Roles of vaccinia virus EEV-specific proteins in intracellular actin tail formation and low pH-induced cell-cell fusion

Article *in* Journal of General Virology · July 1998

DOI: 10.1099/0022-1317-79-6-1415 · Source: PubMed

5 authors, including:

Friedrich Frischknecht
Heidelberg University
287 PUBLICATIONS **8,649** CITATIONS

SEE PROFILE

Michael Way
The Francis Crick Institute
312 PUBLICATIONS **14,337** CITATIONS

SEE PROFILE

Michael Hollinshead
University of Cambridge
146 PUBLICATIONS **8,997** CITATIONS

SEE PROFILE

Geoffrey L Smith
University of Oxford
461 PUBLICATIONS **31,429** CITATIONS

SEE PROFILE

Journal of General Virology (1998), **79**, 1415–1425. Printed in Great Britain

Roles of vaccinia virus EEV-specific proteins in intracellular actin tail formation and low pH-induced cell–cell fusion

Christopher M. Sanderson,[1] Friedrich Frischknecht,[2] Michael Way,[2] Michael Hollinshead[1] and Geoffrey L. Smith[1]

[1] Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

[2] European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, 69117 Heidelberg, Germany

During vaccinia virus (VV) morphogenesis intracellular mature virus (IMV) is wrapped by two additional membranes to form intracellular enveloped virus (IEV). IEV particles can nucleate the formation of actin tails which aid movement of IEVs to the cell surface where the outer IEV membrane fuses with the plasma membrane forming cell-associated enveloped virus (CEV) which remains attached to the cell, or extracellular enveloped virus (EEV) which is shed from the cell. In this report, we have used a collection of VV mutants lacking individual EEV-specific proteins to compare the roles of these proteins in the formation of IEV and IEV-associated actin tails and fusion of infected cells after a low pH shock. Data presented here show that p45–50 (A36R) is not required for IEV formation or for acid-induced cell–cell fusion, but is required for formation of IEV-associated actin tails. In contrast, gp86 (A56R), the virus haemagglutinin, is not required for formation of either IEV or IEV-associated actin tails. Data presented also confirm that p37 (gene F13L), gp42 (B5R) and gp22–24 (A34R) are needed for formation of IEV-associated actin tails and for cell–cell fusion after low pH shock. The phenotypes of these mutants were not affected by the host cell type as similar results were obtained in a range of different cells. Lastly, comparisons of the phenotypes of VV strains Western Reserve, ΔA34R and ΔA36R demonstrate that actin tails are not required for low pH-induced cell–cell fusion.

Introduction

Vaccinia virus (VV) is a poxvirus that was used as the vaccine to eradicate smallpox (Fenner *et al.*, 1988) and is the most extensively studied member of the orthopoxvirus genus (Moss, 1996). Like other poxviruses, VV replicates in the cytoplasm using its own enzymes for transcription and DNA replication and produces large, enveloped virions.

Assembly of VV particles is complex and produces two structurally distinct infectious virions termed intracellular mature virus (IMV) and extracellular enveloped virus (EEV). IMV represents the majority of infectious virus and has been the form of virus studied by most investigators due to its abundance, ease of purification and robust structure. IMV and EEV bind to different cellular receptors (Vanderplasschen & Smith, 1997), enter cells by different mechanisms (Vanderplasschen *et al.*, 1998) and EEV, unlike IMV, is resistant to neutralization by antibody (Ichihashi, 1996; Vanderplasschen *et al.*, 1997). IMV particles are formed by the envelopment of the viral genome in membranes that were proposed originally to be synthesized *de novo* (for review see Dales & Pogo, 1981), but more recently were reported to be derived from the intermediate compartment between the endoplasmic reticulum and the Golgi stacks (Sodeik *et al.*, 1993). After maturation, some IMV particles are wrapped by a double membrane (Ichihashi *et al.*, 1971; Morgan, 1976; Payne & Kristensson, 1979) derived from tubular endosomes (Tooze *et al.*, 1993) or the *trans*-Golgi network (Hiller & Weber, 1985; Schmelz *et al.*, 1994) to form an intracellular enveloped virus (IEV). IEV particles move to the cell surface where the outer membrane fuses with the plasma membrane to form cell-associated enveloped virus (CEV) which remains on the cell surface (Blasco & Moss, 1992), or EEV which is released from the cell. The movement of IEV can be assisted by the polymerization of actin tails (Cudmore *et al.*, 1995, 1996), similar to those described for *Shigella*, *Listeria* and *Rickettsia* (for reviews see Tilney & Tilney, 1993; Cossart & Kocks, 1994; Cossart, 1995); however, EEV are still formed without actin tail formation (Wolffe *et al.*, 1997; Mathew *et al.*, 1998). EEV may also be formed by budding of IMV through the plasma

Author for correspondence: Geoffrey L. Smith.
Fax +44 1865 275501. e-mail glsmith@molbiol.ox.ac.uk

