Replacing the SCR domains of vaccinia virus protein B5R with EGFP causes a reduction in plaque size and actin tail formation but enveloped virions are still transported to the cell surface

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A vaccinia virus (VV) recombinant is described in which the outer envelope of extracellular enveloped virus (EEV), cell-associated enveloped virus (CEV) and intracellular enveloped virus (IEV) is labelled with the enhanced green fluorescent protein (EGFP) derived from Aequorea victoria. To construct this virus, EGFP was fused to the VV B5R protein from which the four short consensus repeats (SCRs) of the extracellular domain had been deleted. Cells infected with the recombinant virus expressed a B5R–EGFP fusion protein of 40 kDa that was present on IEV, CEV and EEV, but was absent from IMV. The recombinant virus produced 2- and 3-fold reduced levels of IMV and EEV, respectively. Analysis of infected cells by confocal microscopy showed that actin tail formation by the mutant virus was reduced by 86% compared to wild-type (WT). The virus formed a small plaque compared to WT, consistent with a role for actin tails in promoting cell-to-cell spread of virus. However, the enveloped virions were still transported to the cell surface, confirming that this process is independent of actin tail formation. Lastly, we compared the mutant virus with a recombinant VV in which the B5R SCR domains were deleted and show that, contrary to a previous report, the plaque size of the latter virus was reduced compared to WT. This observation reconciles an inconsistency in the field and confirms that viruses deficient in formation of actin tails form small plaques.

Introduction

Vaccinia virus (VV) morphogenesis produces four different virions called intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV) (Moss, 1996). Morphogenesis commences in the cytoplasm of infected cells in areas termed virus factories. IMV is the first infectious form of virus and represents the majority of infectious progeny. Once formed, IMV particles are transported from the virus factories to sites where they are wrapped by intracellular membranes (Ichihashi & Dales, 1971; Morgan, 1976; Payne & Kristenson, 1979) derived from the early endosomes (Tooze et al., 1993) or trans-Golgi network (Hiller & Weber, 1985; Schmelz et al., 1994). The transport of IMV requires microtubules and the A27L gene product on the IMV surface (Sanderson et al., 2000). The wrapped particles contain two more membranes than IMV and are called intracellular enveloped virus (IEV). IEV particles were proposed to be transported to the cell surface by the polymerization of actin tails (Cudmore et al., 1995). However, more recently, it was proposed that actin tails are formed only once virions have reached the cell surface (van Eijl et al., 2000) and the transport of IEV to the cell surface was shown to require microtubules (Hollinshead et al., 2001; Ward & Moss, 2001) and the F12L gene product (van Eijl et al., 2002). After IEV particles reach the cell surface the outer membrane fuses with the plasma membrane to form CEV (Blasco & Moss, 1991). Actin tails are then polymerized beneath the CEV particle in a process dependent on the A36R protein (Sanderson et al., 1998; Wolfe et al., 1998; Röttger et al., 1999) to drive virions into surrounding cells or into the extracellular environment to form EEV. With one exception (Herrera et al., 1998), all mutant viruses unable to induce actin tail formation form a reduced size plaque (Cudmore et al., 1995; Wolfe et al., 1997, 1998; Roper et al., 1998; Sanderson et al., 1998, 2000).
EEV is important for long-range virus spread while representing only the minority of total infectivity. The EEV outer envelope contains five proteins absent from IMV, one of which is called B5R.

The B5R gene encodes a 42 kDa type I membrane glycoprotein that is present on the surface of cells and EEV particles (Engelstad et al., 1992; Isaacs et al., 1992), and a 35 kDa form of unknown function that is present in the cell culture supernatant (Martinez-Pomares et al., 1993). The B5R extracellular domain contains four 50–70 amino acid short consensus repeats (SCRs) similar to those found in complement control proteins. Deletion of the B5R protein produced a small plaque and attenuation in vivo (Engelstad & Smith, 1993; Wolffe et al., 1993). Electron and confocal microscopy showed that morphogenesis of the B5R deletion mutant virus (vA5R) was arrested after the formation of IMV particles, so that few IEV, CEV and EEV particles or actin tails were formed (Engelstad & Smith, 1993; Wolffe et al., 1993; Sanderson et al., 1998; Röttger et al., 1999).

