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Article *in* Journal of General Virology · February 2002

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The vaccinia virus F12L protein is associated with intracellular enveloped virus particles and is required for their egress to the cell surface

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The vaccinia virus (VV) F12L gene encodes a 65 kDa protein that is expressed late during infection and is important for plaque formation, EEV production and virulence. Here we have used a recombinant virus (vF12LHA) in which the F12L protein is tagged at the C terminus with an epitope recognized by a monoclonal antibody to determine the location of F12L in infected cells and whether it associates with virions. Using confocal and electron microscopy we show that the F12L protein is located on intracellular enveloped virus (IEV) particles, but is absent from immature virions (IV), intracellular mature virus (IMV) and cell-associated enveloped virus (CEV). In addition, F12L shows co-localization with endosomal compartments and microtubules. F12L did not co-localize with virions attached to actin tails, providing further evidence that actin tails are associated with CEV but not IEV particles. In vΔF12L-infected cells, virus morphogenesis was arrested after the formation of IEV particles, so that the movement of these virions to the cell surface was inhibited and CEV particles were not found. Previously, virus mutants lacking IEV- or EEV-specific proteins were either unable to make IEV particles (vΔF13L and vΔB5R), or were unable to form actin tails after formation of CEV particles (vΔA36R, vΔA33R, vΔA34R). The F12L deletion mutant therefore defines a new stage in the morphogenic pathway and the F12L protein is implicated as necessary for microtubule-mediated egress of IEV particles to the cell surface.

Introduction

The study of vaccinia virus (VV) morphogenesis is complicated by the formation of four different forms of virion: intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell associated enveloped virus (CEV) and extracellular enveloped virus (EEV) (Appleyard *et al.*, 1971; Ichihashi *et al.*, 1971; Blasco & Moss, 1992). The CEV and EEV particles are structurally indistinguishable, but whereas CEV is retained on the surface of the infected cell, EEV is released. EEV is important for the long-range spread of virus in cell culture (the formation of comet-shaped plaques) (Appleyard *et al.*, 1971) and *in vivo* (Payne, 1980). CEV is needed for the efficient cell-to-cell spread of virus (Blasco & Moss, 1992) and is driven by a growing actin tail (Cudmore *et al.*, 1995; van Eijl *et al.*, 2000; Hollinshead *et al.*, 2001) into surrounding cells. IMV represents the majority of infectious progeny, but is retained within the cell until cell lysis. IMV particles are sensitive to complement (Vanderplasschen *et al.*, 1998), and are efficiently neutralized by antibody. However, IMV particles are physically robust and are therefore well suited to mediate virus spread between hosts. Lastly, IEV particles are formed from IMV by wrapping with a double layer of membrane derived from either early endosomes (Tooze *et al.*, 1993) or *trans*-Golgi network (Schmelz *et al.*, 1994). IEV move to the cell surface in a process dependent on microtubules (Hollinshead *et al.*, 2001; Ward & Moss, 2001) rather than on actin tails as proposed originally (Cudmore *et al.*, 1995). At the cell surface the outer IEV membrane fuses with the plasma membrane to produce a CEV, which may either be retained or released as EEV (Blasco & Moss, 1991). For a recent diagram of VV morphogenesis see Hollinshead *et al.* (2001).

Five proteins have been identified as specific to the EEV particles and absent from IMV. These are F13L (Hirt *et al.*, 1986), B5R (Engelstad *et al.*, 1992; Isaacs *et al.*, 1992), A33R (Roper *et al.*, 1996), A34R (Duncan & Smith, 1992) and A56R

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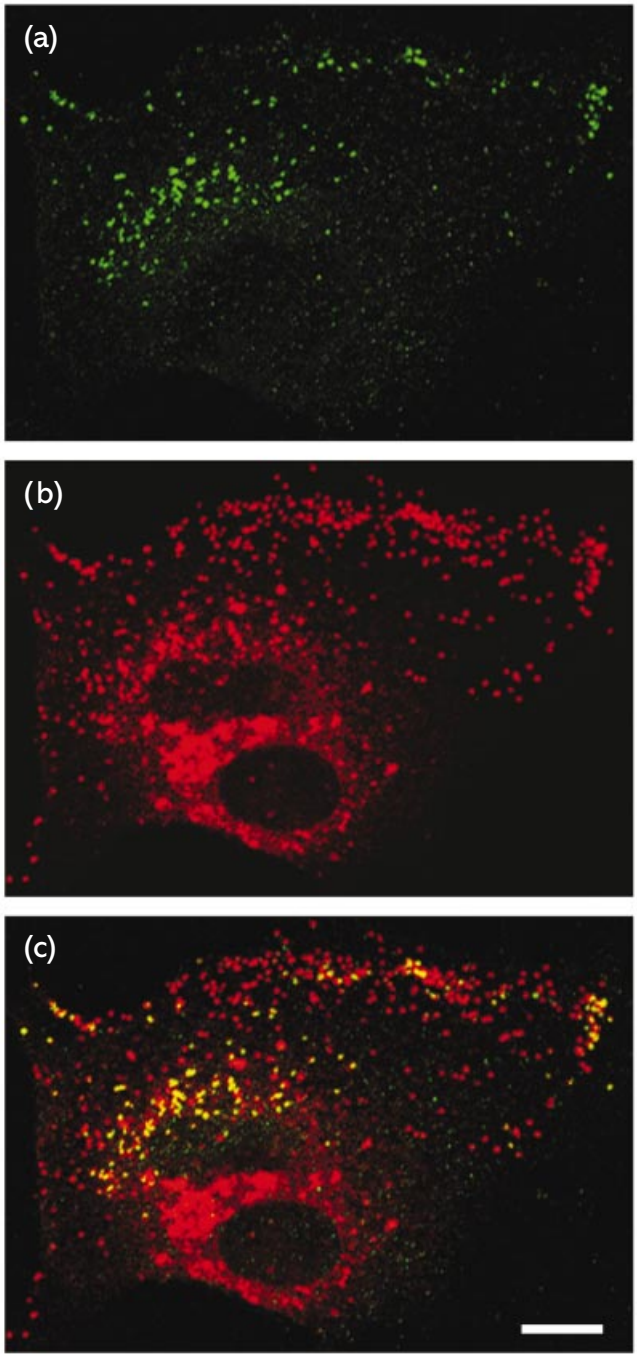

Fig. 1. Location of F12L protein in infected cells. BS-C-1 cells infected with vF12LHA at 1 p.f.u. per cell were fixed and permeabilized at 12 h p.i. and then stained with mAb HA.11 (a) and mAb 19C2 (b) as described in Methods. The merged image of (a) and (b) is shown in (c). Scale bar, 10 μm. Note the perinuclear staining of B5R in the Golgi and the absence of staining for vF12HA in this region of the complementary image. Images shown were reconstructed from two optical z-sections.

(the virus haemagglutinin) (Payne & Norrby, 1976). In addition, the A36R protein (Parkinson & Smith, 1994) is present in IEV but not IMV, CEV or EEV particles (van Eijl *et al.*, 2000). Lastly, the F12L protein was identified recently as a 65 kDa protein that is synthesized predominantly late during infection and is conserved in chordopoxviruses. A deletion mutant lacking F12L did not produce actin tails, had a small plaque size, produced 7-fold reduced levels of EEV and was highly attenuated *in vivo* (Zhang *et al.*, 2000). Although low amounts of the F12L protein co-purified with EEV in density gradients (Zhang *et al.*, 2000), a direct physical interaction with these enveloped virions has not been demonstrated.