0001-5379

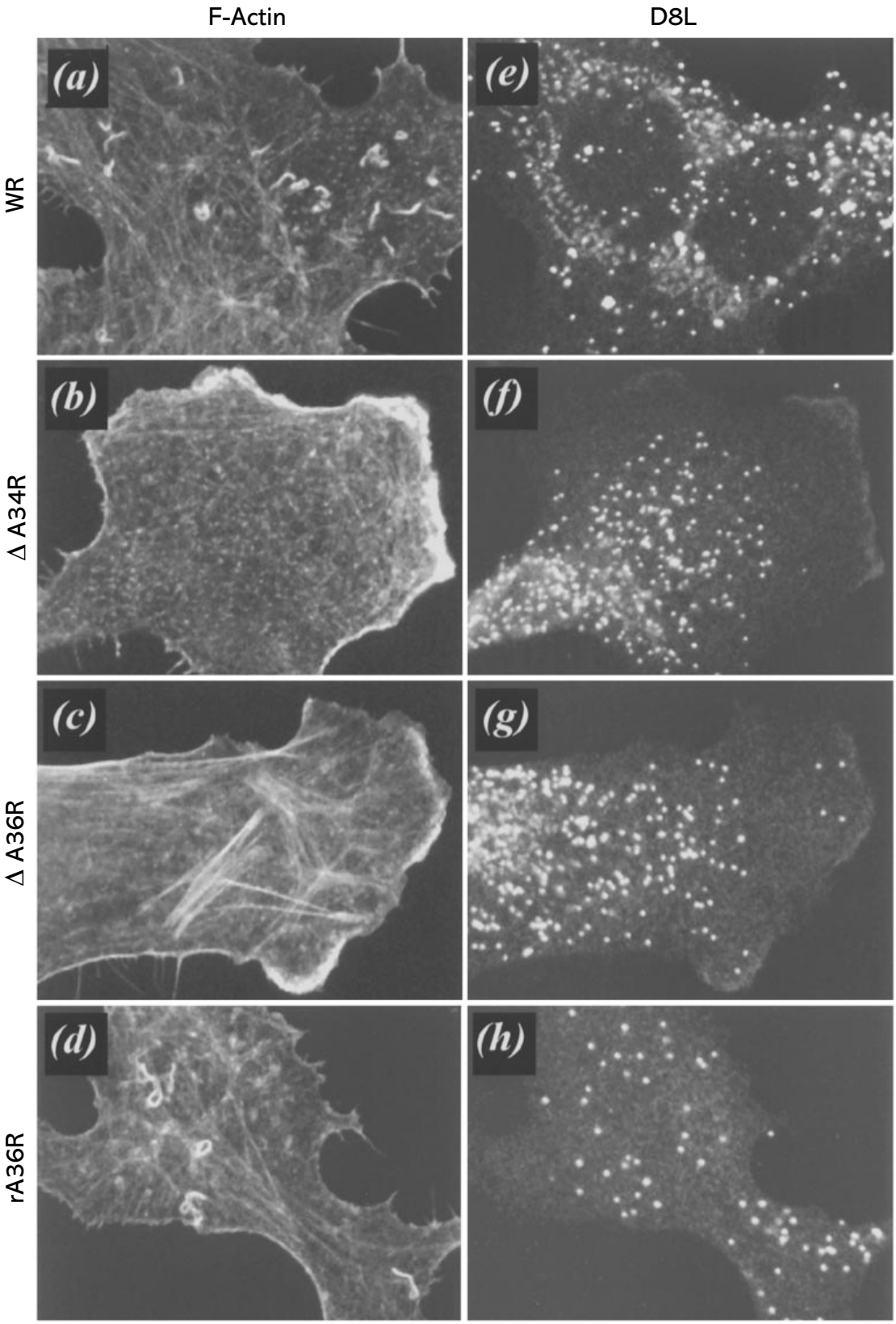

Fig. 1. Distribution of F-actin and VV particles in BS-C-1 cells infected with WR (*a* and *e*), ΔA34R (*b* and *f*), ΔA36R (*c* and *g*) or rA36R (*d* and *h*) at 10 p.f.u. per cell. At 18 h p.i. viral particles were stained with MAb AB1.1 against the VV D8L protein (*e–h*), while TRITC–phalloidin was used to stain F-actin (*a–d*).

membrane, as observed late in infection with the IHD-W strain of VV (Tsutsui, 1983).

Six VV genes have been identified that encode proteins contained within the outer membrane of EEV particles. These are: A56R [haemagglutinin (HA) gp86] (Payne & Norrby, 1976; Shida, 1986), F13L (p37) (Hirt *et al.*, 1986), B5R (gp42) (Engelstad *et al.*, 1992; Isaacs *et al.*, 1992), A34R (gp22–24) (Duncan & Smith, 1992), A36R (p45–50) (Parkinson & Smith, 1994) and A33R (gp23–28) (Roper *et al.*, 1996). None of these proteins (excluding A33R, which has not yet been investigated) affect the formation of IMV, but they have differing affects on IMV wrapping, actin tail formation, EEV release and low pH-induced cell–cell fusion. F13L (Blasco & Moss, 1991) and B5R (Engelstad & Smith, 1993; Wolffe *et al.*, 1993) are required for the wrapping of IMV by intracellular membranes. Viruses lacking A34R (McIntosh & Smith, 1996; Wolffe *et al.*, 1997) or the short consensus repeat domains of B5R (Mathew *et al.*, 1998) enable IEV formation and enhanced release of EEV, but prevent actin tail formation. This suggests that actin tail formation and retention of CEV particles on the cell surface may involve common viral components.

Cell–cell fusion can be induced by brief incubation of VV-infected cells at low pH and requires the p14 protein (gene A27L) that is present on the surface of IMV (Gong *et al.*, 1989, 1990). Fusion also requires the formation of enveloped viruses (IEV, CEV and EEV) since it does not occur with mutant viruses lacking F13L and B5R (Blasco & Moss, 1991; Wolffe *et al.*, 1993). Interestingly, fusion is also blocked in ΔA34R-infected cells (Duncan, 1992; Wolffe *et al.*, 1997), although this virus produces EEV particles (McIntosh & Smith, 1996).

In this study, we have used a collection of mutant viruses lacking individual EEV-specific proteins to study the roles of these proteins in the formation of actin tails and correlate this with the ability to retain CEV particles on the cell surface and cause low pH-induced cell–cell fusion.

Methods

■ **Cells and viruses.** Monkey kidney BS-C-1 cells were grown in Dulbecco's modified Eagle's medium [DMEM with 10% foetal bovine serum (FBS)]. HeLa cells were grown in minimum essential medium (MEM) containing 10% FBS.

The sources of VV strains WR and IHD-J were described previously (Alcamí & Smith, 1992). VV strain WR mutants lacking genes F13L (p37) (Blasco & Moss, 1991), B5R (gp42) (Engelstad & Smith, 1993), A34R (gp22–24) (McIntosh & Smith, 1996) and A36R (p45) (Parkinson & Smith, 1994) have been described previously. A VV strain WR mutant lacking A56R (gp86) was constructed by transient dominant selection (G. L. Smith, unpublished data). For simplicity the mutant viruses are referred to here with names indicating the gene which has been deleted, namely ΔF13L, ΔA34R, ΔA36R, ΔA56R and ΔB5R. Viruses were grown and titrated by plaque assay on BS-C-1 cells as described previously (Mackett *et al.*, 1985).

■ **Immunocytochemistry**

(i) **BSC-1 cells.** Virus was adsorbed for 1 h on ice using 10 p.f.u. per cell. After adsorption, non-adherent viruses were washed away and cells were incubated in MEM containing 2·5% FBS at 37 °C. At the indicated times cells were fixed and processed for immunocytochemistry as described previously (Herzog *et al.*, 1994). Monoclonal antibody (MAb) AB1.1 (diluted 1:300), directed against the IMV surface protein encoded by gene D8L (Parkinson & Smith, 1994), was used for staining VV particles. Bound MAb was revealed with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-mouse IgG (Sigma). F-actin was stained with tetramethylrhodamine B isothiocyanate (TRITC)–phalloidin (Sigma). Images were recorded using a Bio-Rad MRC 1024 confocal laser scanning microscope and processed using Adobe Photoshop software.

(ii) **HeLa cells.** HeLa cells were infected at 1 p.f.u. per cell and processed for immunofluorescence 10 h post-infection (p.i.) as described previously (Cudmore *et al.*, 1995) using MAb C3 directed against the IMV surface protein encoded by gene A27L (Rodriguez *et al.*, 1985) to detect VV particles. Images were collected using a cooled CCD camera with the OpenLab Software (Improvision) and subsequently processed using Adobe Photoshop.

For kinetic analyses of viral infections, BS-C-1 cells were seeded onto glass coverslips to give well isolated cells to facilitate the analyses of virus-induced cell projections (Sanderson *et al.*, 1998). For each virus 30 cells were analysed at each time-point. Viral factories were identified by staining with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) to visualize DNA as described previously (Sanderson *et al.*, 1996). Actin tails were scored qualitatively as the presence or absence of virus particle-associated tails in each cell. For the determination of virus particles on the surface of infected cells, cells were fixed in 4% formaldehyde but were not permeabilized before being stained with rat MAb 19C2 (diluted 1:8) (Schmelz *et al.*, 1994) which recognizes the B5R protein. Bound antibody was detected with FITC-conjugated $F(ab')_2$ rabbit anti-rat IgG (Serotec). Cells were scored positive for virus-induced cell projections only when two or more projections were observed per cell. Images were collected as above.

