Protein B5 is required on extracellular enveloped vaccinia virus for repulsion of superinfecting virions

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Vaccinia virus (VACV) spreads across cell monolayers fourfold faster than predicted from its replication kinetics. Early after infection, infected cells repulse some superinfecting extracellular enveloped virus (EEV) particles by the formation of actin tails from the cell surface, thereby causing accelerated spread to uninfected cells. This strategy requires the expression of two viral proteins, A33 and A36, on the surface of infected cells and upon contact with EEV this complex induces actin polymerization. Here we have studied this phenomenon further and investigated whether A33 and A36 expression in cell lines causes an increase in VACV plaque size, whether these proteins are able to block superinfection by EEV, and which protein(s) on the EEV surface are required to initiate the formation of actin tails from infected cells. Data presented show that VACV plaque size was not increased by expression of A33 and A36, and these proteins did not block entry of the majority of EEV binding to these cells. In contrast, expression of proteins A56 and K2 inhibited entry of both EEV and intracellular mature virus. Lastly, VACV protein B5 was required on EEV to induce the formation of actin tails at the surface of cells expressing A33 and A36, and B5 short consensus repeat 4 is critical for this induction.

INTRODUCTION

Vaccinia virus (VACV) is a member of the genus Orthopoxvirus; it replicates in the cytoplasm (Moss, 2007) and produces multiple distinct virions (Smith et al., 2002; Condit et al., 2006). The first particle formed, intracellular mature virus (IMV), is surrounded by a single lipid membrane (Hollinshead et al., 1999) and represents the majority of progeny virus that remains intracellular until cell lysis. However, some IMV are wrapped by membrane cisternae from early endosomes or the trans-Golgi network to form intracellular enveloped virus (IEV). IEV move on microtubules to the cell periphery (Geada et al., 2001; Hollinshead et al., 2001; Rietdorf et al., 2001; Ward & Moss, 2001) and then fuse with the plasma membrane to form a cell-associated enveloped virus (CEV) that remains attached to the cell surface. VACV protein A36 accumulates in the plasma membrane beneath CEV (van Eijl et al., 2000) and is phosphorylated by Src kinases (Frischknecht et al., 1999; Scaplehorn et al., 2002) to induce actin polymerization (Cudmore et al., 1995) that pushes the CEV away from the cell surface. An extracellular enveloped virus (EEV) is formed when CEV is released into the extracellular medium. Viral proteins A36 (Parkinson & Smith, 1994; van Eijl et al., 2000) and F12 (Zhang et al., 2000; van Eijl et al., 2002) are associated with IEV and CEV membranes and are absent from IMV and EEV. In contrast, proteins A33 (Roper et al., 1996), A34 (Duncan & Smith, 1992), B5 (Engelstad et al., 1992; Isaacs et al., 1992), F13 (Blasco & Moss, 1991) and A56 (Payne & Norrby, 1976) are associated with IEV, CEV and EEV. The A56 protein can also form a complex with VACV protein K2 (Turner & Moyer, 2006) and VACV complement control protein (VCP) (DeHaven et al., 2010) and thereby recruit these proteins to the EEV particle (DeHaven et al., 2011). CEV promotes cell-to-cell spread of virus by the induction of actin tails from the cell surface beneath newly synthesized virions, and EEV promotes the dissemination of virus in cultured cells and in vivo (Smith et al., 2003).

VACV strain Western Reserve (WR) produces new virions by 5–6 h post-infection (p.i.) and the infectious cycle is complete by 12–15 h (Payne & Kristenson, 1979). Despite

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Two supplementary figures are available with the online version of this paper.
this, VACV WR spreads rapidly across susceptible cells at a rate of 1 cell every 1.2 h (Doceul et al., 2010). This rapid spread is not attributable to actin-tail formation from the surface of cells producing new virions (Stokes, 1976; Hiller et al., 1979) or to virus-induced cell motility (Sanderson et al., 1998b; Valderrama et al., 2006) because both of these phenomena are induced at only 5–6 h p.i., too late to explain the rapid spread observed. Furthermore, virus-induced cell motility is a property of only some VACV strains and plaque size does not correlate with induction of cell motility. VACV strain modified virus Ankara (MVA) does not induce cell motility, but can still form large plaques on some cell lines (Drexler et al., 1998; Okeke et al., 2006). Notably, insertion of the F11L gene into MVA restores virus-induced cell motility but makes no difference to the plaque size (Zwilling et al., 2010). Therefore, these phenomena cannot explain how VACV spreads so rapidly.