None of the EEV- and IEV-specific proteins are required for the formation of IMV, but they have differing roles thereafter. In the absence of F13L (Blasco & Moss, 1991) or B5R (Engelstad & Smith, 1993; Wolffe *et al.*, 1993), the wrapping of IMV by intracellular membranes to form IEV is reduced or abolished and therefore subsequent stages of morphogenesis are inhibited. Deletion or repression of A33R (Roper *et al.*, 1998), A34R (Duncan & Smith, 1992; McIntosh & Smith, 1996; Wolffe *et al.*, 1997; Sanderson *et al.*, 1998) or A36R (Parkinson & Smith, 1994; Sanderson *et al.*, 1998; Wolffe *et al.*, 1998) enables IEV, CEV and EEV to be formed, but cell-to-cell virus spread is reduced and these mutants form a small plaque. Enhanced levels of EEV are released when gene A33R (Roper *et al.*, 1998) or A34R (McIntosh & Smith, 1996) is deleted, but without A34R the EEV has a reduced infectivity. Enhanced levels of EEV are also formed when the A34R (Blasco *et al.*, 1993) or B5R proteins (Herrera *et al.*, 1998; Mathew *et al.*, 1998, 2001) are mutated. Loss of the A36R (Parkinson & Smith, 1994) or F12L (Zhang *et al.*, 2000) genes caused a reduction in EEV formation. For all these mutants the production of intracellular actin tails is reduced or abolished, providing a direct correlation between actin tail formation and virus cell-to-cell spread. The only IEV or EEV-specific protein not required for normal morphogenesis is A56R (Ichihashi *et al.*, 1971; Sanderson *et al.*, 1998) (G. L. Smith, unpublished data).

In this paper, we have characterized the F12L protein further by determining its location in infected cells and analysing virus morphogenesis with the F12L deletion mutant. Data presented show that the F12L protein, like the A36R protein, is present only on IEV particles and absent from IMV, CEV and EEV. In the absence of F12L, IEV particles are made, but these do not move to the cell surface, implying a role for F12L in this transport process.

Methods

■ **Cells, viruses and drugs.** Monkey kidney BS-C-1 cells, rabbit kidney (RK_{13}) and HeLa cells were grown in minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS) (GibcoBRL). The VV strain Western Reserve (WR) mutants lacking the F12L gene (vΔF12L) and containing the F12L protein tagged at the C terminus with a peptide epitope recognized by monoclonal antibody (mAb) HA.11 (vF12LHA) were described previously (Zhang *et al.*, 2000). N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine (IMCBH) was used at 10 μg/ml and was kindly provided by R. Wittek, University of Lausanne, Switzerland. Nocodazole and brefeldin A were each purchased from Sigma and used at 33 μM and 10 μg/ml, respectively.

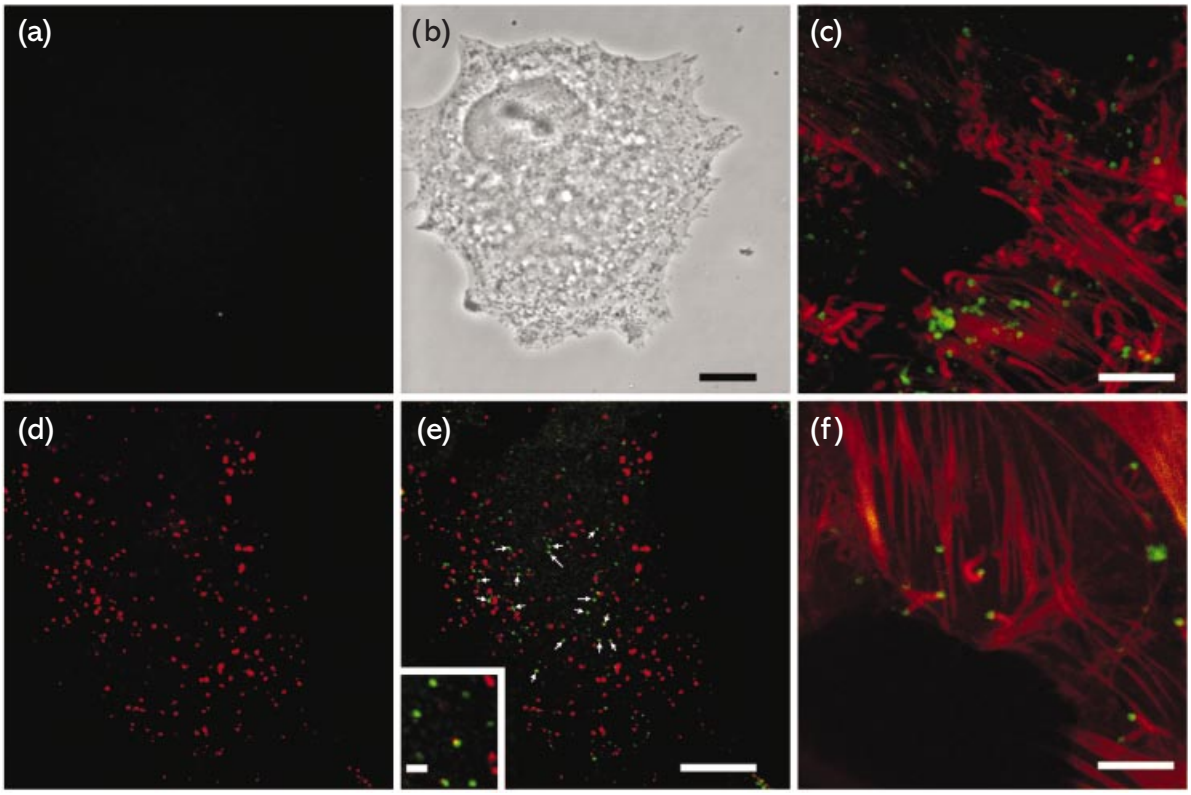

Fig. 2. The F12L protein is not exposed on the cell surface. BS-C-1 cells were infected with vF12LHA at 1 p.f.u. per cell and at 12 h p.i. were stained with mAb HA.11 (a) or mAb 19C2 (d) prior to membrane permeabilization. (b) The cell stained in (a) was examined under phase contrast microscopy. (c) RK_{13} cells infected as for (a) were fixed and permeabilized and then stained with mAb HA.11 and TRITC–phalloidin to detect F12LHA and actin, respectively. A merged image is shown. Note the absence of staining for HA.11 on actin tails. (e) After staining the live cell shown in (d) with mAb 19C2, the cell was permeabilized and then stained with mAb HA.11 and the image obtained was merged with (d) to show the location of intracellular F12L and surface B5R staining. Note the separate locations of the F12LHA and B5R (inset). (f) Cells infected as in (c) were permeabilized and stained with mAb 19C2 and TRITC–phalloidin to detect B5R and actin, respectively. A merged image is shown. Note the presence of B5R-positive virions on the tips of actin tails. Single optical sections are shown. Scales bars: (a, b) 10 μm; (c, f) 5 μm; (d, e) 10 μm; inset in (e) 1 μm.

■ **Fluorescent microscopy.** Cells growing on glass microscope slides (Chance Proper) were infected at 1 p.f.u. per cell with the indicated virus. At the stated times post-infection (p.i.), cells were washed in PBS and fixed in 4% paraformaldehyde (PFA) as described (van Eijl *et al.*, 2000). Cells were blocked and permeabilized for 5 min at room temperature (RT) in PBS containing 0·1% saponin and 10% FBS. Alternatively, infected cells were fixed by incubation for 1 min at −20 °C in acetone and dried in air for 30 min. Cells were stained with rat mAb 19C2 (Schmelz *et al.*, 1994) (diluted 1/50 in PBS containing 0·1% saponin and 10% FBS), which recognizes the VV B5R protein, mouse mAb HA.11 (BAbCo, Berkley, California) (diluted 1/400), which recognizes the HA epitope at the C terminus of F12LHA, mouse mAb AB1.1 (Parkinson & Smith, 1994), which recognizes VV protein D8L (diluted 1/50), rat mAb alpha tubulin (Serotec) (diluted 1/10) or rhodamine-conjugated mouse mAb against the transferrin receptor (Boehringer Mannheim) (diluted 1/50). Bound mAbs were detected by a goat anti-rat IgG fluorescein isothiocyanate (FITC)- or goat anti-mouse IgG tetramethylrhodamine B isothiocyanate (TRITC)-conjugated secondary antibody (Stratech Scientific) (diluted 1/100). F-actin was visualized with TRITC-conjugated phalloidin (Sigma) (diluted 1/100). Cells were washed twice in PBS and once in water, and then mounted in Mowiol–DAPI mounting medium (Sanderson *et al.*, 1996). Images were viewed using a Bio-Rad MRC 1024 confocal laser-scanning microscope, collected using Lasersharp software and processed using Adobe Photoshop.