■ **Electron microscopy.** BS-C-1 cells were infected with ΔA36R at 10 p.f.u. per cell for 24 h, then washed with ice-cold PBS and fixed in 0·5% glutaraldehyde in 200 mM sodium cacodylate (pH 7·4) for 30 min at room temperature. After fixation cells were washed in water and post-fixed in 1% osmium tetroxide and 1·5% potassium ferrocyanide for 60 min at room temperature. Samples were then washed in water, incubated overnight at 4 °C in Mg^{2+}-uranyl acetate, washed in water, dehydrated in ethanol and flat embedded in Epon. Sections were cut parallel to the surface of the dish, lead citrate was added as a contrast agent, and the sections were examined in a Zeiss Omega EM 912 electron microscope.

■ **Low pH-induced cell–cell fusion assay.** Monolayers of BS-C-1 cells were infected at 10 p.f.u. per cell (as indicated) at 37 °C for 1 h, washed three times in MEM containing 2·5% FBS and incubated in fresh MEM containing 2·5% FBS for 20 h at 37 °C. Cells were then washed in PBS (neutral pH), incubated in PBS (pH 5) for 3 min at 37 °C, washed in MEM containing 2·5% FBS and incubated in fresh MEM containing 2·5% FBS at 37 °C for 3 h. Cell morphology was photographed under phase contrast microscopy using an Olympus CK2 microscope.

Results

Analyses of EEV proteins needed for actin tail formation

To investigate the viral proteins required for the polymerization of actin on IEV particles the formation of actin tails

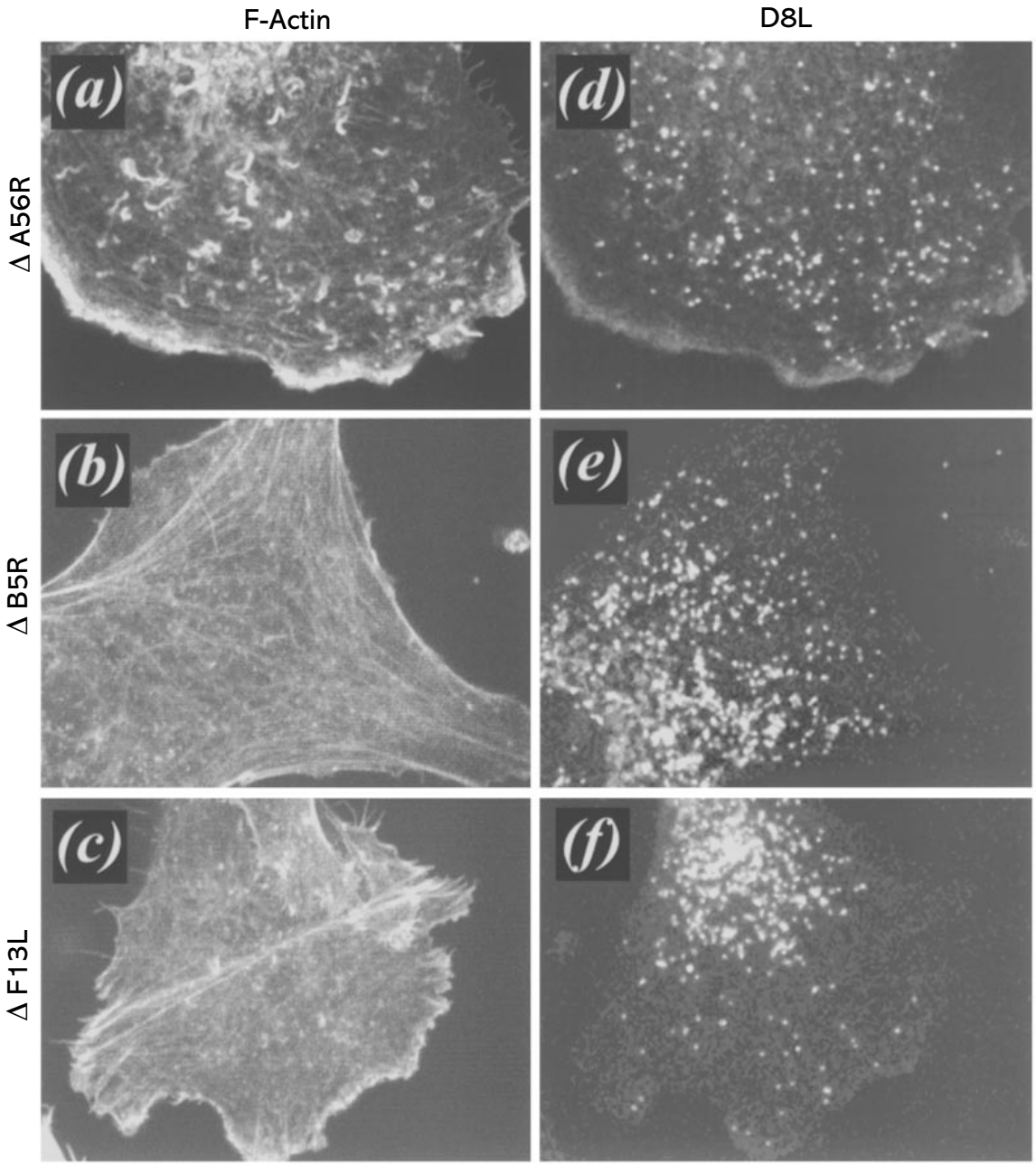

Fig. 2. Distribution of F-actin and VV particles in BS-C-1 cells infected with ΔA56R (*a* and *d*), ΔB5R (*b* and *e*) or ΔF13L (*c* and *f*) at 10 p.f.u. per cell. At 18 h p.i. viral particles were stained with MAb AB1.1 against the VV D8L protein (*d–f*), while TRITC–phalloidin was used to stain F-actin (*a–c*).

was analysed using a collection of VV mutants which lack individual EEV proteins. The formation of VV-associated actin tails in infected BS-C-1 cells was assessed by staining VV particles with MAb AB1.1, directed against the D8L gene product (Parkinson & Smith, 1994), and actin tails with TRITC–phalloidin. Numerous virus particles were visible in cells infected with each virus (Fig. 1*e–h*). However, while actin tails were detected in cells infected with WR virus (Fig. 1*a*), they were absent in cells infected with ΔA34R (Fig. 1*b*) or ΔA36R (Fig. 1*c*). The failure of ΔA34R-infected cells to form actin tails confirmed a recent report (Wolffe *et al.*, 1997). To be certain that the A36R protein was required for actin tail formation, a revertant virus (rA36R) in which the A36R gene had been re-inserted into the deletion mutant was examined. This virus was able to induce actin tail formation (Fig. 1*d*). Analyses of other viruses lacking specific EEV proteins showed that cells infected with ΔB5R (Fig. 2*b*) and ΔF13L (Fig. 2*c*) failed to make actin tails, confirming previous reports (Blasco & Moss, 1992; Cudmore *et al.*, 1995), whereas cells infected with ΔA56R induced actin tail formation (Fig. 2*a*). These data show

that A56R is the only EEV-specific protein examined that is dispensable for induction of actin-tail formation.