Several studies have reported mutagenesis of the B5R protein to investigate the function of particular domains. Deletion of one or more of the SCRs caused inhibition of actin tail formation, a small plaque phenotype and enhanced levels of EEV (Mathew et al., 1998). A similar study, in which all four SCR domains were deleted, reported that the plaque size was normal although actin tail formation was inhibited (Herrera et al., 1998). The transmembrane region of B5R was sufficient for targeting the protein to the trans-Golgi network (Lorenzo et al., 1999; Ward & Moss, 2000) and the transmembrane and cytoplasmic tail of B5R was sufficient to direct incorporation of human immunodeficiency virus gp120 into EEV (Katz et al., 1997). Consistent with this, targeting of B5R to the endoplasmic reticulum (ER) resulted in virions deficient in B5R with a phenotype similar to vA5R infection (Mathew et al., 1999). Further analysis showed that the cytoplasmic tail was dispensable for virus replication in cell culture (Lorenzo et al., 1999; Mathew et al., 2001) but affected the rate of B5R transport to the cell surface and the level of B5R on the surface (Mathew et al., 2001). Additionally, the cytoplasmic tail was reported to mediate retrograde transport of B5R from the plasma membrane to the trans-Golgi network (Ward & Moss, 2000). Recently, the enhanced green fluorescent protein (EGFP) from *Aequorea victoria* has been fused to the VV B5R protein so that processes such as viral morphogenesis can be monitored in real time (Hollinshead et al., 2001; Ward & Moss, 2001). These studies showed that IEV particles move along microtubules at rates of 40–98 µm/min rather than being transported by actin polymerization as is seen with intracellular bacteria (Cudmore et al., 1995, 1996). Here a further characterization of vB5R–EGFP, a recombinant VV in which the B5R SCR domains have been replaced with the EGFP, is reported. We demonstrate that vB5R–EGFP expresses and incorporates a 40 kDa chimaeric B5R–EGFP protein into the membranes used to wrap IMV to form IEV and into virus particles wrapped by those membranes. The vB5R–EGFP virus does not make actin tails but nonetheless virus particles are present on the cell surface, indicating actin-independent transport. Infection with vB5R–EGFP produced a plaque size intermediate between that formed by wild-type (WT) virus and vA5R, and gave reduced levels of EEV. A virus lacking all four SCR domains (Herrera et al., 1998) also showed a reduced plaque size, contrary to a previous report, but consistent with the reduced actin tail formation by that virus.

**Methods**

- **Cells and viruses.** CV-1, TK 143B and RK13 cells were grown in minimum essential medium (MEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco BRL). D98OR and BS-C-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. A VV strain Western Reserve (WR) mutant with 75% of the B5R coding region deleted (vA5R) has been described elsewhere (Engelstad & Smith, 1993). The VV WR mutant lacking SCRs 1–4 (vB5R–ASCRO) was kindly provided by Stuart Isaacs and has been described elsewhere (Herrera et al., 1998). VV infections of cells were carried out in DMEM containing 2% FBS and cells were incubated at 37 °C in a 5% CO₂ atmosphere.

- **Plasmid construction.** Construction of an in-frame mutation in which the four SCR domains of the B5R ORF were replaced with the EGFP ORF was achieved by splicing by overlap extension (Horton et al., 1989) and gene cloning. pSTH2 (Engelstad et al., 1992) contains the entire B5R gene and flanking sequences cloned into pUC13 and was used as a template for a PCR to generate two fragments, which included the 5’ and 3’ ends of the B5R ORF. For details of oligonucleotides used see Hollinshead et al. (2001). Briefly, a 387 bp fragment corresponding to the 5’ end of the B5R gene and including the B5R signal peptide, and a 579 bp fragment encoding the 3’ end of the B5R protein including the stalk region, transmembrane domain and the cytoplasmic tail were fused to the EGFP ORF (Clontech) and assembled into a single gene in which the EGFP ORF replaced the SCR domains of B5R. This was then cloned into pSH17 (Hughes et al., 1991) to form pB5R–EGFP. The fidelity of the chimaeric gene was confirmed by sequencing.