Live cells were stained with mAb 19C2 and mAb HA.11 (diluted 1/50 and 1/200, respectively in cell medium) as described (van Eijl *et al.*, 2000). Cells treated with the microtubule depolymerizing drug nocodazole were lysed for 15 s with 0·1% Triton X-100 in PBS before fixation.

■ **Preparation of cells for electron microscopy.** BS-C-1 cells were infected at 1 p.f.u. per cell. After 1 h the inoculum was replaced with MEM–2·5% FBS and at the indicated times p.i. the cells were washed with ice-cold PBS, fixed in 0·5% glutaraldehyde in 200 mM sodium cacodylate (pH 7·4) for 30 min at room temperature. Where indicated, infected cells were incubated with horseradish peroxidase (HRP) (10 mg/ml) for 60 min before fixation and subsequently were reacted with 1 mg/ml diaminobenzidine for 30 min at room temperature. All cells were washed in water, and post-fixed in 1% osmium tetroxide and 1·5% potassium ferrocyanide for 60 min at room temperature. After being washed in water and then incubated in Mg^{2+} uranyl acetate overnight at 4 °C, the cells were washed again in water and then dehydrated in graded ethanol and flat-embedded in Epon. Sections were cut parallel to the surface of the dish and collected onto slot grids; lead citrate was added as a contrast agent.

■ **Image collection and data processing.** All sections were examined in a Zeiss Omega 912 electron microscope equipped with a Proscan cooled slow-scan charge-coupled device camera (1024 by 1024 pixels). Digital images were captured with the integrated Soft Imaging Software (SIS) image analysis package and processed using Adobe Photoshop.

Results

Location of the F12L protein

The F12L protein is required for the formation of virus-induced actin tails, a normal plaque size, and virulence, but although the F12L protein co-purified with EEV preparations on sucrose density gradients it was not shown that the protein is a component of virions (Zhang *et al.*, 2000). The A36R protein was also shown to co-purify with EEV preparations (Parkinson & Smith, 1994), but subsequent analysis by confocal and immunoelectron microscopy showed the protein was absent from CEV and present only on IEV (van Eijl *et al.*, 2000). Therefore, the location of the F12L protein was investigated by these techniques using the virus vF12LHA, which is recognized by mAb HA.11 at its C terminus. This chimaeric protein is functional during morphogenesis because vF12LHA produced a normal plaque size (Zhang *et al.*, 2000) and actin tails (see Figs 2c and 7b).

Cells infected with vF12LHA were stained with mAbs HA.11 (Fig. 1a) and 19C2 (Fig. 1b) against the F12LHA and B5R proteins, respectively, and analysed by confocal microscopy. The F12LHA protein was present on particulate structures that were of a size (600–700 nm) and distribution consistent with enveloped virions. An IEV particle of 350–400 nm would appear larger due to the presence of primary and secondary antibodies and fluorescent flare. The proposal that F12LHA was associated with enveloped virions was supported by co-localization of these structures with B5R, a protein present on IEV and CEV particles as well as the membranes used to wrap IMV particles. Merging these images (Fig. 1c) showed that a proportion of the enveloped virions were labelled by both F12L and B5R, but others were B5R-positive only. Another difference in the distribution of the F12L and B5R proteins was the presence of B5R but not F12L in the perinuclear region that represents Golgi.

A possible explanation for B5R-positive, F12LHA-negative virions is that the F12L protein is present on only IEV and not CEV particles. This was investigated further by staining live cells with mAb HA.11 (Fig. 2a, b). Phase contrast microscopy

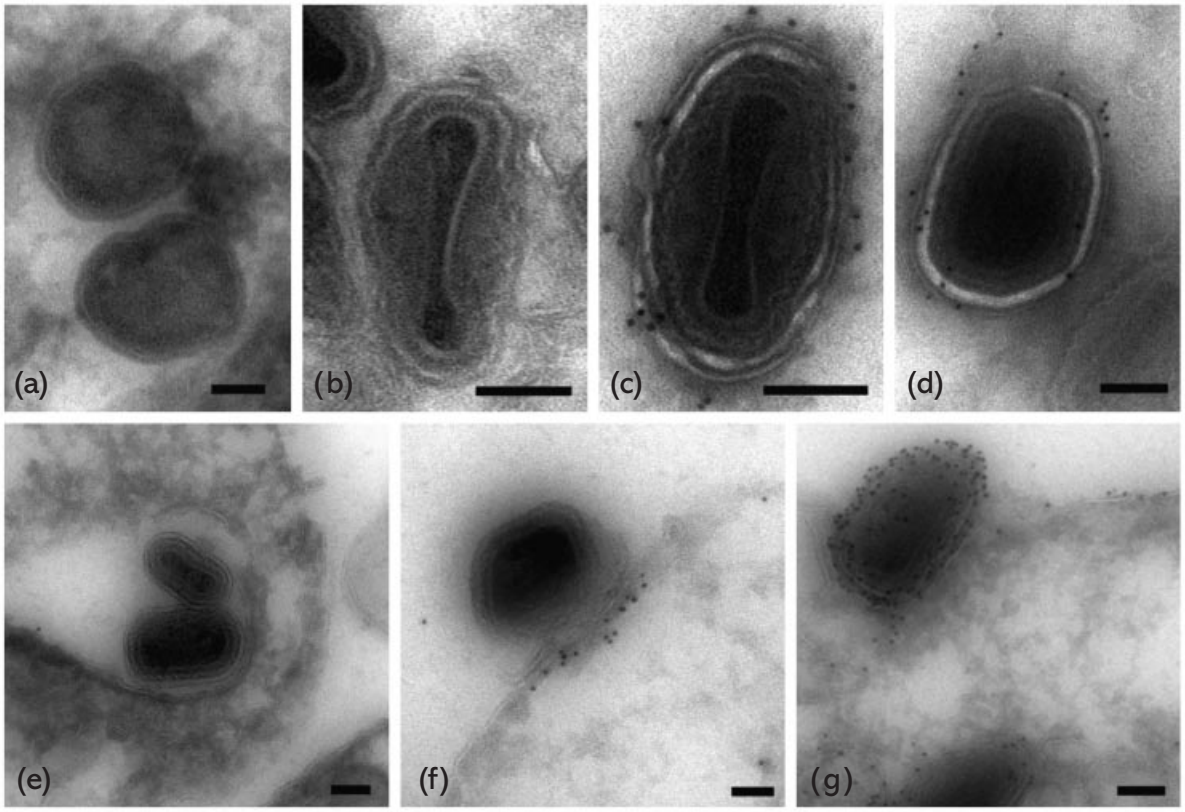

Fig. 3. Immunoelectron microscopy. BS-C-1 cells infected with vF12LHA at 1 p.f.u. per cell were processed for immunoelectron microscopy at 12 h p.i. and cryosections were stained with mAb HA.11 as described in Methods. Representative images of immature virions (a), IMV (b), IEV (c, d) and CEV particles (e) are shown. In (f) and (g) VV strain WR-infected cells were processed as for (a) except that the cryosections were stained with mAb 6.3 (van Eijl *et al.*, 2000) to detect the A36R protein (f) or mAb 19C2 to detect the B5R protein (g). Scale bars, 100 nm.