As host components are required for the formation of actin tails on intracellular bacteria (Welch *et al*., 1997), it was possible that loss of VV-induced actin tail formation could be due to either the virus genotype or the relative availability of host cell factors. To address this, actin tail formation was examined in several different cell lines infected with the different viruses. Fig. 3 shows images of HeLa cells infected with the mutant viruses and stained with phalloidin to detect F-actin (green signal) and MAb C3 against the VV p14 protein to show virus particles (red signal). As previously noted (Cudmore *et al*., 1995), actin tails are visible with a single virus particle at the tip in WR-infected cells (Fig. 3*a*). Similarly, in cells infected with ΔA56R many virus-associated actin tails are visible (Fig. 3*b*). In contrast, cells infected with viruses ΔF13L, ΔB5R, ΔA34R or ΔA36R failed to make actin tails although numerous virus particles were present throughout the cell (Fig. 3*c–f*). This result matches that observed in BS-C-1 cells and several other cell types examined (C2-C12, CV-1 and TK$^-$143) (data not shown), confirming that it is the virus genotype rather than the host cell that determines if actin tails are formed. However, it was noticeable that the number of actin tails varied with cell type and HeLa cells produced appreciably more intracellular actin tails than BS-C-1 cells (compare Fig. 1*a* and 1*d* and Fig. 2*a* with Fig. 3*a* and 3*b*).

It was possible that the lack of actin tails seen after infection with some viruses was due to altered kinetics of tail formation rather then an absolute inhibition. To test this possibility, the formation of actin tails was examined in cells infected with either WR, ΔA34R, ΔA36R or ΔB5R. In each case 30 cells were examined at 2 h intervals between 8 and 18 h p.i. (Fig. 4). As a control for the efficiency of infection, the formation of cytoplasmic factories containing virus DNA was also examined by staining with DAPI. Cells infected with each virus contained cytoplasmic factories by 10 h p.i., but only WR-infected cells contained virions with actin tails. Although ΔA34R- and ΔA36R-infected cells failed to produce actin tails, most cells contained surface virions by 12 h p.i. The slight delay in the appearance of surface virions compared to WR suggested that although actin tails are not essential for EEV formation they may accelerate the dispersal of particles to the cell surface. It was also evident that not all ΔA34R-infected cells contained surface virions; this might reflect the release of ΔA34R$^-$ virus from the cell surface rather than its retention as CEV (McIntosh & Smith, 1996).

Another parameter that was examined was the formation of extended projections from cells that appear late during infection with VV (Sanderson *et al*., 1998). The formation of these projections requires the polymerization of actin (Sanderson *et al*., 1998) and therefore it was possible that in situations where the polymerization of actin to form intracellular actin tails was inhibited, there might be enhanced formation of cell projections late during infection. However, examination of cells infected with WR, ΔB5R, ΔA34R or ΔA36R showed that there appeared to be no direct correlation between the formation of cellular projections and intracellular actin tails (Fig. 4). Although virus-induced cell projections appeared a little sooner with ΔB5R and ΔA34R, a similar proportion of cells had developed cellular projections by 18 h p.i.

ΔA36R forms both IEV and CEV particles

The production of EEV by ΔA36R is reduced 3–5-fold compared to wild-type (Parkinson & Smith, 1994); however, it was not determined whether the EEV formed was derived from IEV or by direct budding of IMV though the plasma membrane (Tsutsui, 1983). The failure of ΔA36R virions to induce actin tail formation might be due to inefficient formation of IEV particles, or an inability to form actin tails on the IEV surface. Therefore ΔA36R-infected cells were examined carefully by transmission electron microscopy to determine if IEV particles were formed (Fig. 5). Numerous virions that are partly or completely wrapped by intracellular membranes were detected (Fig. 5*a, b*) and CEV particles were observed frequently on the surface of cells. However, these particles were never found on the end of virus-induced microvilli (Fig. 5*c*) as occurs when IEV particles containing actin tails reach the cell surface of WR-infected cells. These data demonstrate that the A36R protein is not required for wrapping of IMV by intracellular membranes but is needed for actin tail polymerization.

Effect of A36R deletion on low pH-induced cell–cell fusion

Viruses which fail to make actin tails (ΔF13L, ΔB5R and ΔA34R) also do not induce low pH-mediated cell–cell fusion (Duncan, 1992; Wolffe *et al*., 1993, 1997) and since ΔA36R also fails to make actin tails, the ability of ΔA36R to induce syncytium formation after low pH shock was examined (Fig. 6). BS-C-1 cells infected with WR, ΔA34R or ΔA36R developed the usual cytopathic effect and did not fuse if incubated at pH 7 (Fig. 6*a–c*). However, if these cultures were briefly exposed to pH 5, cells infected with WR and ΔA36R fused (Fig. 6*d, f*), whereas those infected with ΔA34R did not (Fig. 6*e*). However, the degree of fusion induced by ΔA36R was less extensive than with WR and this difference was more apparent if cells were infected at lower m.o.i. (3 p.f.u. per cell) (data not shown). It appeared, therefore, that there was no complete correlation between the ability of viruses to form actin tails and induce cell–cell fusion after low pH shock.

Discussion

This paper describes the roles of EEV proteins in the formation of IEV and IEV-associated actin tails, and the fusion of infected cells after exposure to low pH. These features have