- **Recombinant virus construction.** The recombinant VV vB5R–EGFP was constructed by transient dominant selection (Falkner & Moss, 1990) using the *E. coli* guanine xanthine phosphoribosyltransferase (Ecogpt) gene as the transient selectable marker. CV-1 cells were infected with VV strain WR at 0.1 p.f.u. per cell and transfected with pB5R–EGFP in the presence of Lipofectin (Gibco BRL) as recommended by the manufacturer. Recombinant virus expressing Ecogpt was selected by three rounds of plaque purification in BS-C-1 cells in the presence of mycophenolic acid (MPA). The MPA-resistant virus was then plaque purified three times on D98R cells in the presence of 6-thioguanine to select against virus expressing the Ecogpt gene. Ecogpt-negative virus containing B5R–EGFP was identified by PCR, and stocks were prepared and titrated by plaque assay on BS-C-1 cells.

- **Immunoblotting.** RK13 cells were infected at 10 p.f.u. per cell and cell extracts were prepared 24 h post-infection (p.i.) as described previously (Parkinson & Smith, 1994). Purified IMV was prepared from Dounce-homogenized infected cells by sucrose density-gradient centrifugation as described by Mackett et al. (1985). EEV was collected from the supernatants of infected cells by centrifugation as described by Mathew et al. (1998). After resolution by SDS–PAGE (10% gel), proteins were transferred to nitrocellulose membranes (Towbin et al., 1979) and...
detected by incubation with specific antibodies. To detect B5R or EGFP the membrane was incubated with rabbit α-B5R (Galmine et al., 1999) diluted 1:2000 or mouse mAb JL-8 (Clontech) diluted 1:1000. To confirm that the cells had been infected equally by each of the viruses the membrane was incubated with mouse mAb AB1.1 directed to the VV D8L gene product (Parkinson & Smith, 1994). Bound antibodies were detected by anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Amersham) diluted 1:1000. Membranes were incubated with the ECL Western blotting detection kit (Amersham) as per the manufacturer’s instructions and exposed to X-ray film (Kodak). Images were collected and processed using Precision Pro Scan HP and Adobe Photoshop software.

- **Plaque phenotype.** The plaque phenotype was examined on BS-C-1 cells under semi-solid (MEM/1.5% carboxymethylcellulose/2.5% FBS) or liquid (MEM/2.5% FBS) overlays. At 3 or 4 days p.i. the medium was removed and the cell monolayers were stained with 0.2% crystal violet in 15% ethanol. Images were collected and processed using Precision Pro Scan HP and Adobe Photoshop software.

- **Infectivity assays.** RK13 cells were infected at 10 p.f.u. per cell and the culture supernatant and infected cells were harvested at 24 h.p.i. The titre of infectious virus in the culture supernatant was measured by clarifying the supernatant fraction via low speed centrifugation (2000 r.p.m., 10 min; Beckman GRP bench top centrifuge) followed by plaque assay on BS-C-1 cells in the presence or absence of mAb 2D5 (Ichihashi, 1996) to neutralize IMV as described previously (Law & Smith, 2001). Virus infectivity present in cells was measured by scavenging the monolayer into PBS, combining this with the pellets obtained from clarifying the culture supernatant (see above), followed by three cycles of freeze–thawing before plaque assay on BS-C-1 cells.