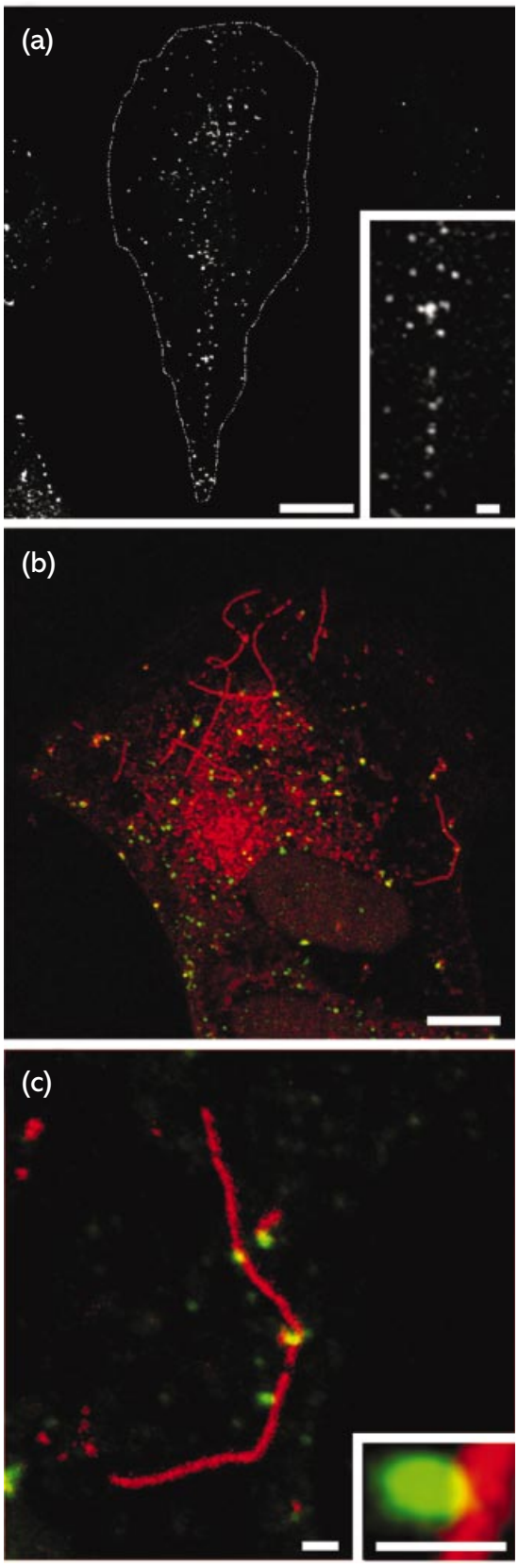

Fig. 4. Association of F12L with microtubules. (a) BS-C-1 cells were infected with vF12LHA at 1 p.f.u. per cell and at 12 h p.i. were permeabilized and stained with mAb HA.11. Bound Ab was detected as described in Methods. The cell outline is indicated. Note the linear array of F12L-positive virions. (b, c) Cells infected as in (a) were treated with nocodazole for 60 min from 11 h p.i. and then cells were treated with Triton-X 100 as described in Methods. Extracted cells were stained with mAbs HA.11 and YL1/2 α-tubulin against microtubules. Scale bars: (a) 10 μm, inset 1 μm; (b) 10 μm; (c) 1 μm, inset 1 μm. Images shown are a single optical section (b, c) or a z-series reconstruction of 10 optical sections (a).

showed an infected cell with aggregates of phase-dense virions in distinct regions beneath the cell surface (Fig. 2b). However, this same live cell was not recognized by mAb HA.11 (Fig. 2a), showing that the F12L protein, or more specifically the C-terminal HA epitope, was not exposed on the surface of the cell or CEV particles. When cells were fixed and then stained with mAb HA.11, punctate intracellular staining was observed (as in Fig. 1a) and if these cells were also stained for actin (Fig. 2c), the typical virus-induced actin tails were observed. Notably, these actin tails did not co-localize with F12LHA, indicating that F12L was not associated with CEV particles. In contrast, when live cells were stained for B5R, punctate cell surface staining was observed (Fig. 2d) representing CEV particles. Moreover, when these same cells were fixed and stained for HA and the images merged (Fig. 2e) the intracellular HA-positive structures were clearly distinct from the B5R-positive structures on the cell surface. If infected cells were stained for B5R and actin (Fig. 2f), actin tails were associated with B5R-positive virions at their tip. This contrasts with the location of F12L. Therefore, F12LHA is not exposed on the cell surface, nor associated with CEV particles.

Immunoelectron microscopy

The location of the F12LHA protein was examined next by immunoelectron microscopy of cryosections of vF12LHA-infected cells (Fig. 3). Staining of sections with mAb HA.11 followed by rabbit anti-mouse IgG and protein A-conjugated gold particles revealed staining on IEV particles (Fig. 3c, d), but not immature virus (Fig. 3a), IMV (Fig. 3b) and CEV (Fig. 3e). Closer examination showed that F12LHA staining of IEV particles was observed predominantly around the outside of the IEV particle rather than between the wrapping membranes (Fig. 3c, d). This distribution is similar to that of the A36R protein except that A36R is also present beneath CEV particles at the cell surface (Fig. 3f) (van Eijl *et al.*, 2000). Other EEV proteins such as B5R (Fig. 3g) stained the circumference of CEV particles, but stained the lumen of the wrapping membranes poorly, possibly due to steric hinderance (data not shown).

The amino acid sequence of F12L revealed no obvious transmembrane domains and so how F12L binds to IEV particles is unclear. However, it is probable that the majority of the F12L polypeptide is located within the cytosol because the multiple potential sites for attachment of *N*-linked carbohydrate are not utilized (Zhang *et al.*, 2000). This is also true for the A36R protein (Parkinson & Smith, 1994), which has the