Instead, rapid VACV spread is due to repulsion of superinfecting EEV particles from the surface of cells expressing proteins A33 and A36 (Doceul et al., 2010). These proteins are expressed both early and late during infection (Parkinson & Smith, 1994; Roper et al., 1996), and are present on the cell surface (Lorenzo et al., 2000; van Eijl et al., 2000) and form a complex (Röttger et al., 1999; Wolff et al., 2001; Ward et al., 2003; Perdigüero & Blasco, 2006). Furthermore, VACV lacking either gene spreads slowly, does not make actin tails and forms a small plaque (Parkinson & Smith, 1994; Roper et al., 1998; Sanderson et al., 1998a). A33 and A36 are sufficient for actin-tail formation upon contact with EEV, because addition of EEV, but not IMV or herpes simplex virus type 1 (HSV-1), to cell lines expressing A33 and A36 induced actin-tail formation from the cell surface to repel EEV particles and accelerate spread to uninfected cells. Consequently, it was shown that EEV can bounce or surf across infected cells to reach uninfected cells without the need to replicate in each cell.

To study this phenomenon further we have investigated: (i) whether expression of A33 and A36 from cells increases the rate of VACV spread (size of plaque); (ii) whether the expression of A33 and A36 can inhibit infection by EEV or IMV, and compared this with the effect of proteins A56 and K2, which also form a complex on the cell surface (Turner & Moyer, 2006) and block IMV entry by binding to components of the IMV entry fusion complex (Wagenaar & Moss, 2007; Wagenaar et al., 2008); and (iii) which proteins on the surface of EEV are needed for interaction with the A33–A36 complex to induce actin-tail formation. Data presented show that plaque size is not affected by the expression of A33 and A36 prior to infection, that the A33–A36 complex does not block infection by IMV or EEV to a significant degree, whereas the A56–K2 complex blocks entry of both viruses, and that the EEV protein B5 is needed for the induction of actin tails from the cell surface.

RESULTS

Effect of A33 and A36 expression in cells on plaque size

Expression of VACV proteins A33 and A36 early during infection is critical for rapid spread (Doceul et al., 2010) and so we wondered whether VACV would spread faster in cells expressing A33/A36 prior to infection. To address this, VACV plaque size was measured in cell lines that expressed either or both of these proteins. HeLa cells expressing A33 and/or A36 were described (Doceul et al., 2010) but these yielded poor VACV plaques that were not easily measurable. Therefore, we created additional cell lines in RK13 and CV-1 cells that expressed A36-v5 and/or A33–HA using lentivirus vectors as described previously (Doceul et al., 2010) and in Methods. Cell lines expressing A33–HA were created first and then these were transduced with vectors expressing A36-v5 and cloned cell lines were isolated as described previously (Doceul et al., 2010). Protein expression was confirmed by immunoblotting with anti-A36 or anti-A33 antibodies (Fig. 1a). Expression levels in these cell lines was comparable to that achieved in the HeLa cells used previously (Doceul et al., 2010) and was slightly less than obtained in cells infected with VACV in the presence of cytosine arabinoside (to eliminate late gene expression, but which increases early gene expression).

To investigate plaque size, monolayers of these cells were infected with VACV strain WR and plaque size was quantified 4 days later. No difference in plaque size was detected between RK13 cells and those expressing A36, A33 or A36–A33 (Fig. 1b). In CV-1 cells expressing A33, there was a slight increase in plaque size compared with parental cells, but no additional increase when A36 was co-expressed (Fig. 1b). This suggests that the small variation in size of plaques is probably attributable to intrinsic differences in the clonal cell lines rather than expression of A33 and A36. Collectively, these data indicate that spread of VACV was not increased in cells constitutively expressing A33 and A36. However, the plaque size of viruses expressing A33 or A36 under only a late promoter or lacking either gene completely was increased on cells expressing A33 or A36, respectively, showing functional complementation (data not shown).