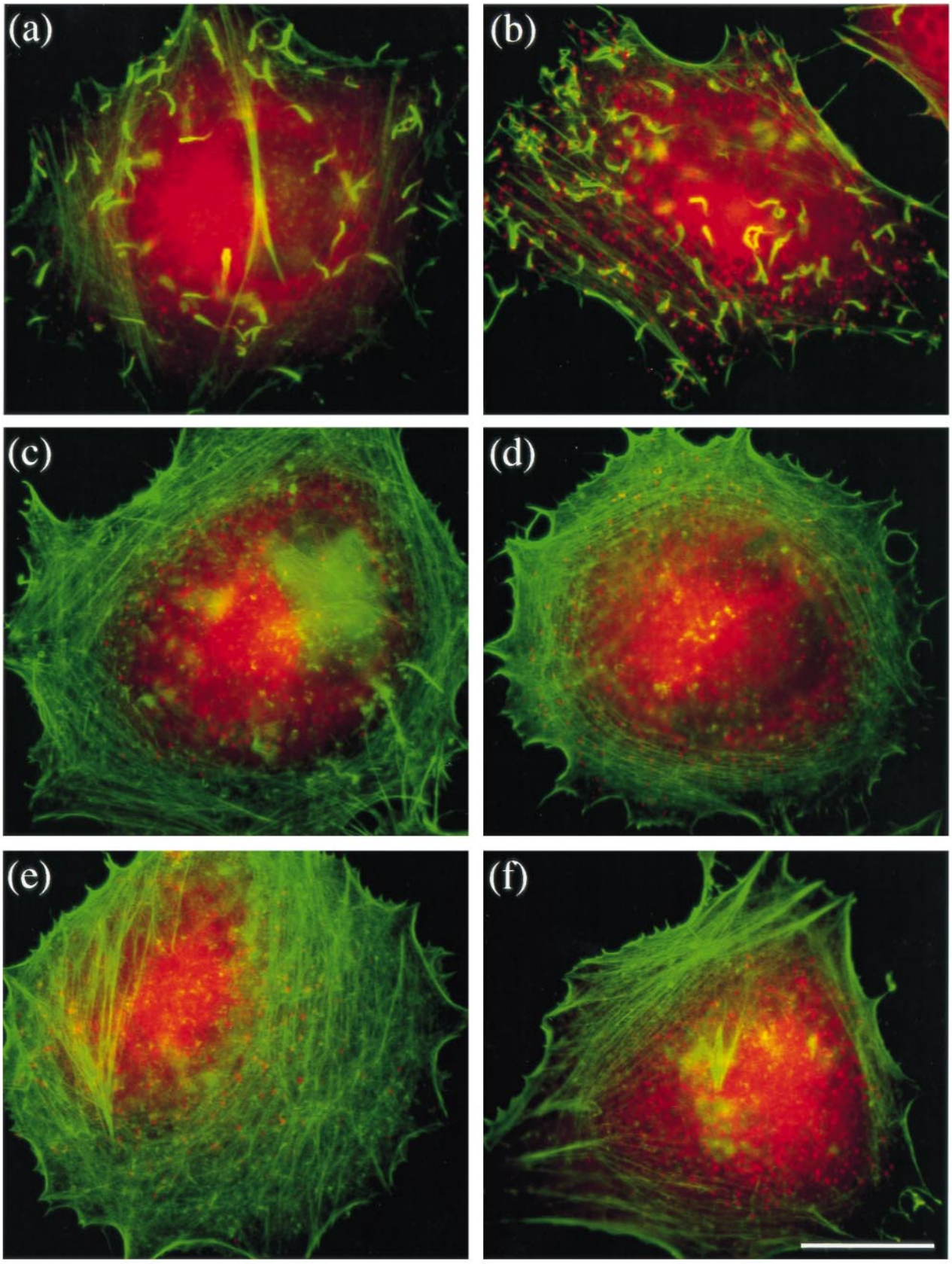

Fig. 3. For legend see facing page.

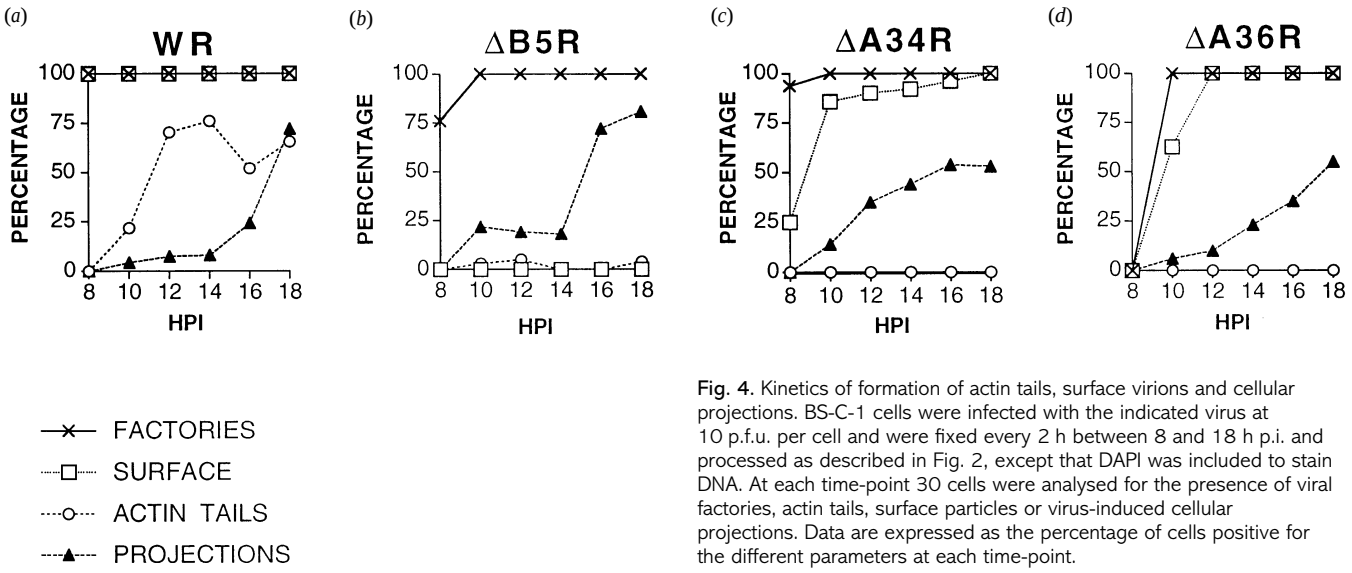

Fig. 4. Kinetics of formation of actin tails, surface virions and cellular projections. BS-C-1 cells were infected with the indicated virus at 10 p.f.u. per cell and were fixed every 2 h between 8 and 18 h p.i. and processed as described in Fig. 2, except that DAPI was included to stain DNA. At each time-point 30 cells were analysed for the presence of viral factories, actin tails, surface particles or virus-induced cellular projections. Data are expressed as the percentage of cells positive for the different parameters at each time-point.

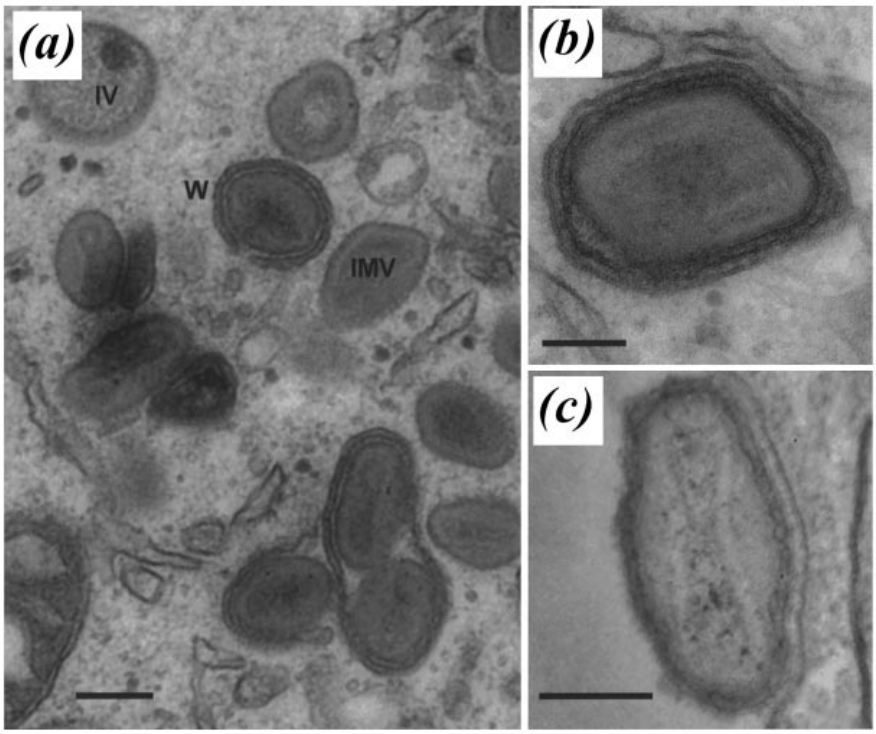

Fig. 5. Transmission electron microscopy of BS-C-1 cells infected with ΔA36R virus. Cells infected with ΔA36R virus at 10 p.f.u. per cell were fixed and processed for electron microscopy 24 h p.i. (*a*) An area of cytoplasm containing immature viral particles (IV), IMV particles (IMV) and particles in the process of wrapping by intracellular membranes (W). (*b*) A fully wrapped IEV particle. (*c*) A CEV particle bound to the outer surface of the plasma membrane. Bars: 200 nm (*a*); 100 nm (*b* and *c*).

been assessed using a collection of virus mutants which lack specific genes.