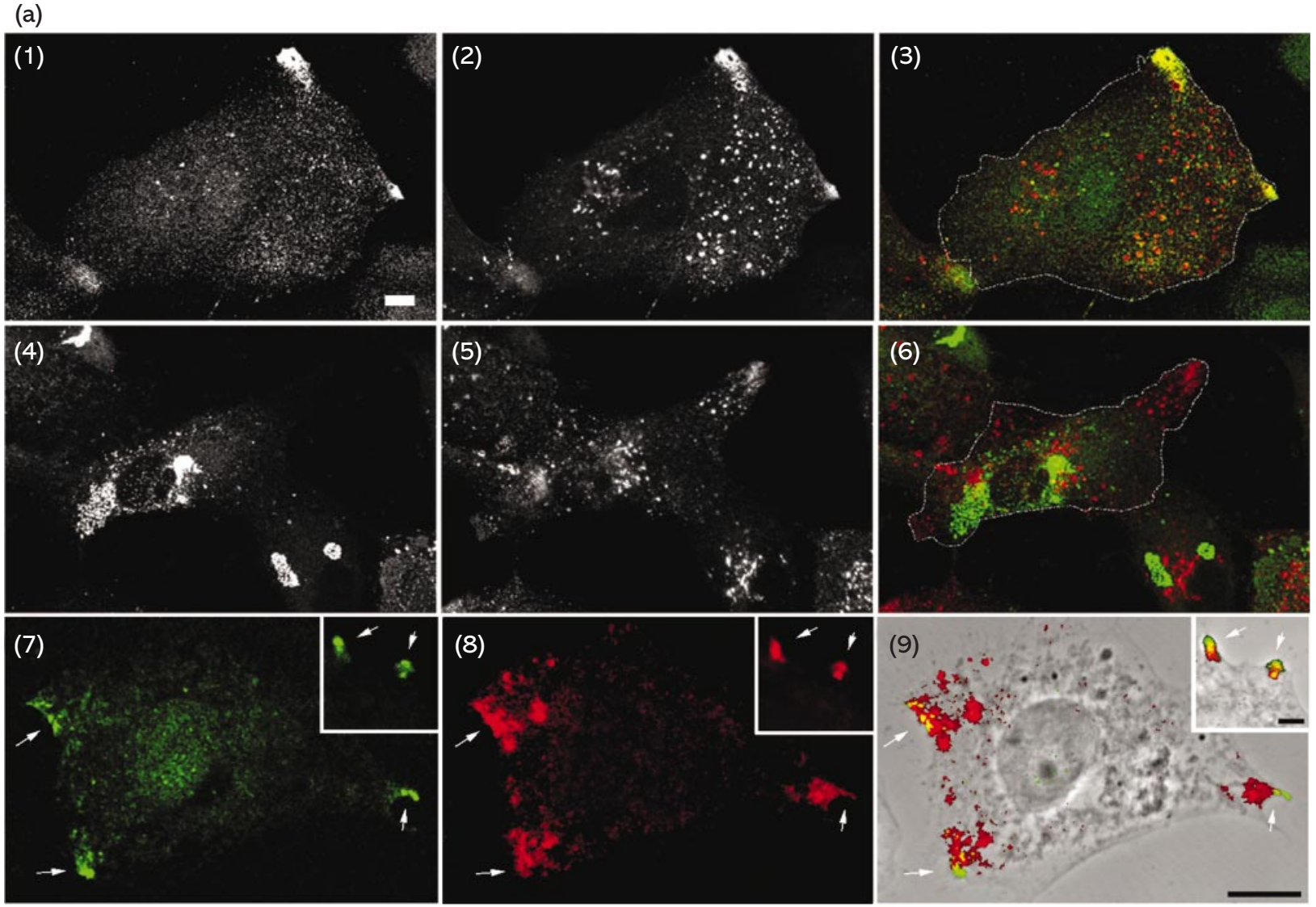

Fig. 5. For legend see facing page.

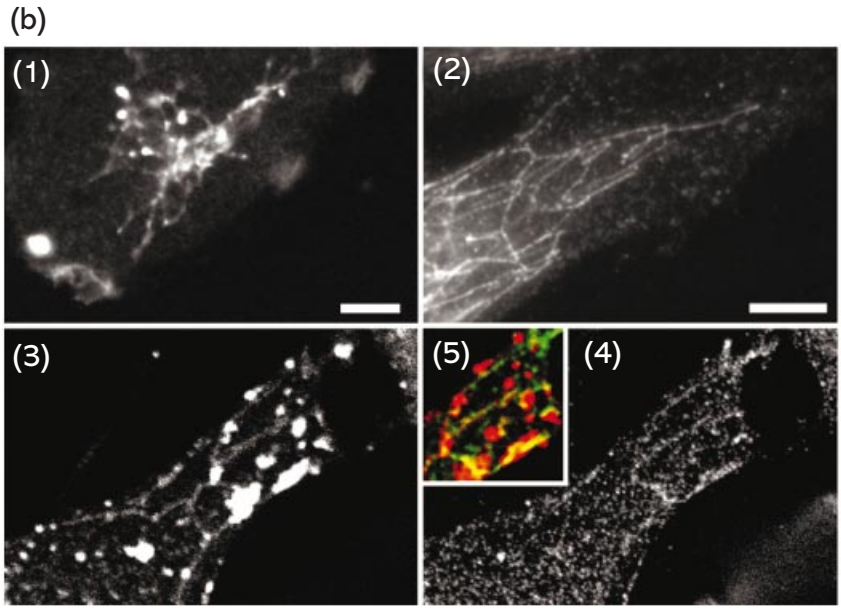

Fig. 5. F12L co-localizes with endosomes. (a) BS-C-1 cells (1–6) or HeLa cells (7–9) were infected with vF12LHA at 1 p.f.u. per cell and at 1 h p.i. were treated with IMCBH for 11 h. Cells were then fixed, permeabilized and stained with mAbs 19C2 (2) and HA.11 (1) or with 19C2 (5) and AB1.1 (4). The images shown in (1) and (2) are merged in (3) and the images shown in (4) and (5) are merged in (6). In panels (7)–(9) cells were incubated for 20 min with HRP before being fixed, permeabilized and stained with MAB HA.11 (7) or reacted with Cy3-tyramine (8). Panel (9) shows a merge of (7) and (8) on a phase contrast image of the same cell. The inserts show higher magnification of HRP- and F12LHA-positive structures. The arrows indicate peripheral distribution of F12LHA and HRP. (b) Brefeldin A causes endosome fusion (1 and 2) HeLa cells were treated with brefeldin A for 15 min at 37 °C and either treated with HRP for 5 min, fixed and then incubated with Cy3-tryamine for 15 min (1) or fixed and stained with rhodamine-conjugated mAb against the transferrin receptor (2). (3 and 4) Cells were infected as in (a) (1)–(6) and at 12 h p.i. were treated with brefeldin A for 15 min, and then fixed, permeabilized and stained with mAbs 19C2 (3) and HA.11 (4). The merged image of part of (3) and (4) is shown in (5). Scale bars, 10 μm. Images shown are a single optical section (Fig. 5a7–9, Fig. 5b1–5) or a z-series reconstruction of 10 optical sections (Fig. 5a1–6).

majority of its polypeptide chain in the cytosol (Röttger *et al.*, 1999; van Eijl *et al.*, 2000).

Taken together, these data are consistent with data obtained by confocal microscopy and indicated that F12LHA was not present on the cell surface nor was it associated with CEV particles.

Co-localization of F12LHA with microtubules

The subcellular localization of F12LHA was examined further by staining infected cells with mAb HA.11 followed by confocal microscopy. In some cases it was noticed that F12LHA-positive intracellular structures seemed to have a linear array (Fig. 4a). This was consistent with these structures being IEV particles that are moving along microtubules to reach the cell surface as shown recently (Hollinshead *et al.*, 2001; Ward & Moss, 2001). The apparent co-localization of F12LHA and microtubules was investigated further by preparing infected cells that had been treated with nocodazole, a microtubule depolymerizing drug, and then extracted with Triton X-100. Under these conditions the microtubules are largely depolymerized and soluble tubulin has been removed, but some filaments remain (Fig. 4b). When these extracted cells were stained with mAbs against F12LHA and α-tubulin, co-localization of microtubules and F12LHA (IEV particles) was seen (Fig. 4c).

The F12L protein co-localizes with endosomes

Fig. 1 shows that although the F12L protein shows some co-localization with B5R, there are also some differences. First, only a proportion of B5R-positive enveloped virions were positive for F12L, and this is explained by F12L being associated with IEV but not CEV particles (Figs 2 and 3). The other difference between the location of F12L and B5R is the presence of B5R but not F12L in perinuclear regions that represent Golgi. To analyse the location of F12L further, vF12LHA-infected cells were treated from 1 h p.i. with IMCBH, which blocks the wrapping of IMV particles. In the presence of this drug, B5R was aggregated in intracellular vesicles and in projections extending away from the cell (Fig. 5a2), indicating that B5R can be transported to the cell periphery even when virus morphogenesis is blocked after IMV formation. Under these conditions, F12L also localized to the peripheral projections (Fig. 5a1) and there was co-localization of B5R and F12L at these sites (Fig. 5a3). The presence of both B5R and F12L in these peripheral regions was not attributable to virus particles at these sites, because in the presence of IMCBH, virions do not disseminate due to IEV

