The US3 kinase of pseudorabies virus leads to activation of the actin regulator cofilin to induce actin cytoskeleton changes

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Introduction

The US3 kinase is conserved amongst all *Alphaherpesvirinae*. We and others have shown that this kinase induces dramatic rearrangements of the actin cytoskeleton, including disassembly of actin stress fibers (resulting in cell rounding) and the formation of cellular projections, which are associated with increased viral spread (Favoreel et al., 2005, PNAS). For the alphaherpesvirus pseudorabies virus (PRV), we have found that the US3-induced changes in the actin cytoskeleton are mediated through p21-activated kinases (PAKs), central regulators in RhoGTPase signaling (Van den Broeke et al., 2009, PNAS). Apart from the involvement of PAKs, relatively little is known on the cellular factors that contribute to US3-mediated actin rearrangements. Cofilin, a member of the ADF/cofilin family, is a central player in actin dynamics and is known to be inactivated through phosphorylation on serine residue 3 (S3) (Moriyama et al., 1996, Genes Cells). Our aim is to investigate whether the US3 protein of the alphaherpesvirus pseudorabies virus (PRV) affects cofilin phosphorylation, and, if so, whether this contributes to the US3-mediated effects on the actin cytoskeleton. We report that US3 leads to strong cofilin dephosphorylation, which is inhibited by a PAK inhibitor, and that overexpression of a phosphomimetic cofilin variant interferes with US3-mediated actin rearrangements.

Results

1. US3 is required for PRV-mediated suppression of cofilin phosphorylation

To investigate whether US3 modulates the activity of cofilin through altered phosphorylation at the critical S3 residue in cofilin, ST cells (seeded at 150.000 c/ml) were inoculated with previously described isogenic NIA3 strains WT PRV, US3null PRV (containing a translational stop codon in US3), or a revertant virus of the latter (MOI 10) (de Wind et al. 1990. J Virol). At 6 hpi, cells were subjected to Western blotting (WB) with antibodies directed against S3 phospho-cofilin and total cofilin. Figure 1A and 1B show that, compared to mock-infected cells, WT and US3rescue PRV infection led to a strong decrease in S3 cofilin phosphorylation, in contrast to US3null PRV. Phospho-S3 cofilin levels in US3null PRV were even increased, albeit not significantly, when compared to mock-infected cells. Concluding, US3 is required and sufficient to suppress S3 phospho-cofilin levels in infected and transfected cells and absence of US3 increases phosphocofilin levels.

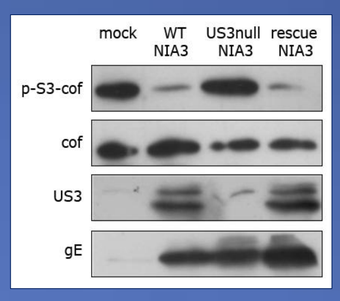

Figure 1A: ST- cells were mock-inoculated or inoculated with WT NIA3 PRV, US3null NIA3 PRV or US3rescue NIA3 PRV. At 6hpi, cells were subjected to Western blotting to detect p-S3-cofilin, total cofilin, US3 and gE.

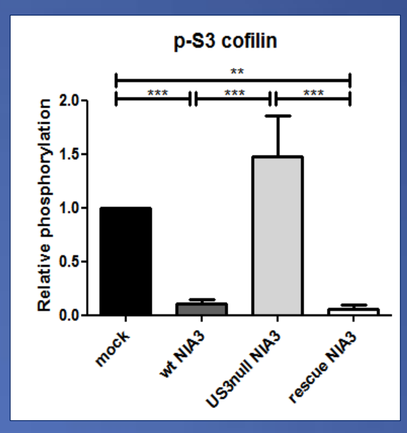

Figure 1B: Relative cofilin phosphorylation levels based on p-S3-cofilin/cofilin ratio (with mock transfection set to 1) represented as means + standard errors of the mean of data from three independent experiments, with ** = P < 0.01 and *** = P < 0.001.

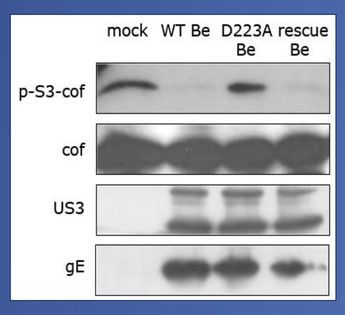

Figure 2A: ST- cells were mock-inoculated or inoculated with WT Be PRV, D223A Be PRV or D223Arescue Be PRV. At 6hpi, cells were subjected to Western blotting to detect p-S3-cofilin, total cofilin, US3 and gE.