Actin tails are formed on IEV but not IMV particles and therefore mutant viruses which fail to make IEV do not induce actin tail formation, as was shown first with ΔF13L (Blasco & Moss, 1992; Cudmore *et al.*, 1995). The data presented here confirm this result and demonstrate that ΔB5R is also unable to induce formation of actin tails, consistent with its defect in wrapping of IMV (Engelstad & Smith, 1993; Wolffe *et al.*, 1993). Recently, Wolffe *et al.* (1997) reported that ΔA34R is

Fig. 3. Formation of actin tails in HeLa cells. Cells were infected with WR (*a*), ΔA56R (*b*), ΔF13L (*c*), ΔB5R (*d*), ΔA34R (*e*) or ΔA36R (*f*) at 1 p.f.u. per cell for 10 h. After fixation, cells were stained with a combination of MAb C3 against the A27L gene product on the surface of IMV particles (red signal), and FITC–phalloidin to show the distribution of F-actin (green signal).

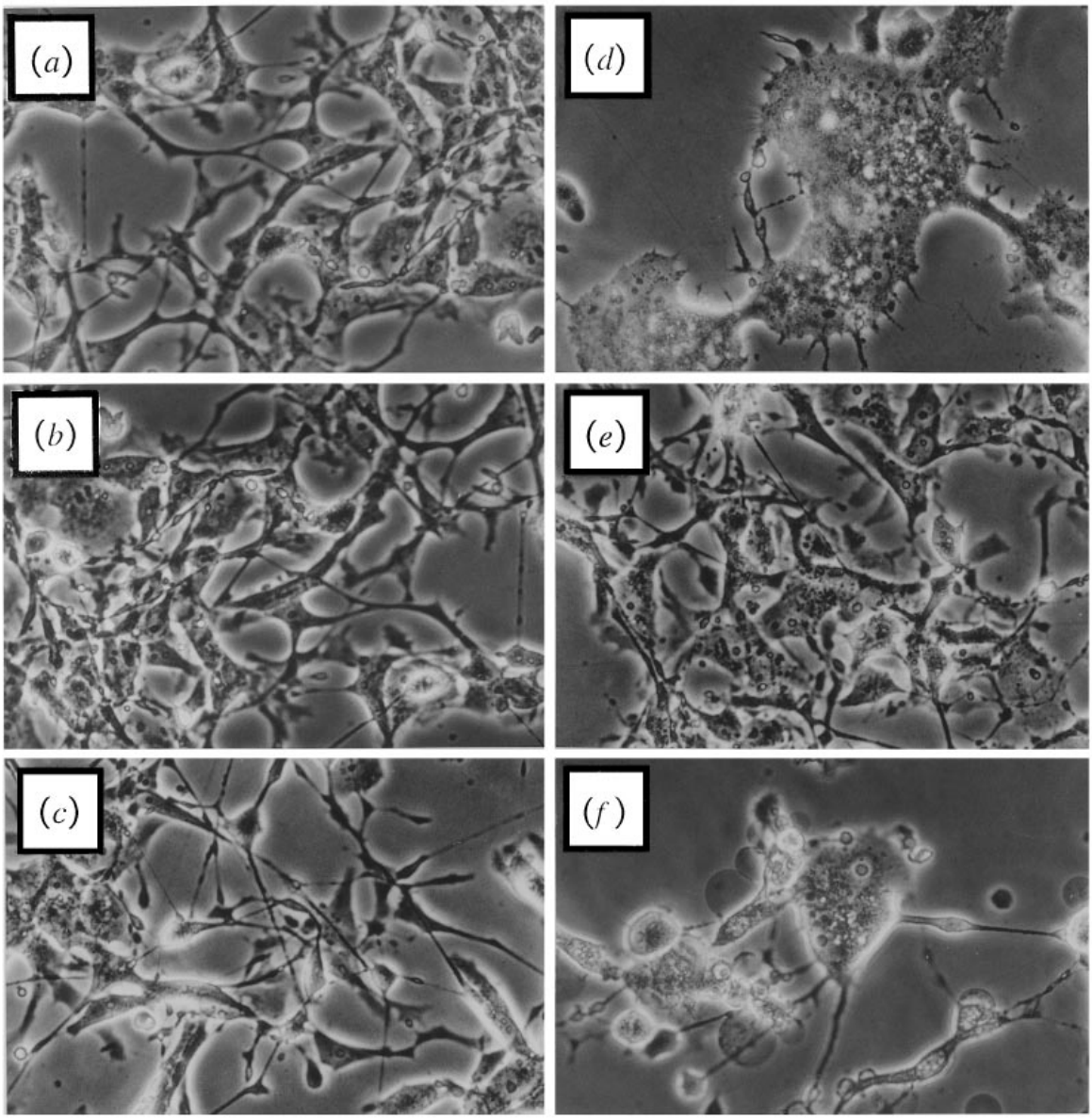

Fig. 6. Virus-induced fusion from within. BS-C-1 cells were infected with either WR (*a* and *d*), ΔA34R (*b* and *e*) or ΔA36R (*c* and *f*) at 10 p.f.u. per cell. At 20 h p.i. cells were incubated for 3 min at pH 7 (*a–c*) or pH 4·5 (*d–f*). Cell morphology was recorded at 24 h p.i. under phase contrast microscopy.

able to form IEV but unable to form actin tails. Here we demonstrate that both ΔA34R and ΔA36R have this phenotype. Virus ΔA36R was known to form reduced levels of EEV compared to wild-type (Parkinson & Smith, 1994) but it was uncertain if this EEV was derived from IEV or from direct budding of IMV through the cell membrane. Electron micrographs presented here demonstrate that ΔA36R does form IEV, but despite this no actin tails are formed. The only EEV protein examined so far which is dispensable for actin tail formation is A56R, the virus HA.

The finding that A36R and A34R are required for actin tail formation is notable given that both proteins are predicted to have only a very short cytoplasmic region which alone seems unlikely to be sufficient to induce the nucleation of actin (Duncan & Smith, 1992; Parkinson & Smith, 1994). This suggests that these proteins may form part of a larger complex of proteins (viral or cellular) or lipoproteins which together facilitate the nucleation of actin on the cytosolic surface of IEV particles. In support of this, viruses expressing a mutated B5R protein lacking one or more short consensus repeats of the extracellular (lumenal) domain are also unable to induce actin polymerization despite enabling IEV formation (Mathew *et al.*, 1998).

The actin tail formation phenotype of the virus mutants

was shown to be determined by the virus genotype rather than the cell type used for infection, since in each cell type examined the phenotype of the mutants was the same. However, it was evident that the number of actin tails varied (HeLa cells gave more than BS-C-1 cells) and this presumably reflects the relative abundance of some cellular factor(s) required for actin polymerization on IEV particles.