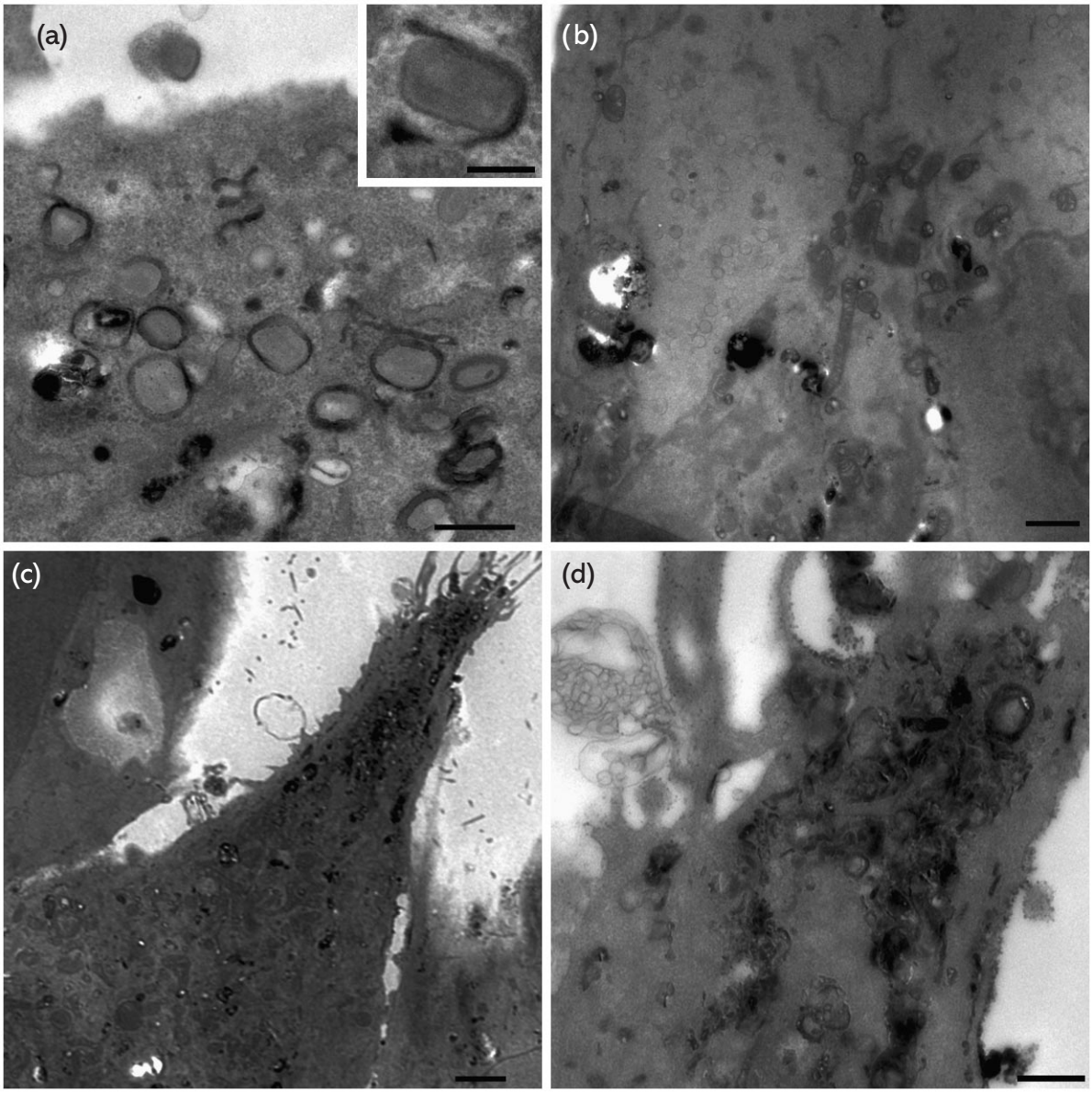

Fig. 6. Endosomal membranes are used to wrap IMV particles. BS-C-1 cells were infected with vF12LHA at 1 p.f.u. per cell and at 11 h p.i. were incubated with HRP for 60 min. Cells were then processed for electron microscopy as described in Methods. (a) IEV particles containing the fluid phase marker between the two outer membranes. The inset shows an IMV particle being wrapped by these membranes. (b) Cells were infected as in (a) and treated with IMCBH at 1 h p.i. for 10 h before being incubated with HRP and processed as in (a). Note the absence of IEV particles. (c, d) Cellular projection contain numerous endosomes. Cells were treated as in (b). Note the concentration of endosomal membranes in the peripheral region of the cell. Scale bars: (a) 0·5 μm, insert 0·2 μm; (b) 1 μm; (c) 2 μm; (d) 0·5 μm.

formation being blocked. This was illustrated by staining IMCBH-treated cells with D8L (Fig. 5a4), an IMV protein present on all virions, and B5R (Fig. 5a5). D8L remained in factories and on a few cytoplasmic virions, but was not transported to the B5R- and F12L-positive peripheral sites (Fig. 5a3, 6). Therefore, F12L can move to peripheral sites of the cell without association with IEVs. To investigate if these peripheral structures were endosomes we incubated infected cells with HRP for 20 min, so that this fluid phase marker would be taken up into endosomes, and then fixed and permeabilized the cells. Cells were stained with anti-HA mAb (Fig. 5a7) and cyanine 3 (cy3)-labelled tyramine (New England Nuclear Life Sciences Products) (Fig. 5a8), which is a substrate for HRP and is deposited at the site of HRP uptake. The cells were examined by fluorescent and phase-contrast microscopy. F12LHA (7) and HRP (8) were located predominantly at peripheral structures