- **Immunofluorescence.** Cells growing on glass coverslips (Chance Proper) were infected at 1 or 10 p.f.u. per cell. At the indicated time p.i. cells were fixed in 4% paraformaldehyde (PFA) in 250 mM HEPES for 20 min on ice. The cells were then blocked and permeabilized in PBS containing 0.1% Triton X-100 and 10% FBS for 30 min. MAbbs 15B6 (Hiller & Weber, 1985) and AB1.1, which recognize the F1L and D8L proteins, respectively, were used to detect virus particles. Bound mAbs were detected by either a fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG antibody, diluted 1:50, or a tetramethylrhodamine B isothiocyanate (TRITC)-conjugated donkey anti-mouse IgG antibody, diluted 1:100 (both from Jackson ImmunoResearch Laboratories). Alternatively, cells were incubated with rabbit α-B5R (Galmine et al., 1999), diluted 1:200, or mouse mAb JL-8, diluted 1:100, at 37 °C for 1 h prior to fixation in 4% PFA. Fixed cells were then blocked and permeabilized in PBS containing 0.1% saponin and 10% FBS for 30 min. Bound mAbs were detected by either a TRITC-conjugated donkey anti-rabbit IgG antibody, diluted 1:100, or TRITC-conjugated donkey anti-mouse IgG antibody, diluted 1:100, (both from Jackson ImmunoResearch Laboratories). TRITC–phalloidin (Sigma) was used to stain for F-actin. 4′,6-Diamino-2-phenylindole (DAPI) was added to the mounting medium to stain for DNA. Cells were analysed using a Zeiss LSM 510 confocal laser-scanning microscope. Images were collected and processed using LSM 510 acquisition and Adobe Photoshop software.

**Results**

**Expression of B5R–EGFP**

The construction of pB5R–EGFP and isolation of vB5R–EGFP are described in Methods and elsewhere (Hollinshead et al., 2001). The genome of vB5R–EGFP was analysed by PCR using oligonucleotides that either flank the B5R gene, or amplify the EGFP ORF, or amplify only SCR domains 2–4 of B5R. The genomes of WT WR and vΔB5R were analysed as controls in parallel. PCR using primers that flank the B5R gene produced DNA fragments of 1·6, 1·7 and 0·9 kb for WT, vB5R–EGFP and vΔB5R, respectively, whereas primers specific for EGFP produced a DNA fragment of 0·7 kb for vB5R–EGFP and no product for the other viruses. Finally, reactions using primers specific for the SCR domains of B5R produced a DNA fragment of 0·5 kb for WT, and no product for the other viruses lacking the SCR domains (supplementary data available in [GV Online (http://vir.sgmjournals.org)]). The sizes of these DNA fragments were consistent with the predicted sizes of 1·670, 1·620, 0·912, 0·714 and 0·493 kb and this confirmed that the chimaeric B5R–EGFP gene was inserted into the B5R locus. The structure of the B5R locus of this virus and the other recombinant viruses used in this study are shown in Fig. 1. The B5R–EGFP chimaeric protein was expected to have a size similar to that of the native B5R protein because 222 amino acids of B5R had been replaced by 238 amino acids of EGFP.

The expression of B5R–EGFP was investigated by immunoblotting infected cell lysates, purified IMV preparations and EEV prepared from culture supernatants (Fig. 2). In contrast to the 42 kDa B5R protein produced by WT-infected cells, infection with vB5R–EGFP resulted in a 40 kDa protein that was detected with a mouse mAb directed to GFP (Fig. 2a) and a-B5R rabbit antiserum directed to the stalk region of B5R (Fig. 2b). After longer exposure using the latter antiserum some lower molecular mass bands were seen (data not shown) as detected with the anti-GFP antibody in Fig. 2b. The B5R–EGFP protein was not detected in association with purified IMV but was expressed and incorporated into EEV, showing that the targeting signals within the transmembrane and cytoplasmic tail functioned correctly. As a control, mouse mAb AB1.1 detected similar levels of D8L in infected cell extracts and in each type of virion. The higher molecular mass species present in the WT B5R and the B5R–EGFP proteins probably represents oligomeric forms of these proteins noted previously (Engelstad et al., 1992; Isaacs et al., 1992). The increased mobility of the B5R–EGFP protein compared to the WT B5R protein is likely to be a consequence of different post-translational modification because, whereas WT B5R contains three potential N-linked glycosylation sites within SCR 2
B5R–EGFP is incorporated into virus particles during infection with vB5R–EGFP

