Short Communication

Pseudorabies virus US3 triggers RhoA phosphorylation to reorganize the actin cytoskeleton

Thary Jacob,1 Céline Van den Broeke,1 Cliff Van Waesberghge,1 Leen Van Troys2 and Herman W. Favoreel1

1Department of Virology, Parasitology, and Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
2Department of Biochemistry, Faculty of Medicine and Health Sciences, Ghent University, Albert Baertsoenkaai 3, 9000 Ghent, Belgium

The conserved alphaherpesvirus serine/threonine kinase US3 causes dramatic changes in the actin cytoskeleton, consisting of actin stress fibre breakdown and protrusion formation, associated with increased virus spread. Here, we showed that US3 expression led to RhoA phosphorylation at serine 188 (S188), one of the hallmarks of suppressed RhoA signalling, and that expression of a non-phosphorylatable RhoA variant interfered with the ability of US3 to induce actin rearrangements. Furthermore, inhibition of cellular protein kinase A (PKA) eliminated the ability of US3 to induce S188 RhoA phosphorylation, pointing to a role for PKA in US3-induced RhoA phosphorylation. Hence, the US3 kinase leads to PKA-dependent S188 RhoA phosphorylation, which contributes to US3-mediated actin rearrangements. Our data suggest that US3 efficiently usurps the antagonistic RhoA and Cdc42/Rac1/p21-activated kinase signalling branches to rearrange the actin cytoskeleton.

The Alphaherpesvirinae represent the largest subfamily of the family Herpesviridae, comprising closely related pathogens of humans and animals. These include herpes simplex virus 1/2 (HSV-1/2; human herpesvirus 1/2) and varicella-zoster virus (VZV; human herpesvirus 1) in humans. Pseudorabies virus (PRV) is a porcine alphaherpesvirus and is often used to study general aspects of alphaherpesvirus biology (Pomeranz et al., 2005). Infection with PRV can lead to dramatic changes in the actin cytoskeleton of the host cell, consisting of actin stress fibre breakdown and protrusion formation (Favoreel et al., 2005). These actin rearrangements are associated with increased viral cell-to-cell spread and depend on the kinase activity of the viral serine/threonine kinase US3 (Favoreel et al., 2005; Van den Broeke et al., 2009a). US3 is conserved within the alphaherpesviruses and comparable US3-induced cytoskeletal changes have been described for several other alphaherpesviruses, including HSV-2 and bovine herpesvirus 1/5 (BHV-1/5) (Brzozowska et al., 2010; Finnen et al., 2010; Ladeña et al., 2011).

Rearrangements of the actin cytoskeleton are generally regulated by Rho GTPase signalling pathways (Hall, 1998). RhoA, Rac1 and Cdc42 are the best characterized Rho GTPases and regulate many actin-driven processes. Activation of RhoA generally leads to actin stress fibre formation, whilst Rac1 and Cdc42 are typically associated with the formation of different actin-based protrusions, most notably filopodia (Cdc42) and lamellipodia (Rac1) (Hall, 1998). Rac1/Cdc42 signalling typically counteracts RhoA signalling and vice versa (Van den Broeke et al., 2014).

We have demonstrated previously that PRV US3 influences the Rac1/Cdc42 signalling branch, via activation of p21-activated kinases (PAKs), downstream effectors of Rac1 and Cdc42. In particular, US3 phosphorylates, and thereby activates, both PAK1 and PAK2, contributing to stress fibre breakdown and protrusion formation (Van den Broeke et al., 2009b).

It is currently not known whether US3 may also affect the opposing RhoA signalling branch. RhoA GTPase activity is regulated via several mechanisms, including phosphorylation at serine residue 188 (S188). S188 phosphorylation of RhoA suppresses RhoA signalling via RhoGDIs (Rho GDP-dissociation inhibitor)-mediated relocalization of (GTP-bound) RhoA from the cellular membrane to the cytoplasm, thereby keeping RhoA away from its site of activity and preventing downstream signalling (Lang et al., 1996; Rolli-Derkinderen et al., 2005). Cyclic AMP (cAMP)-dependent protein kinase A (PKA) has been reported to phosphorylate RhoA at position S188 in several cell types (Dong et al., 1998; Ellerbroek et al., 2003; Jones & Palmer, 2012; Lang et al., 1996; Lapetina et al., 1989; Quil- liam et al., 1991; Tkachenko et al., 2011). This is of
particular interest as the US3 protein kinase homologues of alphaherpesviruses such as HSV-1 and VZV have been shown (i) to functionally overlap with cellular PKA with regard to cellular substrates and/or (ii) trigger activation of cellular PKA during infection (Benetti & Roizman, 2004; Benetti et al., 2003; Erazo & Kinchington, 2010; Munger & Roizman, 2001; Ogg et al., 2004).