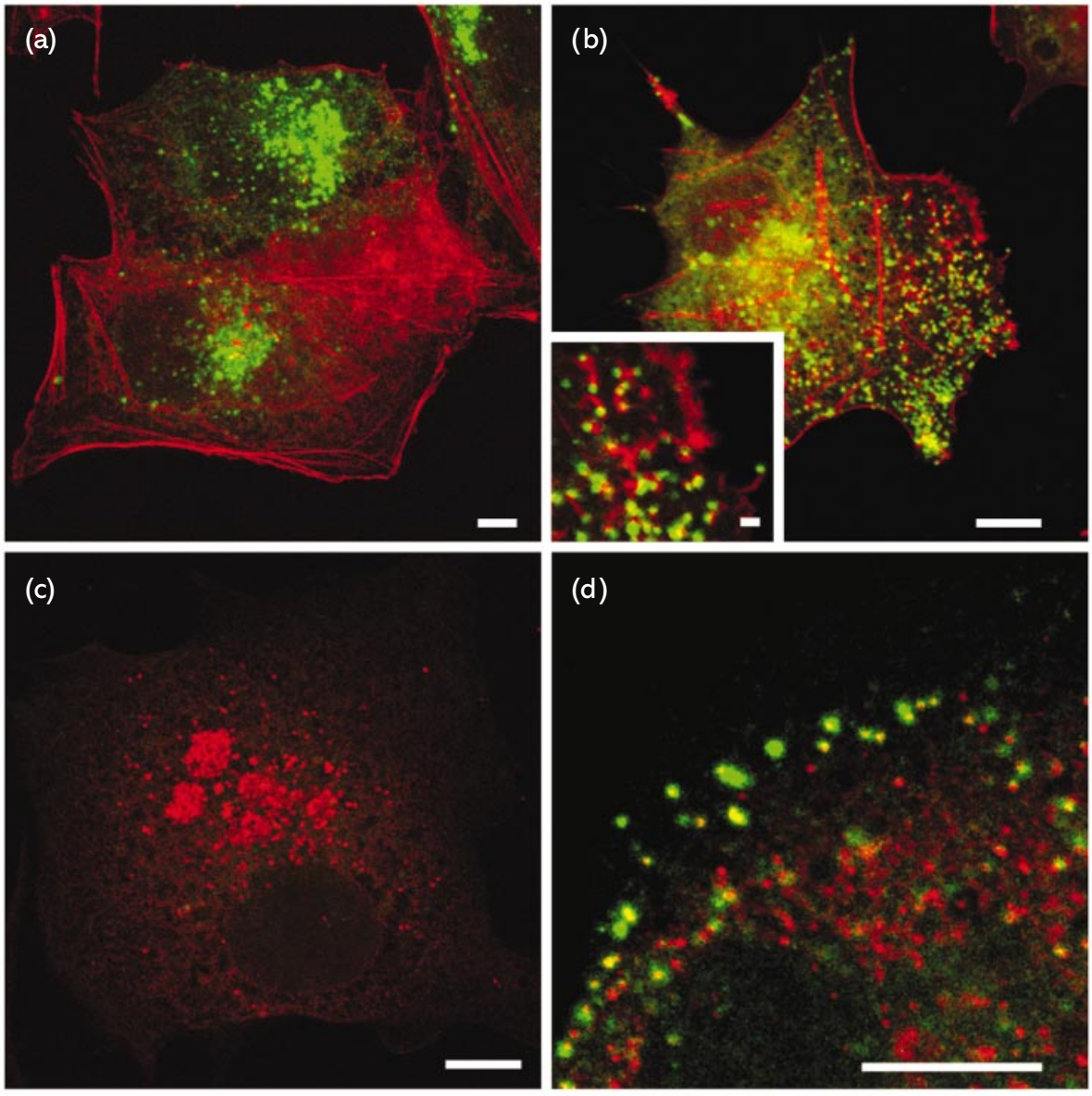

Fig. 7. vΔF12L-infected cells lack CEV particles. BS-C-1 cells infected with vΔF12L (a, c), vF12LHA (b) or vΔA36R (d) at 1 p.f.u. per cell for 12 h. (a, b) Cells were permeabilized and then stained with mAb 19C2 (green) and with TRITC–phalloidin (red). The insert in (b) shows virus-tipped actin tails. (c, d) Live cells were stained with mAb 19C2 and then fixed and permeabilized and stained with mAb AB1.1 (red). Note the absence of cell surface B5R in (c) and its presence in (d). Scale bars: (a–d) 10 μm, inset 1 μm. Images shown are each a z-series reconstruction of 13 optical sections.

that were largely co-incident (Fig. 5a9). The inserts show a higher magnification of the F12LHA- and HRP-positive structures at the cell periphery.

The location of F12L in relation to endosomes was also analysed by incubation of cells with HRP followed by cytochemistry and electron microscopy (Fig. 6). In cells infected with vF12LHA for 12 h, HRP was taken up into endosomes and also stained the lumen between the two outer membranes of IEV (Fig. 6a). The insert shows a higher magnification of a virion undergoing wrapping by these membranes. The presence of this marker within the membranes wrapping these virions is consistent with previous work (Tooze *et al.*, 1993) and provides evidence that the wrapping of IMV particles can utilize membranes from a post-Golgi compartment. Treating vF12LHA-infected cells with IMCBH to block IEV formation, followed by addition of the fluid phase marker, prevented the formation of these labelled virions (Fig. 6b). Infected cells treated with IMCBH showed clustering of F12L and B5R in cellular processes near the cell periphery (Fig. 5a, b). Examination of such processes following uptake of HRP identified

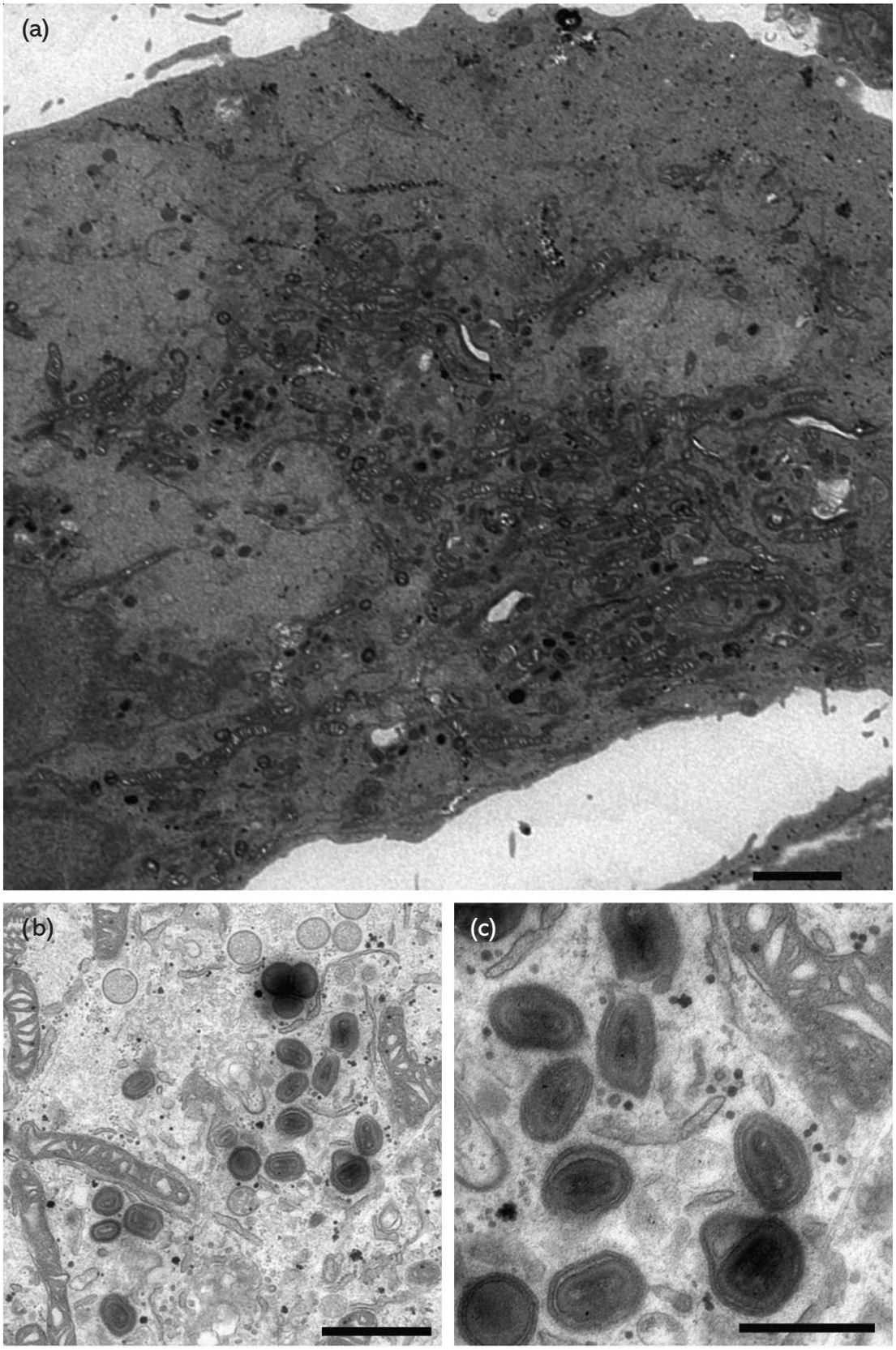

Fig. 8. Electron microscopy of vΔF12L-infected cells. BS-C-1 cells infected with vΔF12L at 1 p.f.u. per cell for 12 h. Cells were then processed for thin section transmission electron microscopy as described in Methods. (a) Low magnification image of cell

numerous endosomes clustered in these areas near the cell periphery (Fig. 6c, d). This provided further evidence for the co-localization of F12L with endosomal compartments.

The relative locations of B5R and F12L were studied further by treating cells with brefeldin A for 15 min, which causes the coalescence of endosomes into a tubular network (Tooze *et al.*, 1993) and is illustrated here by labelling cells with either HRP (Fig. 5b1) or rhodamine-conjugated mAb to the transferrin receptor (Fig. 5b2). Note the reticular pattern of fused endosomes. In vF12LHA-infected cells, B5R was present in Golgi aggregates (bright staining) but also in endosome compartments as for the transferrin receptor (Fig. 5b3). These were co-incident with F12L staining (Fig. 5b4, 5), confirming co-localization of F12L and endosomes.