The immunoblotting data suggested that the B5R–EGFP protein was incorporated into enveloped virus particles, and therefore this was investigated further by analysis of the distribution of B5R–EGFP in infected cells. Cells were infected with vB5R–EGFP and analysed by confocal microscopy. In Fig. 3 a peripheral region of a cell including a cellular projection (arrow) is shown. Many particulate structures were visible that were positive for EGFP (Fig. 3b). To investigate if these were virions, the infected cells were also stained with DAPI to detect virus DNA within virus particles (Fig. 3a). Merging these images showed that the majority of EGFP-positive structures were also positive for DNA and therefore represented virions (Fig. 3c). The few EGFP-positive and DAPI-negative structures may represent intracellular membranes that have not wrapped an IMV particle. Many of the B5R–EGFP particles (Fig. 3e) also co-localized with another IEV, CEV and EEV protein, F13L (Hiller & Weber, 1985) (Fig. 3d, f). Not all the EGFP-positive structures were positive for F13L because B5R is a glycoprotein that is co-translationally inserted into the ER before transport to the Golgi, whereas F13L associates with membranes due to acylation and so is transported to the Golgi via a different mechanism. These data are consistent with immunoelectron microscopy that showed that B5R–EGFP was incorporated into IEV and CEV, but not IMV, particles (Hollinshead et al., 2001).

B5R–EGFP is exposed on the surface of infected cells

The location of B5R–EGFP was examined further in infected BS-C-1 cells that were either stained with rat mAb 19C2, directed to B5R, mouse mAb JL-8, which recognizes EGFP, or by virtue of the EGFP fluorescence (Fig. 4). At 12 h p.i. cells were fixed and permeabilized prior to addition of the antibody to reveal the distribution of B5R and B5R–EGFP within the infected cell (Fig. 4a, b). Alternatively, cells were incubated with antibody before fixation and permeabilization and this showed the presence of B5R and B5R–EGFP on the cell surface (Fig. 4c, d). Cells infected with WR WT (Fig. 4a, c) showed the characteristic B5R staining of perinuclear areas (probably Golgi) and intracellular and surface virus particles. A similar profile was seen with vB5R–EGFP (Fig. 4b, d), confirming that these virions were transported to the cell surface. Note the clustering of virions near cellular projections (arrows). As a control, infection with vΔB5R failed to show staining because the antigen recognized by this antibody is absent (data not shown).

Plaque phenotype of vB5R–EGFP

Mutations in the SCR domains of the B5R protein were reported to have variable effects on the virus plaque size...
(Herrera et al., 1998; Mathew et al., 1998) and therefore we investigated the plaque phenotype of vB5R–EGFP. In BS-C-1 cells, vB5R–EGFP (Fig. 5c) gave a plaque size intermediate between that of WT WR (Fig. 5a) and vΔB5R (Fig. 5d). This was consistent with the phenotype of VV mutants in which 1, 2 or 3 of the SCR domains had been deleted (Mathew et al., 1998). However, W-B5RΔSCR\textsubscript{1-4}, a VV WR mutant lacking all four SCR domains, was reported to form a plaque of a size equivalent to WT (Herrera et al., 1998). This result was different to the phenotype reported for other SCR mutants and vB5R–EGFP reported here, and therefore we also investigated the size of W-B5RΔSCR\textsubscript{1-4} in parallel with the other viruses (Fig. 5b). Contrary to the previous report, we found that the plaque phenotype of W-B5RΔSCR\textsubscript{1-4} was smaller than WT, a phenotype consistent with other mutants in which SCR domains had been deleted.