Here, we investigated whether expression of the US3 protein of the alphaherpesvirus PRV affected RhoA phosphorylation at S188 and, if so, whether this contributed to the US3-mediated effects on the actin cytoskeleton.

We report that expression of US3 leads to increased RhoA S188 phosphorylation, that overexpression of a non-phosphorylatable S188A RhoA mutant interferes with the US3-mediated actin rearrangements and that inhibition of PKA interferes with the ability of US3 to trigger RhoA phosphorylation.

To investigate whether PRV infection leads to RhoA S188 phosphorylation and, if so, whether this depends on US3 expression, porcine ST cells (seeded at 150 000 cell ml\(^{-1}\); cultured as in Geenen et al., 2005) were mock inoculated or inoculated with WT PRV or isogenic US3null PRV

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**Fig. 1.** Expression of US3 during infection or transfection triggers RhoA-S188 phosphorylation. (a, b) ST cells were mock inoculated or inoculated with WT PRV (strain NIA3) or isogenic US3null PRV. (a) At 6 h p.i., cell lysates were subjected to Western blotting to detect P-S188-RhoA (P-RhoA), total RhoA, US3, gE or tubulin. (b) Relative RhoA phosphorylation levels (P-S188-RhoA : RhoA ratio with mock infection set to 1) from three independent repeats. Data represent means ± SEM; ***P<0.001 (determined by one-way ANOVA and Tukey’s post test). (c, d) ST cells were mock-transfected or transfected with a eukaryotic expression vector encoding PRV US3 or kinase-inactive PRV US3 (‘kin mut’, with a point mutation in the predicted ATP-binding site, K136G). (c) At 24 h post-transfection, cell lysates were subjected to Western blotting to detect total P-S188-RhoA (P-RhoA), total RhoA, US3 and tubulin expression levels. (d) Relative RhoA phosphorylation levels (P-S188-RhoA : RhoA ratio with mock transfection set to 1) from three independent repeats. Data represent means ± SEM; *P<0.05, **P<0.01 (determined by one-way ANOVA and Tukey’s post test). Western blot panels were obtained from separate gels, but were derived from the same sample. All gels were run with the same amount of sample, and were blotted, incubated with antibodies and detected together in parallel.
(a) pcDNA3.1

(b) pcDNA3.1
Fig. 2. Overexpression of non-phosphorylatable S188A-RhoA or, to a lesser extent, WT RhoA interferes with the ability of US3 to cause actin rearrangements. (a) ST cells were transfected with empty vector pcDNA3.1 alone or cotransfected with US3 together with pcDNA3.1 or either HA-tagged WT RhoA or S188A RhoA. At 24 h post-transfection, cells were fixed and stained for US3 (Texas red, red), HA-RhoA (Alexa Fluor 647, purple), actin (phalloidin–FITC, green) and nuclei (Hoechst 33342, cyan). White squares in (a) indicate zoomed areas in (b). (b) Cells marked by white arrowheads demonstrate cells with the US3 phenotype (cell rounding and stress fibre breakdown) that were scored positive in Fig. 3(a).

NIA3 (containing a translational stop codon in US3) (Baskerville, 1973; de Wind et al., 1990). At 6 h post-inoculation (p.i.), cells were lysed and subjected to SDS-PAGE and Western blotting to detect levels of phospho (P)-S188-RhoA (Abcam), total RhoA (Santa Cruz), viral proteins US3 (antibody kindly provided by LeighAnne Olsen and Lynn Enquist, Princeton University, Princeton, NJ, USA) and gE (13D12; Nauwynck & Pensaert, 1995), and loading control tubulin (Abcam) (Fig. 1a). P-S188-RhoA and RhoA band intensities were measured with the ‘Analyse gels’ option in ImageJ, and P-S188-RhoA ratios were normalized to mock levels (Fig. 1b). Figure 1(a, b) shows that WT PRV infection triggered a substantial increase in RhoA phosphorylation, compared with mock-infected or US3null PRV-infected cells. A time-course assay showed that RhoA phosphorylation could be detected from early in infection, gradually increasing up to 6 h p.i. and reaching a plateau from then onwards (Fig. S1, available in the online Supplementary Material). Hence, PRV infection induced US3-mediated RhoA S188 phosphorylation. In addition, transfection of a WT US3-encoding eukaryotic expression plasmid (Geenen et al., 2005) in ST cells (seeded at 100 000 cells ml⁻¹) was sufficient to trigger S188 RhoA phosphorylation, whilst transfection of kinase-inactive US3 (K136G mutation in the ATP-binding site) (Deruelle et al., 2007; Van den Broeke et al., 2009a) did not (Fig. 1c, d). We concluded that expression of US3 led to S188 RhoA phosphorylation in infected and transfected ST cells.