Effect of A33–A36 complex on entry of VACV IMV or EEV

It was clear that some EEV added to cells expressing A33–A36 induced actin tails (Doceul et al., 2010) and are repelled to find new cells to infect, but what proportion of EEV enter the cell or are repelled was unclear. To address this, a recombinant VACV (rVACV) expressing luciferase, vLuc-WR, was constructed using established methodology (Mackett et al., 1984). This virus was used to monitor viral entry/early gene expression by measuring luciferase activity shortly after addition of IMV or EEV to cells. Cell line 293EACK13D expressing A56–K2 was also studied because
expression of A56–K2 at the cell surface inhibits IMV entry by preventing fusion of virus particles with the plasma membrane (Wagenaar & Moss, 2009). A substantial decrease in luciferase activity was detected after addition of vLuc-WR IMV or EEV to 293EACK13D cells compared with the parental HEK293 cells (Fig. 2a), showing that the A56–K2 complex blocks infection by both IMV and EEV. This result is consistent with both virions having to fuse the IMV membrane with the cell membrane. For EEV this occurs after removal of the outer viral membrane upon contact with glycosaminoglycans on the cell surface (Law et al., 2006). In contrast to the A56–K2 complex, A33–A36 did not reduce IMV or EEV entry to a detectable degree (Fig. 2b). Furthermore, virus cores inside cells were detected by electron microscopy within 30 min of infection with EEV irrespective of whether the A33–A36 complex was present (see Fig. S1, available in JGV Online).

Proteins on superinfecting EEV particles needed to induce actin-tail formation

The formation of actin tails at the surface of cells expressing A33 and A36 is triggered by EEV but not IMV or HSV-1 (Doceul et al., 2010). Thus, a specific EEV surface molecule(s) must engage the A33–A36 complex leading to actin-tail formation. To determine which EEV surface protein(s) is needed, VACV mutants lacking A33, A34, A56 or B5 individually were tested. Fresh EEV from these mutants was added to cells expressing A33–A36 and the formation of actin tails was measured. The F13 protein is also associated with EEV but is located on the inner surface of the EEV membrane (Husain et al., 2003) and so

Fig. 1. Ectopic expression of A33 and/or A36 does not affect virus spread. (a) Expression of A33 and/or A36 in the RK13 and CV-1 cell lines. Cell extracts were analysed by immunoblotting with antibodies against A33, A36 and tubulin. (b) Expression of A33 and A36 does not affect VACV plaque size in RK13 and CV-1. Cell monolayers were infected with VACV WR for 4 days and the diameter of 12 plaques was measured (see Methods). The relative plaque size is expressed as a percentage of the size obtained for the parental cell line. Data shown are the mean ± SD, n=3.

Fig. 2. Expression of A33 and A36 does not prevent VACV entry. (a) HEK293 cells and EACK cells (HEK293 cells expressing A56 and K2) were infected with vLuc-WR EEV or IMV. Cells were lysed 1 h after infection and the luciferase activity was determined. (b) The same experiment was performed with the CV-1 v5, A33, A33–A36-7 and A33–A36-8 cell lines. Data are expressed in relative luciferase units (RLU) per 10^3 cells. The results shown are the mean ± SD, n=4, and are representative of three experiments.
is unable to bind an extracellular ligand. Consequently, this mutant was excluded from the analysis. These mutant viruses produce different amounts of EEV (Smith et al., 2002) and so it was necessary to compare the number of actin tails produced against the number of EEV particles bound to the cell surface. EEV were quantified by fixing virions bound to cells, permeabilizing the EEV membrane and staining with an anti-F13 mAb (as described in Methods). Data obtained show that EEV lacking A56 and A33 induced actin tails as efficiently as wild-type EEV (Fig. 3). In contrast, EEV lacking A34 or B5 did not induce actin tails.

A34 is a type II membrane glycoprotein that contains a C-type lectin-like domain in its extracellular domain (Duncan & Smith, 1992) and B5 a type I membrane glycoprotein with an extracellular domain composed of four short consensus repeats (SCRs) (Takahashi-Nishimaki et al., 1991; Engelstad et al., 1992; Isaacs et al., 1992). A34 and B5 interact with each other (Röttger et al., 1999; Earley et al., 2008; Perdiguerò et al., 2008; Roberts et al., 2009) and A34 is required for the incorporation of B5 in EEV (Earley et al., 2008; Perdiguerò et al., 2008; Roberts et al., 2009). Furthermore A34 glycosylation, trafficking and stability depend on B5 (Breiman & Smith, 2010). Given the mutual dependence of A34 and B5, it was impossible to determine from the above data whether A34, B5 or both proteins were required to trigger actin-tail formation. Therefore, mutations in B5 and A34 that influence EEV formation were also investigated.

For A34, a K151E mutation caused enhanced release of EEV (Blasco et al., 1993; McIntosh & Smith, 1996), explaining why VACV strain IHD-J, which contains this mutation, released more EEV than strain WR (Payne, 1979). Using IHD-J EEV, actin-tail formation on cells expressing A33–A36 was normal (Fig. 3). This showed this mutation does not affect actin-tail formation and VACV strains WR and Lister (Doceul et al., 2010), can induce actin tails on cells expressing the A33–A36 proteins.