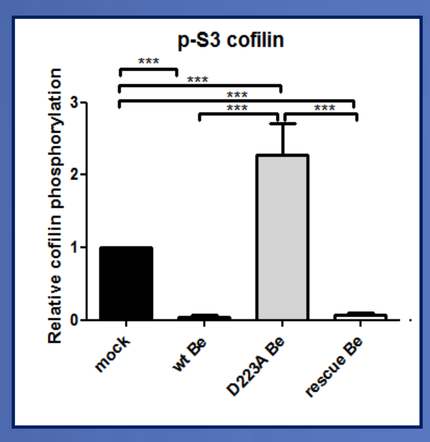

Figure 2B: Means + standard errors of relative cofilin phosphorylation level (with mock transfection set to 1) represented as means + standard errors of the mean of data from three independent experiments, with *** = P < 0.001.

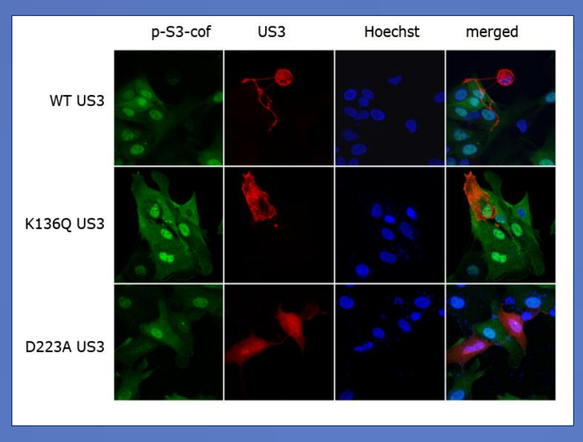

Figure 2C: ST-cells were transfected with constructs encoding WT US3 or kinase dead variants K136Q US3 or D223A US3. 24h post transfection, cells were fixed and stained for p-S3-cof (green), US3 (red) and nuclei (blue).

2. US3 kinase activity is required to suppress cofilin phosphorylation

To assess involvement of the kinase activity of US3 in suppressing cofilin phosphorylation, cells were inoculated (MOI 10) with a PRV strain expressing a kinase-inactive US3 protein (D223A Be) (Coller et al., 2008, Traffic; Van den Broeke et al., 2009, Virology). At 6hpi, S3 phospho-cofilin vs total cofilin levels were evaluated by WB (Figure 2A&B). D223A Be did not suppress S3 phospho-levels of cofilin, while the revertant virus acted like WT virus. D223A Be showed increased phosphorylation of cofilin compared to mock-infected cells. US3 kinase function importance for cofilin dephosphorylation was confirmed in transfection assays (Figure 2C), using plasmids encoding WT and D223A US3 (Geenen et al., 2005. Virology; Van den Broeke et al., 2009, Virology) and other kinase inactive K136Q US3 (pHF61). In conclusion, the kinase activity of US3 is required to suppress S3 phosphorylation of cofilin.

3. Constitutively inactive S3D cofilin interferes with US3-mediated cell actin rearrangements

If US3-mediated effects on cofilin activity levels are important for PRV US3-induced rearrangements of the actin cytoskeleton, one would expect that expression of a constitutively inactive S3D mutant of cofilin (S3) will interfere with these rearrangements. To assess this, ST cells were co-transfected with US3 and constructs expressing previously described GFP fusions of wild type cofilin, S3D cofilin or S3A cofilin (Leyman et al., 2009, Mol Biol Cell). At 24h post transfection, cells were scored for US3-mediated effects on the actin cytoskeleton, consisting of actin stress fiber disassembly (assessed by cell rounding) and cell projection formation. Phosphomimetic S3D cofilin, but not wild type or S3A cofilin, significantly suppressed the ability of US3 to induce actin rearrangements (Figure 3A&B). Hence, expression of phosphomimetic S3D cofilin interferes with the ability of US3 to induce actin rearrangements.

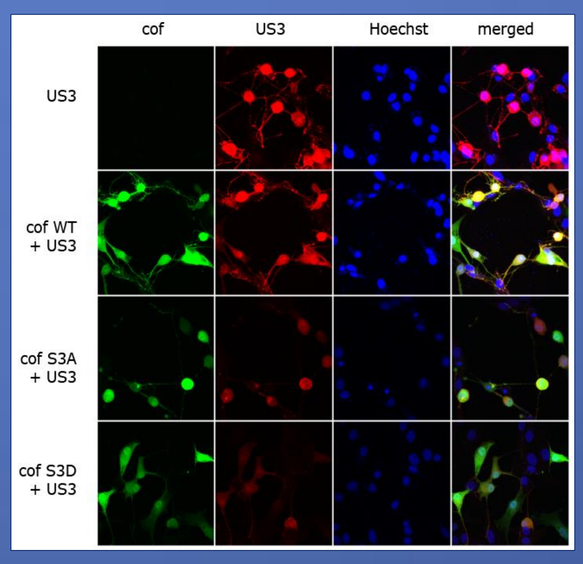

Figure 3A: ST cells were transfected with either US3 alone or US3 together with GFP-tagged WT cofilin, S3A cofilin or S3D cofilin. At 24h post transfection, cells were fixed, stained and analyzed for expression of cofilin (GFP - green), US3 (red) and nuclei (blue).

