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The vaccinia virus A27L protein is needed for the microtubule-dependent transport of intracellular mature virus particles

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The vaccinia virus (VV) A27L gene encodes a 14 kDa protein that is required for the formation of intracellular enveloped virus (IEV) and, consequently, normal sized plaques. Data presented here show that A27L plays an additional role in VV assembly. When cells were infected with the VV WR32-7/Ind 14K, under conditions that repress A27L expression, transport of intracellular mature virus (IMV) from virus factories was inhibited and some IMV was found in aberrant association with virus crescents. In contrast, other VV mutants (vΔB5R and vΔF13L) that are defective in IEV formation produce IMV particles that are transported out of virus factories. This indicated a specific role for A27L in IMV transport. Induction of A27L expression at 10 h post-infection promoted the dispersal of clustered IMV particles, but only when microtubules were intact. Formation of IEV particles was also impaired when cells were infected with WR32-7/14K, a VV strain expressing a mutated form of the A27L protein; however, this mutation did not inhibit intracellular transport of IMV particles. Collectively, these data define two novel aspects of VV morphogenesis. Firstly, A27L is required for both IMV transport and the process of envelopment that leads to IEV formation. Secondly, movement of IMV particles between the virus factory and the site of IEV formation is microtubule-dependent.

Introduction

Vaccinia virus (VV), a member of the *Poxviridae*, is a large DNA virus that replicates within the cytoplasm of the host cell. VV morphogenesis is initiated within specialized areas of the cytoplasm called virus factories (Dales & Siminovitch, 1961; Joklik & Becker, 1964; Morgan, 1976). The first virus structures to form are crescent-shaped membranes that contain virus proteins. The origin of these membranes and the number of lipid bilayers they contain is contentious. Two models have been proposed: the first concludes that virus crescents contain a single lipid bilayer formed by *de novo* membrane synthesis (Dales & Mosbach, 1968). The second suggests that virus crescents are composed of two lipid bilayers that are derived from the intermediate compartment of the cell (Sodeik *et al.*, 1993). Recent ultrastructural data showing that virus crescents contain only one lipid bilayer appear to support a model of *de novo* membrane synthesis (Hollinshead *et al.*, 1999); however, the mechanism by which these membranes form and the method of protein recruitment remain unclear.

As morphogenesis progresses, virus crescents extend to form spherical immature virus (IV) particles that in turn condense to form infectious intracellular mature virus (IMV). To date, 11 VV proteins are known to be associated with the IMV membrane (Takahashi *et al.*, 1994; Jensen *et al.*, 1996). Of these, the protein (D13L) that is sensitive to rifampicin (Tartaglia *et al.*, 1986; Baldick & Moss, 1987; Zhang & Moss, 1992), A17L (Rodriguez *et al.*, 1995, 1997; Wolffe *et al.*, 1996) and A14L (Rodriguez *et al.*, 1997) are required for the formation of IMV particles. In addition to facilitating IMV assembly, the A17L protein also functions to bind A27L protein on the IMV surface (Rodriguez *et al.*, 1993). Although most IMV particles remain within the cytoplasm until cell death, some IMV particles become enveloped by membranes of the *trans*-Golgi network (TGN) (Hiller & Weber, 1985; Schmelz *et al.*, 1994) or tubular endosomes (Tooze *et al.*, 1993) to form intracellular enveloped virus (IEV).

Components within the outer membrane of IEV particles promote the polymerization of actin tails that are thought to

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assist IEV particle movement and enhance infection of neighbouring cells (Cudmore *et al.*, 1995, 1996). When IEV particles reach the cell surface their outer membrane fuses with the plasma membrane, exposing infectious cell-associated enveloped virus (CEV) on the surface of the cell. In the case of the Western Reserve (WR) strain of VV, the majority of enveloped virus particles remain attached to the cell surface as CEV, and only a small percentage of enveloped virions are released from the cell as extracellular enveloped virus (EEV). The outer membrane of CEV or EEV particles contains six virus-encoded proteins that are not found in IMV 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). Deletion of the F13L or B5R genes or repression of A27L gene expression reduces dramatically the formation of IEV particles (Rodriguez & Smith, 1990*a*; Blasco & Moss, 1991; Engelstad *et al.*, 1992).