Many mutations have been described for B5, including deletion of domains, point mutations and domain swaps with other VACV proteins (Herrera et al., 1998; Lorenzo et al., 1998; Mathew et al., 1998, 1999, 2001; Grosenbach et al., 2000; Newsome et al., 2004; Roberts et al., 2009; Breiman & Smith, 2010; Lorenzo et al., 2012). To determine which SCRs were needed for actin-tail formation, EEV made...
by mutant viruses lacking one or more SCR was added to cells and actin tails were quantified (Fig. 4). This showed that SCR4 was needed for actin-tail formation. To address this further we studied a P189S mutation within this domain. Katz et al. (2002) showed that the P189S mutation caused an increased release of EEV by a virus lacking the A36 protein...
et al. (2002) and its introduction into wild-type VACV caused a small-plaque phenotype, increased EEV formation, a failure to induce actin tails from the cell surface of infected cells, and a reduction in virulence (Katz et al., 2003). Later, this mutation was reported to reduce phosphorylation of A36 by src kinases and cause loss of actin-tail formation (Newsome et al., 2004). However, these studies measured actin-tail formation during the exit of newly synthesized virions, rather than during superinfection. To address whether this mutation also affected actin-tail formation during superinfection, a rVACV containing the P189S mutation was constructed (for details see Methods) and the replication and properties of this virus, vB5P189S, were studied (Fig. 5).

Characterization of rVACV vB5P189S

Immunoblotting showed that the P189S mutation did not affect B5 stability, and analysis of plaque size revealed that vB5P189S formed plaques twice as big as those formed by vΔB5R and one-third the size of plaques formed by VACV WR (Fig. 5a, b). Electron microscopy demonstrated that vB5P189S IMV were formed and wrapped normally to form IEV (Fig. 5c), unlike vΔB5R, which is defective in IMV wrapping and therefore produces little EEV (Engelstad & Smith, 1993; Wolfe et al., 1993). vB5P189S also induced virus-tipped actin projections at the cell surface, although these were fewer than on WR-infected cells. To quantify CEV and actin tails, infected cells were stained with anti-B5 or anti-D8 mAb and phalloidin (Fig. 5d, e). vB5P189S produced similar numbers of CEV at 8 and 16 h p.i. to VACV WR (Fig. 5d), but the number of actin tails were reduced (Fig. 5e) and only 25 % of infected cells showed between one and five actin tails. In contrast, with VACV WR >50 % of cells made >20 actin tails per cell (Fig. 5e). This mutation therefore reduces actin-tail formation substantially but does not eliminate it. Lastly, the release of infectious virus into the medium was measured and data are expressed as the percentage of total infectivity (virus present in cells and culture medium) that was released into the medium (Fig. 5f). This showed that the proportion of total virus represented by extracellular virus was enhanced about fourfold compared with the wild type. These data are broadly in agreement with the study of Katz et al. (2003) and the later study from Newsome et al. (2004), except that actin-tail formation is not completely inhibited, just reduced significantly.

B5 P189S reduces actin-tail formation by EEV on cells expressing A33–A36

The ability of vB5P189S EEV to induce actin tails from the surface of cells expressing A33–A36 was then examined. This mutation caused a substantial reduction in actin tails (Fig. 4), but did not eliminate their formation, consistent with the observation on the surface of cells producing new virions (Fig. 5c).

Incorporation of A34 and B5 into EEV particles of mutant viruses

The interdependence of A34/B5 for trafficking and incorporation into EEV made it necessary to check incorporation of these proteins into the mutant EEV. Cells infected by each mutant virus expressed the IMV protein F13 at levels comparable to wild-type WR (Fig. 6a). B5 containing SCR2 was detected by mAb to this domain, and A34 was expressed at similar levels by all viruses except vΔA34 (Fig. 6a). Note that the glycosylation profile of A34 was different in cells infected with this deletion virus (Fig. 6a) as reported previously (Breiman & Smith, 2010). In EEV particles, A34 was present in WR and IHD-J and also the mutants lacking B5 SCRs (Fig. 6b), consistent with another study (Perdiguerro et al., 2008) showing that A34 and B5 can interact through the SCRs and the C-terminal region of B5. B5P189S was also incorporated into EEV,
consistent with this mutation not affecting B5–A34 interaction (Perdiguero et al., 2008). Collectively, these data indicate that the impaired ability of vSCR0, vSCR1, vSCR1–3 and vBP189S EEV to induce actin tails upon addition to cells expressing A33–A36 is caused by the deletion or mutation of B5 SCR4. These data also indicate that CEV-mediated induction of actin tails from the cell surface during virus exit, and actin-tail formation from the surface of cells binding superinfecting EEV each require protein B5.