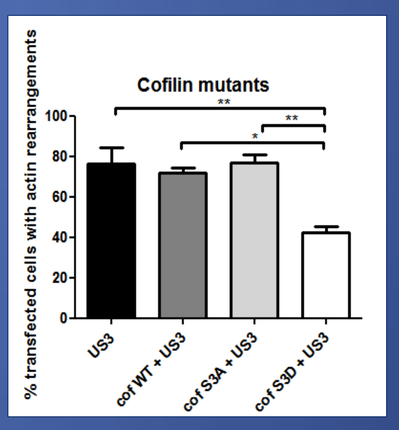

Figure 3B: percentage of transfected cells displaying actin rearrangements, as assessed by cell rounding and the formation of cell projections (means + standard errors of the mean, data from three independent experiments), with * = P < 0.05 and ** = P < 0.01.

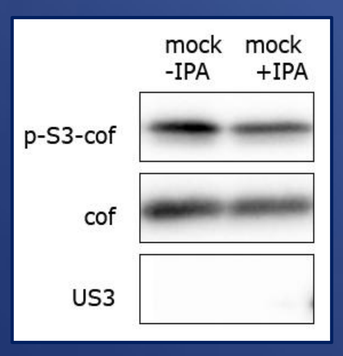

Figure 4A: ST cells treated with or without 33nM of the group I PAK inhibitor IPA-3, were mock-inoculated. At 6hpi, cells were lysed and analyzed for levels of p-S3-cofilin, total cofilin and US3.

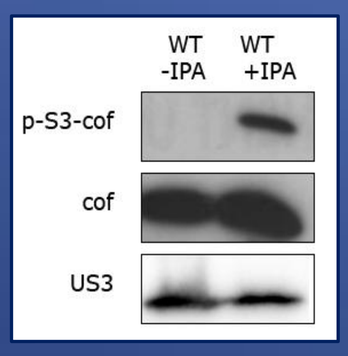

Figure 4B: ST cells treated with or without 33nM of the group I PAK inhibitor IPA-3, were inoculated with WT PRV. At 6hpi, cells were lysed and analyzed for levels of p-S3-cofilin, total cofilin and US3.

4. PAK is involved in the US3-mediated dephosphorylation of cofilin

The ability of US3 to induce actin rearrangements has been shown to depend on the ability of US3 to phosphorylate and thereby activate group I p21-activated kinases (PAKs). As a consequence, the PAK inhibitor IPA-3 is able to inhibit the US3-mediated actin rearrangements (Van den Broeke et al., 2010, Trends Cell Biol). Hence, we investigated whether IPA-3 is able to revert the US3-mediated suppression of S3 cofilin phosphorylation. To this end, ST cells were either or not inoculated with WT PRV in the absence or presence of 33nM IPA-3. At 6hpi, cells were lysed and subjected to WB and detection of p-S3-cofilin, total cofilin, gE and US3 expression. Addition of IPA-3 did not affect US3 expression or total cofilin protein levels, but restored the phospho-S3 cofilin signal in PRV-infected cells (Figure 4A&B). Hence, PAK activity is required for US3-mediated suppression of S3 cofilin phosphorylation.

Conclusions

US3 of pseudorabies virus (PRV) leads to activation (dephosphorylation) of the central actin regulator cofilin. Mutations that impair US3 kinase activity and the group I p21-activated kinase inhibitor IPA-3 inhibited US3-mediated cofilin activation. Additionally, expression of phosphomimetic S3D cofilin significantly suppressed the ability of US3 to cause cell projections and cell rounding. Concluding, the US3 kinase of PRV leads to activation (dephosphorylation) of cofilin and cofilin contributes to US3-mediated actin rearrangements.

Acknowledgements

Thary Jacob is supported by a PhD grant from the Agency for Innovation by Science and Technology in Flanders (IWT-Vlaanderen). We thank C. Van Waesberghe for technical assistance, L.A. Olsen and L. Enquist (Princeton University, USA) for the mouse monoclonal anti-US3 antibodies, G.A. Smith (Northwestern University, USA) for PRV strains GS847 and GS976, the ID-DLO (The Netherlands) for PRV NIA-3 strains, and J. Chernoff (Fox Chase Cancer Center, USA) for IPA-3.