generated by alanine substitution of residues 6–10, preventing binding of 3A to host-protein GBF1 (Lanke *et al.*, 2009).

Transfection of bicistronic vectors. BHK-21 cells were transfected with pRF, pRFMDVF, pRHCVF and pREMCVF plasmid DNA using polyethylenimine (PEI). The DNA : PEI mix was incubated at room temperature for 10 min and supplemented with complete Dulbecco's modified Eagle's medium (DMEM, Sigma) prior to addition to the cells. After 6 h the media was removed and replaced with complete DMEM. Cells that were treated with a titration of PIK93 (1–5 μM) (Sigma) were pre-treated 2 h prior to transfection, and PIK93 was replaced each time the media was changed.

RNA transcription of replicons. Details of transcription of pGFP-pac replicons (Tulloch *et al.*, 2014) are described elsewhere (Bentham *et al.*, 2012; Herod *et al.*, 2015). RNA was purified using a Zymogen RNA clean and concentrate kit according to the manufacturer's instructions. CVB3 and HCV replicons were treated in a similar way. CVB3 replicon DNA was linearized with *Mlu*I (NEB) and HCV replicon DNA was first linearized with *Xba*I (NEB), prior to transcription.

RNA transfection/electroporation. GFP-pac-WT, GFP-pac-GNN/Δ3D and Rib-Fluc-CB3/T7 (-3A) RNAs were transfected into BHK-21 or HeLa cells using Lipofectin (Life Technologies) according to the manufacturer's instructions as described by Herod *et al.* (2015). PIK93 (1–5 μM), or CMPD (3) or (7) (0.5–20 μM), was added to the cells 2 h prior to transfection, or at the time of transfection, respectively, and replaced once the transfection mix was added to the cells. Electroporation of SGR-Luc-GFP JFH-1 RNA into Huh 7.5 cells was undertaken as described by Ross-Thriepland *et al.* (2015) and Stewart *et al.* (2015).

IncuCyte analysis of replication. Expression of GFP in transfected cells was measured and analysed using an IncuCyte Dual Colour ZOOM FLR (Essen Bioscience) as described previously (Forrest *et al.*, 2014;

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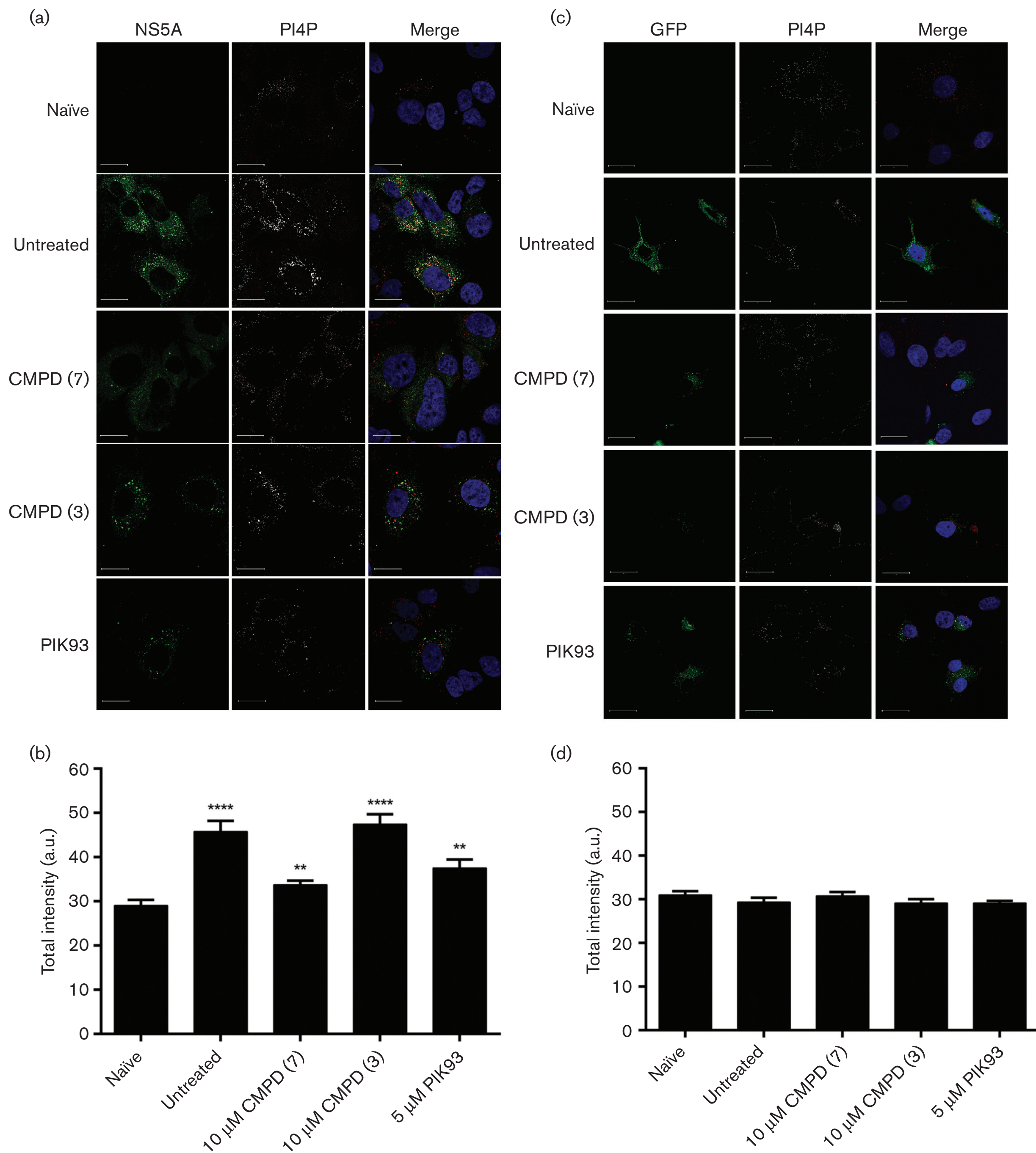