The formation of actin tails on IEV particles imparts motility (3 μm/min) (Cudmore *et al*., 1995, 1996) that aids their dispersal through the cell to the plasma membrane. However, the ability of ΔA34R (Wolffe *et al*., 1997) (and Figs 1–3), ΔA36R (Figs 1–3) and the mutants lacking the SCR domains of B5R (Mathew *et al*., 1998) to make EEV despite not making actin tails indicated that actin tails are not essential for IEV dispersal and EEV release. Rather, the function of actin tails might be to speed up this process, as suggested by the kinetic measurements of virus appearance at the cell surface (Fig. 4), and to enable the direct spread of CEV into adjacent cells on the tip of specialized cell surface microvilli (Cudmore *et al*., 1995). Evidently there are at least three ways that EEV can be formed: actin tail-dependent or -independent movement of IEV particles, and direct budding of IMV through the plasma membrane (Tsutsui, 1983). Possibly these have different roles in different situations or cell types.

Is there a correlation between actin tail formation and EEV release or plaque size? Concerning EEV release, the mutants that are unable to form actin tails can produce either enhanced levels of EEV, e.g. ΔA34R (×25-fold) (McIntosh & Smith, 1996) or the B5R SCR domain mutants (×70-fold) (Mathew *et al*., 1998), or reduced levels of EEV, e.g. ΔA36R (3–5-fold lower) (Parkinson & Smith, 1994). Also, IHD-J forms both actin tails and enhanced levels of EEV. Plainly no correlation can be made. However, for plaque size, each mutant which is deficient in actin tail formation produces a small plaque. For viruses which fail to make IEV, e.g. ΔB5R and ΔF13L, this is presumably due to the greatly diminished levels of CEV and EEV, but ΔA34R, ΔA36R and the B5R SCR domain mutant viruses make IEV and EEV yet the plaque size is small. Conversely, when actin tails are made, e.g. ΔA56R, plaque size is normal (G. L. Smith unpublished data). Actin tail formation might therefore be required for normal plaque size and a possible reason might be that actin tails facilitate the direct spread of CEV into adjacent cells on the tip of specialized cell surface microvilli (Cudmore *et al*., 1995). Although enveloped virus is important for virus spread to neighbouring cells (Rodriguez & Smith, 1990; Blasco & Moss, 1991), the absolute level of CEV is not critical for plaque size since the IHD-J virus, which retains little CEV, makes a plaque of similar size to WR which makes high CEV and low EEV.

Concerning cell–cell fusion, the data of Fig. 6 show that there is no correlation between the formation of actin tails and ability to undergo low pH-induced cell–cell fusion: WR virus makes actin tails and induces fusion, ΔA36R induces fusion but does not make actin tails, and ΔA34R neither makes tails nor induces fusion. There also appears to be no direct correlation between cell–cell fusion and the release or retention of the majority of enveloped virus (EEV or CEV): some viruses which produce enhanced levels of EEV, such as IHD-J and the B5R SCR domain mutants, induced cell–cell fusion after low pH shock but another virus, ΔA34R, does not (Blasco & Moss, 1991; Wolffe *et al*., 1997; Mathew *et al*., 1998) (this paper and data not shown). For ΔA34R the inability to induce cell–cell fusion might reflect the diminished infectivity of ΔA34R$^-$ EEV (McIntosh & Smith, 1996) or the requirement for this protein in release of IMV from the wrapping membrane (Wolffe *et al*., 1997). Virus ΔA36R can induce fusion but this is less extensive than seen with the other viruses; possibly this might reflect reduced levels of EEV (Parkinson & Smith, 1994) and/or CEV, which was not examined.

The exact mechanism whereby p14 mediates fusion is uncertain. However, the recent demonstration that low pH disrupts the EEV outer membrane (Vanderplasschen *et al*., 1998) suggests that this treatment would expose p14 present on the surface of the IMV particles contained within CEV particles emerging from infected cells or EEV particles which rebind to the cell surface.

Lastly, we have examined the formation of cellular projections which are produced by extending lamellipodia late during infection (Sanderson *et al*., 1998). The formation of extending lamellipodia requires actin polymerization, and therefore the production of these structures might be influenced by the formation of actin tails on IEV particles since this would affect the pool of actin available for polymerization. However, cell projection formation was found to be unaffected by actin tail formation. Instead the number of these projections that formed late during infection was influenced by the cell type (data not shown).

In summary, we have characterized a collection of virus mutants lacking specific EEV proteins for the formation of IEV, IEV-associated actin tails and cell–cell fusion. Of the five EEV proteins examined, only A56R is dispensable for actin tail formation. Loss of F13L or B5R prevents IEV formation, and loss of A34R or A36R prevents the formation of actin tails on IEV. Actin tail formation is not required for retention of CEV or for acid-induced cell–cell fusion.

Note added in proof. The requirement for the A36R protein in actin tail formation is also reported by E. J. Wolffe, A. S. Weisberg & B. Moss (1998). Role for the vaccinia virus A36R outer envelope protein in the formation of virus-tipped actin-containing microvilli and cell-to-cell spread. *Virology* (in press).

This work was supported by the grants from The Wellcome Trust and the Medical Research Council.

References

Alcamí, A. & Smith, G. L. (1992). A soluble receptor for interleukin-1β encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. *Cell* **71**, 153–167.

Blasco, R. & Moss, B. (1991). Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-Dalton outer envelope protein. *Journal of Virology* **65**, 5910–5920.

Blasco, R. & Moss, B. (1992). Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. *Journal of Virology* **66**, 4170–4179.

Cossart, P. (1995). Actin based bacterial motility. *Current Opinion in Cell Biology* **7**, 94–101.

Cossart, P. & Kocks, C. (1994). The actin-based motility of the facultative intracellular pathogen *Listeria monocytogenes*. *Molecular Microbiology* **13**, 395–402.

Cudmore, S., Cossart, P., Griffiths, G. & Way, M. (1995). Actin-based motility of vaccinia virus. *Nature* **378**, 636–638.

Cudmore, S., Reckmann, I., Griffiths, G. & Way, M. (1996). Vaccinia virus: a model system for actin-membrane interactions. *Journal of Cell Science* **109**, 1739–1747.

Dales, S. & Pogo, B. G. T. (1981). Biology of poxviruses. In *Virology Monographs*, pp. 1–101. Edited by D. W. Kingsbury & H. zur Hausen. Berlin: Springer-Verlag.

Duncan, S. A. (1992). *Analysis of three vaccinia virus genes, one of which is essential for plaque formation*. D. Phil., University of Oxford, Oxford, UK.

Duncan, S. A. & Smith, G. L. (1992). Identification and characterization of an extracellular envelope glycoprotein affecting vaccinia virus egress. *Journal of Virology* **66**, 1610–1621.

Engelstad, M. & Smith, G. L. (1993). The vaccinia virus 42 kDa envelope protein is required for envelopment and egress of extracellular virus and for virulence. *Virology* **194**, 627–637.

Engelstad, M., Howard, S. T. & Smith, G. L. (1992). A constitutively expressed vaccinia virus gene encodes a 42 kDa glycoprotein related to complement control factors that forms part of the extracellular envelope. *Virology* **188**, 801–810.