If RhoA S188 phosphorylation is important for US3-induced actin rearrangements, one would expect that overexpression of WT RhoA may interfere with US3-induced actin rearrangements, as accumulated levels of RhoA may exceed the ability of US3 to inactivate it. Overexpression of a non-phosphorylatable S188A-RhoA mutant, which could not be inactivated through phosphorylation, would then be expected to interfere to an even larger extent with US3-induced actin rearrangements. To test this, ST cells (seeded at 100 000 cells ml⁻¹) were transfected with empty vector pcDNA3.1 alone or cotransfected with US3 together with pcDNA3.1 or either haemagglutinin (HA)-tagged WT RhoA or S188A-mutated RhoA (as described in Rolli-Derkinderen et al., 2005). At 24 h post-transfection, cells were fixed using 3 % paraformaldehyde, permeabilized with Triton X-100, and fluorescently labelled with antibodies against US3, the HA tag (Sigma-Aldrich) and a probe against actin (phalloidin–FITC; Invitrogen). Finally, cells were counterstained for nuclei (Hoechst 33342; Invitrogen) (Fig. 2a, b).

Figure 3(a) summarizes results from three independent experiments where each time 200 cotransfected cells (both high US3 and RhoA expressing cells) per condition were scored for cell rounding (actin stress fibre disassembly) and cell projection formation [white arrows on the merged views of Fig. 2(b) indicate cells that were scored positive]. Western blot assays of total cell lysates 24 h post-transfection detecting US3, RhoA-HA and tubulin expression indicated that US3 expression was similar in all samples (Fig. 3b). Our data showed that overexpression of RhoA interfered with the ability of US3 to trigger actin rearrangements. Importantly, co-expression of S188A RhoA with US3 caused a significantly stronger suppression of US3-mediated actin rearrangements compared with co-expression of WT RhoA with US3. Hence, non-phosphorylatable S188A RhoA more potently suppressed the ability of US3 to induce actin rearrangements, supporting a role for US3-triggered RhoA S188 phosphorylation in these actin rearrangements.

To investigate whether US3 phosphorylated RhoA directly, a kinase assay was performed according to published literature (Lang et al., 1996; Van den Broeke et al., 2009b). In short, 1 μg recombinant RhoA (Sigma-Aldrich) was added to 1 μg recombinant glutathione S-transferase (GST)-tagged US3 or 2500 U positive control PKA catalytic subunit (New England BioLabs) in kinase buffer (50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT and 20 μM ATP), each reaction containing a total volume of 20 μL. The lot of GST-US3 we used exhibited kinase activity on the PAK1 substrate (data not shown), in line with our previous publication (Van den Broeke et al., 2009b). After 30 min incubation at 30 °C, samples were boiled in loading buffer and used for SDS-PAGE/Western blotting. Whilst this assay confirmed that active PKA directly phosphorylated RhoA, US3 did not induce detectable RhoA phosphorylation, suggesting that US3 may not directly phosphorylate RhoA and that a cellular kinase may be involved (Fig. 4a).

US3 of HSV-1 has been reported to trigger PKA activation (Benetti & Roizman, 2004; Benetti et al., 2003; Munger & Roizman, 2001). To investigate whether this is also the case in PRV, ST cells were either mock inoculated or inoculated with WT PRV or US3null PRV. At 6 h p.i., cells were lysed and subjected to Western blotting to detect levels of phospho-PKA substrates (Cell Signalling; 6921), viral proteins US3 and gE, and loading control tubulin (Fig. 4b). WT PRV infection resulted in substantially increased...
phospho-PKA substrate signal, whilst this was considerably less prominent for US3null PRV infection.

Next, we examined whether PRV-mediated RhoA phosphorylation depended on PKA activity. To this end, ST cells were mock inoculated or inoculated with WT PRV in the presence or absence of 50 μM cell-permeable PKA inhibitor PKI (14-22 myristoylated; Tocris Bioscience), added from 30 min before viral inoculation. At 6 h p.i., cells were lysed and subjected to SDS-PAGE and Western blotting to detect levels of P-S188-RhoA, total RhoA, phospho-PKA substrates, viral proteins US3 and gE, and loading control tubulin (Fig. 4c, d). Phospho-PKA substrate signal confirmed increased PKA activity during PRV infection. PKI largely suppressed this PRV-induced phospho-PKA substrate signal, confirming that this signal specifically correlated with increased PKA activity (Fig. 4c). Interestingly, PKI eliminated PRV-induced RhoA phosphorylation, indicating that PRV-triggered RhoA phosphorylation depended on PKA activity (Fig. 4c, d). Hence, our data indicated that US3 indirectly triggered RhoA phosphorylation, via the cellular PKA kinase.