The above result also resolved an inconsistency in the field. Hitherto, all VV mutants in which IEV or EEV proteins had been mutated or deleted, except W-B5RΔSCR\textsubscript{1-4}, had a plaque size that could be correlated with the ability of the virus to induce actin tail formation. Deletion of F13L, B5R, A34R, A36R, A33R or F12L all reduced plaque size due to the reduction or inhibition of actin tail formation (introduction). The only exception to this rule had been the W-B5RΔSCR\textsubscript{1-4} mutant that was reported to form a normal plaque size but not to make actin tails. However, the plaque phenotype in our hands was consistent with the reported inhibition of actin tail formation. In view of this observation, we re-investigated actin tail formation by this virus and the vB5R–EGFP mutant described here (Fig. 6). Cells were infected with these viruses and the number of virus-tipped actin tails was quantified at 14 h p.i. Actin tails were revealed by staining cells with TRITC–phalloidin and virions were stained with mAb AB1.1 directed against the D8L gene product. The number of virus-tipped actin tails in 10 cells was counted from confocal images of infected cells. Compared to WR-infected cells, the number of actin tails seen in cells infected with vΔB5R, vB5R–EGFP and W-B5RΔSCR\textsubscript{1-4} was reduced by 98%, 86% and 88%, respectively. The more severe inhibition of actin tail formation by the deletion mutant vΔB5R is consistent with this virus
having the smallest plaque phenotype. Mutants vB5R–EGFP and W-B5RΔSCR produced more actin tails than the deletion mutant, consistent with these mutants having a slightly increased plaque size.

**Growth properties of vB5R–EGFP**

The plaque phenotypes of WR, vΔB5R, vB5R–EGFP and W-B5RΔSCR were also investigated under liquid overlay conditions.
Fig. 5. Plaque size (a–d) and comet formation (e–h) resulting from infection of BS-C-1 cells with WR WT (a, e), W-B5R∆SCR1-4 (b, f), vB5R-EGFP (c, g) and vΔB5R (d, h). Cells were incubated under semi-solid (a–d) or liquid (e–h) medium, for 3 or 4 days, respectively.

Fig. 6. Virus-tipped actin tail formation. At 14 h p.i. with the indicated viruses, VV particles were identified by mAb AB1.1, which recognizes D8L, or by virtue of the EGFP while TRITC-phalloidin was used to detect F-actin. Samples were analysed by confocal microscopy and reconstructed Z-series of images of different cell sections were examined. The number of virus-tipped actin tails per cell ± SEM (n = 10) is shown.

(Fig. 5e–h). This showed that vB5R–EGFP and W-B5R∆SCR1-4 formed comets and that for W-B5R∆SCR1-4 these were more pronounced than WR. The formation of comets is attributable to release and unidirectional spread of EEV and virus strains that produce more EEV form more pronounced comets. This suggested that vB5R–EGFP produced EEV at similar levels to WR and W-B5R∆SCR1-4 released more EEV than WR, as noted earlier (Herrera et al., 1998). This was investigated further for vB5R–EGFP.

BS-C-1 cells were infected with 10 p.f.u. per cell and the levels of intracellular and extracellular virus were determined at 24 h p.i. Under these conditions, the infectious virus titre in cells infected with vB5R–EGFP was reduced approximately 2-fold compared to WR, and the titre of virus in the supernatant was reduced 3-fold compared to WR (Fig. 7a). Therefore, the proportion of total infectivity that was released into the culture medium was similar for WR (0–21%) and vB5R–EGFP (0–14%). In comparison, only 0–026% of total infectivity was released from cells infected by vΔB5R, consistent with the previous report that the titre of extracellular virus produced by vΔB5R was reduced approximately 10-fold compared to WT (Engelstad & Smith, 1993). The result with vB5R–EGFP contrasted with other mutants in which the SCR domains had been deleted, where there was a 10- to 50-fold increase in EEV (Herrera et al., 1998; Mathew et al., 1998), but was in accord with another report where the extracellular domain of the B5R protein was replaced with coding sequences from the VV haemagglutinin and that resulted in decreased levels of EEV (Mathew et al., 2001).

To determine the proportion of virus in the supernatant that was EEV, rather than IMV that had been released due to
Growth of vB5R–EGFP in tissue culture. (a) The titre of infectious virus associated with infected cells (shaded bars) or present in the culture supernatant (hatched bars) 24 h.p.i. with the indicated viruses was determined by plaque assay in triplicate. Data shown are ±SEM (n = 3). (b) The percentage of infectious virus present in the culture supernatant that was resistant to the IMV-neutralizing mAb 2D5 was also determined by plaque assay in triplicate ±SEM (n = 3).