DISCUSSION

The rapid spread of VACV from cell to cell requires early expression of proteins A33 and A36 on the cell surface, contact of this complex by a CEV/EEV and polymerization of actin to drive superinfecting EEV away towards uninfected cells. In this paper we have investigated additional features of this phenomenon and addressed the following questions: (i) does the constitutive expression of A33–A36 in cell lines make VACV spread faster than it does in normal cells?; (ii) does this complex prevent the cells being infected by the majority of EEV particles?; and (iii) which components of the EEV particle are needed for the interaction with the A33–A36 complex to induce actin polymerization?

Early expression of A33–A36 is crucial for rapid spread, and so, if these proteins were already present on cells before infection, the spread might be accelerated because there would be no delay between infection and when these proteins are present at sufficient level to induce actin tails after contact with superinfecting EEV. However, measurement of plaque size in cells expressing A33–A36 showed no increase over controls. While these data do not show an increase under the conditions tested, the optimal expression level and ratio of A33/A36 remain unknown and it is possible that cells expressing higher levels might be better at inducing actin tails upon addition of EEV. It is also possible that although the A33–A36 complex is sufficient for induction of actin polymerization by EEV, there could be other virus proteins that influence the efficiency of this process, perhaps by stabilizing the complex.

To investigate whether the A33–A36 complex influenced virus entry, an rVACV expressing luciferase under an early promoter was used. Measurement of luciferase expression early after infection with IMV or EEV showed no reduction in cells expressing the A33–A36 complex compared to parental cells. In addition electron microscopy showed virus cores inside the cytosol shortly after infection. Even though the majority of EEV enter cells expressing the A33–A36 complex, the A33–A36 complex is important for rapid spread, and viruses lacking either gene or expressing either gene only late during infection form small plaques (Parkinson & Smith, 1994; Roper et al., 1998; Doceul et al., 2010). Evidently, the rapid spread to uninfected cells could be achieved by only a small percentage of total EEV. In comparison, a cell line expressing the A56–K2 complex (kindly provided by B. Moss, NIH, Bethesda, MD, USA) that binds the IMV entry fusion complex (Wagenaar & Moss, 2009) was also studied. Consistent with previous reports, this cell line blocked infection by IMV, and we show here that it also blocks infection by EEV. The latter result is logical because after an EEV particle has lost its outer envelope either after contacting glycosaminoglycans (GAGs) on the cell surface (Law et al., 2006) or following endocytosis and acidification (Schmidt et al., 2011), the IMV particle must still fuse with the cell membrane and the presence of the A56–K2 complex would block this. Deletion of either K2 or A56 induces a fusogenic plaque phenotype but the plaque size is similar to the wild type, indicating this does not impact on virus spread (Law & Smith, 1992; Zhou et al., 1992; Law et al., 2002). These data indicate that it is the A56–K2 complex that prevents superinfection, and the function of the A33–A36 complex is to promote rapid spread.

To address which EEV protein is needed to engage the A33–A36 complex, mutants lacking EEV proteins were tested for actin polymerization on A33–A36 cells. This showed that A33 and A56 were not required, but A34 and B5 were. Given that the A34 protein is needed for efficient incorporation of B5 into EEV (Earley et al., 2008; Perdiguero et al., 2008; Roberts et al., 2009), the phenotype of the vAA34 EEV could be due to loss of B5. Analysis of additional mutants was consistent with this proposal, and SCR4 of B5 was implicated. This was also supported by analysis of a B5 protein bearing a P189S mutation in SCR4 which had a defect in actin-tail polymerization. B5 and A34 were incorporated into EEV particles of these mutants and so the defect was not attributable to lack of A34. Previously it was shown that the P189S mutant was not able to induce actin-tail formation at the surface of cells producing new virions and that B5 SCR4 was required for actin polymerization (Katz et al., 2003; Newsome et al., 2004). Our data on the induction of actin tails during release by the P189S mutant and during superinfection are broadly in agreement with these reports, although the defect in actin-tail formation is not absolute either during virus exit or during superinfection. It is notable that the A34–B5 complex is not only important for actin-tail induction by CEV or EEV, but it is also important for the disruption of the EEV envelope upon contact with GAGs on the cell surface (Law et al., 2006; Roberts et al., 2009).