In line with this, it has been reported that PKA-induced RhoA S188 phosphorylation leads to changes in cell morphology that correspond well with US3-induced actin rearrangements. For example, in epithelial SH-EP cells, addition of forskolin, which increases PKA activity via increased intracellular cAMP levels, resulted in PKA-mediated RhoA S188 phosphorylation, and consequent cell retraction and formation of long, branched cell projections that resembled US3-induced cell projections (Dong et al., 1998).

Phosphorylation and activation of PAK, another effect of US3 signalling (Van den Broeke et al., 2009b), has also been associated with US3-like actin rearrangements, stress fibre disassembly and cell projection formation (Zhao et al., 1998). Hence, US3 both suppresses RhoA signalling via RhoA phosphorylation and triggers Cdc42/Rac1 signalling via PAK phosphorylation, and both appear to contribute to the US3-induced actin rearrangements. Why does US3 interfere with both pathways? In this context, it is interesting that there is negative feedback between RhoA signalling and Cdc42/Rac1/PAK signalling, and reciprocal control of both branches of signalling has been reported in different cellular settings (Kozma et al., 1997; Leeuwen et al., 1997; Sander et al., 1999; Xie et al., 2008; Yamaguchi et al., 2001). For example, mesenchymal and amoeboid migration rely on antagonistic reciprocal control of RhoA and Rac signalling (Parri & Chiarugi, 2010). Relevant to this work, RhoA activation promotes formation of stress fibres, whereas PAK activation destabilizes stress fibres (Etienne-Manneville & Hall, 2002; Van Aelst & D’Souza-Schorey, 1997). By simultaneously triggering both PAK activating signalling and RhoA inactivating signalling, US3 seems to usurp this feedback system very efficiently.

Interestingly, a US3-like phenotype of actin rearrangements has also been described for the F11 protein of vaccinia virus, a poxvirus (Arakawa et al., 2007). F11 also exerts its effect via suppression of the RhoA signalling axis, albeit via a different mechanism than US3. F11 interferes with the Rho GTPase-activating protein myosin-9A (Handa et al., 2013), thereby blocking the interaction of RhoA with its downstream effectors ROCK and mDia (Valderrama et al., 2006). Several other viruses have also been reported to mediate RhoA inactivation at some
point in their replication cycle to lead to successful infection or spread, such as human cytomegalovirus (human herpesvirus 5) (Frampton et al., 2007), simian virus 40 (Stergiou et al., 2013), hepatitis C virus (Brazzoli et al., 2008), Epstein–Barr virus (human herpesvirus 4) (Loesing et al., 2009) and human immunodeficiency virus (del Real et al., 2004). To the best of our knowledge, the current study is the first report describing virus-triggered phosphorylation of RhoA.

To conclude, US3 expression leads to PKA-dependent RhoA S188 phosphorylation, which contributes to the US3-induced actin rearrangements. The combination of activation of PAK signalling and suppression of RhoA signalling may provide the ideal signalling conditions to generate the robust US3-induced cytoskeletal rearrangements.

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**Fig. 4.** US3-induced RhoA phosphorylation is PKA-dependent. (a) *In vitro* kinase assay: recombinant RhoA was added to either recombinant US3-GST or cAMP-dependent PKA catalytic subunit (‘PKA CS’) in kinase buffer. After incubation, samples were subjected to SDS-PAGE/Western blotting to detect levels of P-S188-RhoA (P-RhoA), RhoA and US3. (b) ST cells were mock inoculated or inoculated with WT PRV or US3null PRV. At 6 h p.i., cell lysates were subjected to Western blotting to detect levels of phospho-PKA (P-PKA) substrate signal, US3, gE and tubulin. (c) ST cells were mock inoculated or inoculated with WT PRV in the presence or absence of PKI, a cell-permeable PKA inhibitor. At 6 h p.i., cell lysates were subjected to Western blotting to detect levels of P-S188-RhoA (P-RhoA), RhoA, phospho-PKA (P-PKA) substrate signal, US3, gE and tubulin. (d) Relative RhoA phosphorylation levels (P-S188-RhoA : RhoA ratio with mock infection set to 1) from three independent repeats. Data represent means ± SEM; **P<0.01, ***P<0.001 (determined by one-way ANOVA and Tukey’s post test). Western blot panels were obtained from separate gels but were derived from the same sample. All gels were run with the same amount of sample, and were blotted, incubated with antibodies and detected together in parallel.
the laboratory of G. Loirand (INSERM, France) for the RhoA mutants.

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