Loss of F12L prevents migration of IEV to cell periphery

Electron microscopy showed that the F12L protein is associated with IEV particles (Fig. 3), but that when the F12L gene is deleted, actin tails are not made and there is a small plaque phenotype (Zhang *et al.*, 2000). The failure to make actin tails might have been due to an inhibition of IEV formation (like vΔF13L), blockage of IEV transport to the cell surface, or inability of the CEV at the surface to induce actin tail formation (like vΔA36R). To investigate these possibilities, vΔF12L-infected cells were stained with B5R and phalloidin (Fig. 7a). In these cells, the B5R (green signal) remained predominantly in the perinuclear regions and was not disseminated to the cell surface. Consistent with the lack of actin tails, the cell actin stress fibres remained prominent. In contrast, in cells infected with vF12LHA (or WR, not shown) B5R was dispersed from the central region and there were aggregates of virions in the cell periphery (Fig. 7b). These cells produced actin tails (Fig. 7b insert). To investigate if B5R reached the cell surface in the absence of F12L, live cells infected with vΔF12L were stained with mAb 19C2, and after fixation and permeabilization the cells were stained for D8L (red signal). Under these conditions no B5R staining was observed (Fig. 7c) showing that CEV particles were not made, and the D8L protein was located close to virus factories with only a few dispersed virions. In comparison, cells infected with vΔA36R produced cell surface B5R and CEV particles, which also contained D8L (Fig. 7d). Therefore, although both vΔF12L and vΔA36R do not make actin tails, these viruses arrest at different stages of the morphogenic pathway.

Virus morphogenesis in vΔF12L-infected cells was examined further by electron microscopy (Fig. 8). In these cells, virus factories were visible (Fig. 8a) and at higher magnification (Fig. 8b) the factories were seen to contain normal IV and IMV particles. In addition, there were many IEV particles clustered near to these factories that had not dispersed to the cell surface (Fig. 8c). The presence of so many IEV particles clustered in this region was unusual and suggested that there was a defect in their dispersal. This was evident when analysing infected cells at lower magnification (Fig. 8a), which showed few particles near the cell periphery and no CEV particles on the cell surface. These data were consistent with the results of confocal microscopy and indicated that VV morphogenesis was arrested after the formation of IEV particles in the absence of the F12L protein.

Discussion

This paper provides a further characterization of the VV F12L protein and its function during virus morphogenesis. The phenotype of the F12L deletion mutant defines a new stage in VV morphogenesis, namely the formation of IEV particles but the failure of these to move to the cell surface.

The location of the F12L protein in infected cells was examined using a recombinant virus in which the F12L protein was tagged at the C terminus with a 9-amino-acid epitope that is recognized by a mAb. Immunoelectron microscopy showed that the F12L protein was present on IEV particles, predominantly near the outer membrane, but it was absent from IV, IMV and CEV (Fig. 3). F12L is therefore the second IEV-specific protein that has been identified. The presence of low amounts of the protein associated with EEV that had been purified on density gradients (Zhang *et al.*, 2000) is reminiscent of the A36R protein that also co-purified with EEV, but which was associated with fragments of cell membranes that remained attached to EEV particles after their detachment from the cell. Within the infected cells, one difference between F12L and A36R was the presence of A36R but not F12L on the cytosolic face of the plasma membrane beneath CEV particles. The position of A36R at this site is consistent with the role of A36R in inducing the polymerization of actin tails to drive CEV particles away from the cell. How F12L is absent from this site while having a similar distribution on IEV particles remains to be determined.

Immunofluorescence showed that the F12LHA protein was not detected on the cell surface, consistent with its absence from CEV particles. However, since the mAb detects only the C terminus of F12LHA, it was uncertain if all the F12L protein was within the cell. The F12L protein has several potential sites for the attachment of *N*-linked carbohydrate that apparently are not used because the protein has a similar electrophoretic mobility after synthesis in the presence or absence of tunicamycin (Zhang *et al.*, 2000). This would be consistent with

showing virus factories and the absence of CEV particles. (b) Virus factory showing presence of immature virions and IMV particles and adjacent IEVs. (c) High magnification image showing clusters of IEV particles close to virus factories. Scale bars: (a) 2 μm; (b) 1 μm; (c) 0·5 μm.

the majority of the F12L polypeptide being within the cytosol, rather than within the lumen of membrane vesicles of the exocytic pathway. The mechanism of association of the F12L protein with IEV particles is unknown. Other virus-encoded proteins that are present in the IEV membranes either have a transmembrane hydrophobic domain (A33R, A34R, A36R, A56R, B5R) or have fatty acids that mediate membrane interaction (F13L). F12L does not have a predicted transmembrane domain and it is unknown if the protein is modified by addition of fatty acids. Another possibility is that F12L associates with IEV via protein–protein interactions with other virus or cell proteins present in the IEV outer membrane. The A36R and F13L proteins have the majority of their amino acids in the cytosol and so might have the greatest chance of interacting with F12L; however, it is known that the A36R deletion mutant is able to move to the cell surface (van Eijl *et al.*, 2000), implying that F12L can function in the absence of the A36R protein.

The F12L protein showed an interesting distribution within infected cells and co-localized with both microtubules and endosomes. The co-localization with microtubules is consistent with their role in the egress of IEV to the cell surface (Hollinshead *et al.*, 2001; Ward & Moss, 2001), and this co-localization, together with the failure of IEV made by the deletion mutant to disseminate to the cell surface, implies that F12L has a role in this microtubule-dependent IEV egress. Computational analyses of the F12L protein with known microtubule-binding proteins did not detect significant amino acid similarity. However, a microtubule-binding domain may exist. Alternatively, F12L might co-localize with microtubules indirectly via interactions with other proteins.

The co-localization of F12L with endosomes was shown by the presence of F12L in areas of the cells that contain endosomes (defined by the uptake of a fluid phase marker) and by its behaviour in infected cells treated with drugs that alter endosome location and appearance. The localization of F12L with endosomes did not require the formation of IEV particles, since when IEV particle formation was blocked by addition of IMCBH the F12L protein was localized to peripheral areas of the cell where no virions were present (Fig. 5a), but which were rich in endosomes (Figs 5 and 6). The membranes used to wrap IMV particles to form IEV particles were shown to be of endosomal origin since they were labelled with a fluid phase marker that was efficiently taken up into endosomes. This observation is consistent with an earlier study (Tooze *et al.*, 1993) and inconsistent with the wrapping membranes deriving from the *trans*-Golgi network (Schmelz *et al.*, 1994). This discrepancy might be explained by use of membranes from both compartments, or due to increased traffic between these intracellular membrane compartments during VV infection such that their distinction is blurred.

The role of microtubules in intracellular transport of virus particles following infection has been studied with other viruses such as herpes simplex virus (Sodeik *et al.*, 1997), but less attention has been paid to the role of microtubules during virus egress. The use of microtubules by vaccinia virus during egress is therefore unusual and we have identified the F12L protein as necessary for this process.

In summary, the F12L protein is shown to be a component of IEV particles, but is absent from other virions. It is required for the egress of IEV particles to the cell surface and the deletion mutant defines a new stage in VV morphogenesis. Preliminary data indicate a co-localization with microtubules, which would be consistent with a unique role for F12L in mediating the transport of IEV particles to the cell surface on microtubules.

This work was supported by the EC grant TMR CT980225, and grants from the UK Medical Research Council and The Wellcome Trust. G.L.S. is a Wellcome Trust Principal Research Fellow. We thank Brendan Murphy for critical reading of the manuscript.

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Received 25 June 2001; Accepted 27 September 2001