Fenner, F., Anderson, D. A., Arita, I., Jezek, Z. & Ladnyi, I. D. (1988). *Smallpox and its Eradication*. Geneva: World Health Organization.

Gong, S. C., Lai, C. F., Dallo, S. & Esteban, M. (1989). A single point mutation of Ala-25 to Asp in the 14,000-M_r envelope protein of vaccinia virus induces a size change that leads to the small plaque size phenotype of the virus. *Journal of Virology* **63**, 4507–4514.

Gong, S. C., Lai, C. F. & Esteban, M. (1990). Vaccinia virus induces cell fusion at acid pH and this activity is mediated by the N-terminus of the 14-kDa virus envelope protein. *Virology* **178**, 81–91.

Herzog, M., Draeger, A., Ehler, E. & Small, V. J. (1994). Immunofluorescence microscopy of the cytoskeleton: double and triple immunofluorescence. In *Cell Biology: A Laboratory Manual*, pp. 355–360. San Diego: Academic Press.

Hiller, G. & Weber, K. (1985). Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. *Journal of Virology* **55**, 651–659.

Hirt, P., Hiller, G. & Wittek, R. (1986). Localization and fine structure of a vaccinia virus gene encoding an envelope antigen. *Journal of Virology* **58**, 757–764.

Ichihashi, Y. (1996). Extracellular enveloped vaccinia virus escapes neutralization. *Virology* **217**, 478–485.

Ichihashi, Y., Matsumoto, S. & Dales, S. (1971). Biogenesis of poxviruses: role of A-type inclusions and host cell membranes in virus dissemination. *Virology* **46**, 507–532.

Isaacs, S. N., Wolffe, E. J., Payne, L. G. & Moss, B. (1992). Characterization of a vaccinia virus-encoded 42-kilodalton class I membrane glycoprotein component of the extracellular virus envelope. *Journal of Virology* **66**, 7217–7224.

McIntosh, A. A. G. & Smith, G. L. (1996). Vaccinia virus glycoprotein A34R is required for infectivity of extracellular enveloped virus. *Journal of Virology* **70**, 272–281.

Mackett, M., Smith, G. L. & Moss, B. (1985). The construction and characterization of vaccinia virus recombinants expressing foreign genes. In *DNA Cloning: A Practical Approach*, pp. 191–211. Edited by D. M. Glover. Oxford: IRL Press.

Mathew, E., Sanderson, C. M., Hollinshead, M. & Smith, G. L. (1998). The extracellular domain of vaccinia virus protein B5R affects plaque formation, EEV release and intracellular actin tail formation. *Journal of Virology* **72**, 2429–2438.

Morgan, C. (1976). Vaccinia virus reexamined: development and release. *Virology* **73**, 43–58.

Moss, B. (1996). *Poxviridae*: the viruses and their replication. In *Fields Virology*, pp. 2637–2671. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott–Raven.

Parkinson, J. E. & Smith, G. L. (1994). Vaccinia virus gene A36R encodes a M_r 43–50 K protein on the surface of extracellular enveloped virus. *Virology* **204**, 376–390.

Payne, L. G. & Kristensson, K. (1979). Mechanism of vaccinia virus release and its specific inhibition by N_1-isonicotinoyl-N_2–3-methyl-4-chlorobenzoylhydrazine. *Journal of Virology* **32**, 614–622.

Payne, L. G. & Norrby, E. (1976). Presence of haemagglutinin in the envelope of extracellular vaccinia virus particles. *Journal of General Virology* **32**, 63–72.

Rodriguez, J. F. & Smith, G. L. (1990). IPTG-dependent vaccinia virus: identification of a virus protein enabling virion envelopment by Golgi membrane and egress. *Nucleic Acids Research* **18**, 5347–5351.

Rodriguez, J. F., Janezcko, R. & Esteban, M. (1985). Isolation and characterization of neutralizing monoclonal antibodies to vaccinia virus. *Journal of Virology* **56**, 482–488.

Roper, R. L., Payne, L. G. & Moss, B. (1996). Extracellular vaccinia virus envelope glycoprotein encoded by the A33R gene. *Journal of Virology* **70**, 3753–3762.

Sanderson, C. M., Parkinson, J. E., Hollinshead, M. & Smith, G. L. (1996). Overexpression of the vaccinia virus A38L integral membrane protein promotes Ca^{2+} influx into infected cells. *Journal of Virology* **70**, 905–914.

Sanderson, C. M., Way, M. & Smith, G. L. (1998). Virus-induced cell motility. *Journal of Virology* **72**, 1235–1243.

Schmelz, M., Sodeik, B., Ericsson, M., Wolffe, E. J., Shida, H., Hiller, G. & Griffiths, G. (1994). Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. *Journal of Virology* **68**, 130–147.

Shida, H. (1986). Nucleotide sequence of the vaccinia virus hemagglutinin gene. *Virology* **150**, 451–462.

Sodeik, B., Doms, R. W., Ericsson, M., Hiller, G., Machamer, C. E., van'Hof, W., van Meer, G., Moss, B. & Griffiths, G. (1993). Assembly of vaccinia virus: role of the intermediate compartment between the endoplasmic reticulum and the Golgi stacks. *Journal of Cell Biology* **121**, 521–541.

Tilney, L. G. & Tilney, M. S. (1993). The wily ways of a parasite: induction of actin assembly by *Listeria*. *Trends in Microbiology* **1**, 25–31.

Tooze, J., Hollinshead, M., Reis, B., Radsak, K. & Kern, H. (1993). Progeny vaccinia viruses and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. *European Journal of Cell Biology* **60**, 163–178.

Tsutsui, K. (1983). Release of vaccinia virus from FL cells infected with IHD-W strain. *Journal of Electron Microscopy* **32**, 125–140.

Vanderplasschen, A. & Smith, G. L. (1997). A novel virus binding assay using confocal microscopy: demonstration that the intracellular and extracellular vaccinia virions bind to different cellular receptors. *Journal of Virology* **71**, 4032–4041.

Vanderplasschen, A., Hollinshead, M. & Smith, G. L. (1997). Antibodies against vaccinia virus do not neutralize extracellular enveloped virus but prevent virus release from infected cells and comet formation. *Journal of General Virology* **78**, 2041–2048.

Vanderplasschen, A., Hollinshead, M. & Smith, G. L. (1998). Vaccinia virus extracellular enveloped virus and intracellular mature virus enter cells by different mechanisms. *Journal of General Virology* **79**, 877–887.

Welch, M. D., Iwamatsu, A. & Mitchison, T. J. (1997). Actin polymerization is induced by Arp2/3 protein complex at the surface of *Listeria monocytogenes*. *Nature* **385**, 265–269.

Wolffe, E. J., Isaacs, S. N. & Moss, B. (1993). Deletion of the vaccinia virus B5R gene encoding a 42-kilodalton membrane glycoprotein inhibits extracellular virus envelope formation and dissemination. *Journal of Virology* **67**, 4732–4741.

Wolffe, E., Katz, E., Weisberg, A. & Moss, B. (1997). The A34R glycoprotein gene is required for induction of specialized actin-containing microvilli and efficient cell-to-cell transmission of vaccinia virus. *Journal of Virology* **71**, 3905–3915.

Received 8 December 1997; Accepted 6 February 1998