Fig. 5. Fluorescence microscopy of (a) Huh7.5 cells stably expressing HCV SGR-Feo-JFH-1 and (c) BHK-21 cells transfected with GFP-pac-WT replicon RNA. Transfected cells were treated with 10 μM CMPD (7), CMPD (3) or 5 μM PIK93. Fixed cells were stained with anti-PI4P antibody (mouse). NS5A was detected by anti-NS5A antibody (sheep). Untransfected/non-stably expressing (i.e. naïve) cells were included as controls. Levels of PI4P expression were measured and presented as total PI4P intensity measured in arbitrary units (a.u.) in (b) Huh7.5 cells and (d) BHK-21 cells. Bars, 20 μm. Data show mean values with SEM (n=10). Statistical analysis was performed using a two-tailed unpaired t-test (**P>0.01, ****P>0.0001).

Herod *et al.*, 2015; Tulloch *et al.*, 2014). After transfection with replicon RNA, the cells were maintained within an IncuCyte housed at 37 °C in a humidified 5 % CO_2 incubator. Cells were monitored hourly for up to 20 h. Images (9) of each well were taken and the number of GFP-expressing cells was counted as green object count per well analysed by an internal algorithm within the IncuCyte ZOOM software.

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Fluorescence microscopy. Huh 7.5 cells, Huh 7.5 cells stably harbouring SGR-feo-JFH-1 (Wyles *et al.*, 2009) (under 0.5 mg ml^{-1} G418 selection) and BHK-21 cells electroporated with GFP-pac replicon RNA (following the aforementioned protocols) were incubated in a humidifying incubator with 5 % CO_2 for 24 or 4 h, respectively. Cells were washed, fixed and permeabilized as described previously (Ross-Thriepland *et al.*, 2015) prior to staining with primary anti-PI4P antibody (mouse, 1 : 100; Echelon) and anti-NS5A (sheep, 1 : 2500) with fluorescently conjugated secondary antibodies – Alexa Fluor 568 rabbit-anti-mouse and Alexa Fluor 488 donkey-anti-sheep (1 : 1000) (Life Technologies). Confocal microscopy images were acquired on a Carl Zeiss 880 upright microscope. Quantification and analysis of the data was undertaken using integrated Zeiss 2.1 software.

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