Discussion

A characterization of a VV mutant in which EGFP replaced the SCR domains of B5R is described. The B5R–EGFP fusion protein was expressed in infected cells as a 40 kDa protein and was incorporated into IEV, CEV and EEV particles, but was absent from purified IMV. The plaques formed by vB5R–EGFP were smaller than those formed by WT virus and, consistent with this, the formation of virus-tipped actin tails was reduced compared with WR. Nonetheless, enveloped virions were transported to the cell surface and EEV was released from the cell.

Katz et al. (1997) showed that fusion of the B5R transmembrane and the cytoplasmic tail to HIV gp120 was sufficient to localize the protein to the wrapping membranes and EEV particles. Consistent with this, immunofluorescence showed that the B5R–EGFP protein co-localizes with particulate intracellular structures that were shown to be enveloped virus particles because they contained DNA and the F13L protein. Like WT B5R, B5R–EGFP is also present in the Golgi and on the cell surface. Together, this demonstrates that B5R–EGFP is incorporated into the wrapping membranes and subsequently into IEV, CEV and EEV particles. The incorporation of B5R–EGFP into virus particles has been investigated by immuno-electron microscopy (Hollinshead et al., 2001).

Actin tail formation by VV is inhibited in mutants in which one or more of the SCR domains of B5R are missing (Herrera et al., 1998; Mathew et al., 1998). In agreement with this observation, actin tail formation by vB5R–EGFP was reduced 86% compared with WR. Nonetheless, CEV particles were detected on the surface of infected cells by immunofluorescence (here) and electron microscopy (Hollinshead et al., 2001). This supports the view that IEV particles utilize microtubules (Hollinshead et al., 2001; Ward & Moss, 2001) rather than the polymerization of actin (Cudmore et al., 1995) for their transport from the site of wrapping to the cell surface.

Consistent with the reduction in actin tail formation, the cell-to-cell spread of vB5R–EGFP was diminished resulting in a smaller plaque phenotype than those formed by WT VV. Another B5R mutant virus in which all the SCR domains were deleted, W-B5RASCRΔ–%, was reported to form a plaque phenotype comparable to WT (Herrera et al., 1998). However, in this study, we found that W-B5RASCRΔ–% had a small plaque tail formation (Mathew et al., 1998) and W-B5RASCRΔ–% now fits with the phenotype of all other VV mutants that have diminished actin tail formation in having a reduced plaque size.

The titres of IMV and EEV made by vB5R–EGFP were reduced 2- and 3-fold compared to WT, but the proportion of
virus released into the supernatant was similar. This contrasted with some other SCR mutants (Herrera et al., 1998; Mathew et al., 1998) that formed 10- to 50-fold more EEV. However, unlike those mutants where B5R sequences were just deleted, the vB5R–EGFP mutant has B5R sequences fused to a foreign protein sequence, and previously where the SCR domains of the B5R protein were fused to the extracellular domain of the vv haemagglutinin, the formation of EEV was reduced (Mathew et al., 2001).

In summary, infection with vB5R–EGFP, a virus in which the SCR domains have been replaced with EGFP, resulted in a significant decrease in actin tail formation and consequently a small plaque phenotype. Furthermore, infection with W-B5RΔSCR1–4, a vv mutant lacking the SCR domains of B5R, also gives a small plaque phenotype, contrary to a previous report. These observations reinforce the view that the ability of vv to spread efficiently from cell to cell is dependent on the formation of actin tails. Lastly, the vB5R–EGFP virus is a useful tool for the study of vv morphogenesis, egress, re-entry and spread. This mutant has been used previously to show that IEV particles move to the cell surface associated with microtubules (Hollinshead et al., 2001). In the future, this mutant may be useful to study the interactions between EEV and the host cell during virus binding and re-entry in vitro, and virus infection in vivo, as has been described recently for the spatial-temporal imaging of bacterial infections (Zhao et al., 2001).

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