Most of the A36 protein is situated in the cytoplasm, whereas A33 has most of its polypeptide outside the cell. So it is likely that A33 acts extracellularly to recognize EEV via B5 SCR4 at the cell surface and A36 is then phosphorylated intracellularly to initiate actin polymerization. A33 and B5 interact with each other but this interaction requires the transmembrane region of B5 (Perdiguero & Blasco, 2006). Data presented here suggest that B5 and A33 might interact even when anchored in different membranes, possibly via the extracellular domains, although direct binding data are needed to confirm this. Interestingly, the X-ray crystal structure of the A33 ectodomain has revealed that A33
contains an unusual C-type lectin like domain (CLTD) that is probably involved in binding ligands (Su et al., 2010).

Lastly, the involvement of A33 in rapid virus spread may help explain other observations. First, antibodies to A33 can help provide protection against orthopoxvirus infection, without neutralizing the EEV particle (Galmiche et al., 1999; Fogg et al., 2004). Second, VACV plaque formation is not inhibited by polyclonal antibody raised against a VACV infection, so VACV can spread from cell to cell in an antibody-resistant manner; but if the A33 protein is absent VACV spread is prevented by antibody (Law et al., 2002). The requirement of A33 to facilitate rapid spread of virus may explain both observations, because binding of antibody to A33 on the cell surface might block spread and therefore reduce the induction of disease. Further, rapid spread of VACV requiring the A33–A36 proteins may explain why spread is normally resistant to antibody and therefore why it becomes sensitive when the A33 protein is absent.

In conclusion, this study provides insight into the mechanism evolved by VACV to repulse superinfecting virions and enhance cell-to-cell spread of the virus. A better understanding of these mechanisms could identify viral targets and lead to the discovery of new viral drugs such as molecules designed to neutralize B5 and A33.

**METHODS**

**Cells and viruses.** BSC-1, HeLa, CV-1 and RK13 cells were grown as described previously (Kerr et al., 1991; Mathew et al., 1999). HEK293 cells expressing A56 and K2 (EACK cells) were described by Wagenaar & Moss (2009). The VACV strains Western Reserve (WR) and International Health Department (IHD)-J (Alcamı´ & Smith, 1992) and A33RHA-forward (5′-CGCGGATCCACATGTAATCTGGAACATCGTATGGGTAGTTCATT-3′) and A33RHA-reverse (5′-GTACACCAGAAAAC-3′) containing BamHI or Ndel sites (underlined) and a haemagglutinin (HA) tag. The PCR product was digested with BamHI and Ndel and ligated into lentivirus vector pdlSurPklB that was derived from pHR-MCS’R’PK and expressing A36-v5 was described earlier (Doceul et al., 2010). A DNA fragment encoding A33–HA was amplified from pcDNA3-A33 with oligonucleotides A33RNA-forward (5′-GGATTCTATCTTCGTTGAC-3′) and A33RNA-reverse (5′-GGATTCTATCTTCGTTGAC-3′) containing BsiEI sites or CEV, respectively, as described by Herrero-Martínez et al. (2005). Cells were permeabilized with Triton X-100 (VWR) when required, blocked in 0.5% BSA and incubated with rat anti-F13 mAb (15B6; Schmelz et al., 1994), rat anti-B5 mAb (19C2; Schmelz et al., 1994) or mouse anti-D8 mAb (AB1.1; Parkinson & Smith, 1994). Secondary Alexa 488- or Alexa 546-conjugated donkey anti-mouse or anti-rat were used to detect bound primary antibody. Actin was visualized with phalloidin labelled with Alexa Fluor 488 or 546 (Molecular Probes). Samples were mounted in Mowiol–DAPI mounting medium. Microscopy was carried out with a Zeiss 510 Meta confocal microscope (Zeiss).

**Spionoculation of EEV and quantification of actin tails.** Fresh EEV were spinoculated onto cells and EEV and actin tails were quantified as described previously (Doceul et al., 2010). The number of cells per coverslip was determined using a Countess automated cell counter (Invitrogen) (n=2) and the number of bound EGFP-positive virions present per cell was counted in five different fields.

**Electron microscopy.** Infected cells were processed as described previously (Doceul et al., 2010) and collected using ANALYSIS version DOCU software (Olympus Soft Imaging Solutions).
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