The A27L protein (p14) is a multifunctional protein that has been implicated in virus attachment (Chung *et al.*, 1998), virus–cell fusion (Doms *et al.*, 1990), cell–cell fusion (Gong *et al.*, 1990), plaque size (Dallo *et al.*, 1987; Rodriguez & Smith, 1990*a*) and the formation of enveloped virions (Rodriguez & Smith, 1990*a*). In this study, two closely related recombinant viruses, WR32-7/14K (Rodriguez & Smith, 1990*a*) and WR32-7/Ind 14K (Rodriguez & Smith, 1990*b*), have been used to characterize further the role of A27L in VV morphogenesis. Both viruses were derived from a variant form of WR (WR32-7) that was isolated from persistently infected Friend erythroleukaemia cells (Dallo & Esteban, 1987). Analyses of the WR32-7 virus identified an 8 MDa deletion in the left terminus of the virus genome, and alterations in the size of three structural proteins with molecular masses of 39, 21 and 14 kDa (Paez *et al.*, 1987). In particular, the protein encoded by gene A27L migrated with an apparent electrophoretic mobility of 15·5 kDa and not 14 kDa as observed for the parental WR virus. Phenotypically, WR32-7 produced smaller plaques and fewer enveloped virions (IEV, CEV and EEV) than the parental WR virus. Genetic analyses of the WR32-7 A27L gene identified a single base change (C to A) resulting in the substitution of Ala-25 by Asp, which was shown to be responsible for the observed changes in both plaque size and electrophoretic mobility (Gong *et al.*, 1989).

After characterization of VV WR32-7, a recombinant virus was generated (WR32-7/14K) in which a wild-type (WT) A27L ORF was inserted into the thymidine kinase (TK) locus of the WR32-7 virus genome under the transcriptional control of the VV late p4b promoter and repressed by the *E. coli* LacI protein and *lac* operator (Rodriguez & Smith, 1990*a*). Consequently, the WT A27L protein was expressed only in the presence of IPTG. To generate a virus that encoded only the 14 kDa A27L protein, the mutated A27L gene contained within the endogenous locus of WR32-7/14K was deleted leaving only the inducible WT A27L ORF within the VV TK locus (Rodriguez & Smith, 1990*b*). Ultrastructural analysis of cells infected with WR32-7/Ind 14K in the absence of IPTG showed that although IMV particles were formed, IEV particles were not (Rodriguez & Smith, 1990*b*). As such, both WR32-7/14K and WR32-7/Ind 14K exhibit a small plaque phenotype in the absence of IPTG and are defective in the formation of enveloped virions. In this report we show that WR32-7/14K and WR32-7/Ind 14K are defective at different stages of VV morphogenesis, demonstrating that A27L plays a multifunctional role in VV assembly. In addition, we show that intracellular movement of mature IMV particles is dependent upon microtubules and A27L gene expression.

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). Chicken embryo fibroblasts (CEF) were prepared from whole embryo digests and grown in minimal essential medium (MEM) with 10% FBS. Confluent monolayers of CEF were maintained at 31 °C for up to 4 weeks, with weekly changes of medium (MEM with 2·5% FBS). Fresh CEF monolayers were prepared the day before use by a 1:3 split and overnight growth in MEM containing 10% FBS. Recombinant VVs WR32-7/14K (Rodriguez & Smith, 1990*a*) and WR32-7/Ind 14K (Rodriguez & Smith, 1990*b*) and WR mutants lacking the F13L (called vRB12 previously, but referred to here as vΔF13L) (Blasco & Moss, 1991) or B5R (vΔB5R) (Engelstad *et al.*, 1992) genes were described previously.

■ **Fluorescent microscopy.** VV was adsorbed onto cells on ice for 1 h using 1 p.f.u. per cell. After adsorption, non-adherent viruses were removed by washing repeatedly and cells were incubated in MEM containing 2·5% FBS at 37 °C. At the indicated times, cells were fixed in 4% formaldehyde–PBS for 15 min at room temperature and immunocytochemistry was performed as described (Herzog *et al.*, 1994). Monoclonal antibody (MAb) AB1.1, which recognizes the VV D8L protein (Parkinson & Smith, 1994), was used (diluted 1:300) for the identification of VV particles, while MAb 19C2 (Schmelz *et al.*, 1994) was used to identify the VV B5R protein (hybridoma culture supernatants were used at a dilution of 1:8). A27L was identified using MAb C3 (Rodriguez *et al.*, 1987) at a dilution of 1:50, while F-actin was visualized 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.

■ **Electron microscopy.** WR32-7/Ind 14K virus (1 p.f.u. per cell) was adsorbed onto BS-C-1 cells on ice for 1 h. Unbound virus was then washed away and cells were incubated at 37 °C in MEM containing 2·5% FBS for 12 h before being washed in 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 sodium cacodylate, 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 on a Zeiss Omega EM 912 electron microscope.

■ **Kinetic analyses of the number of cytoplasmic virions.** BS-C-

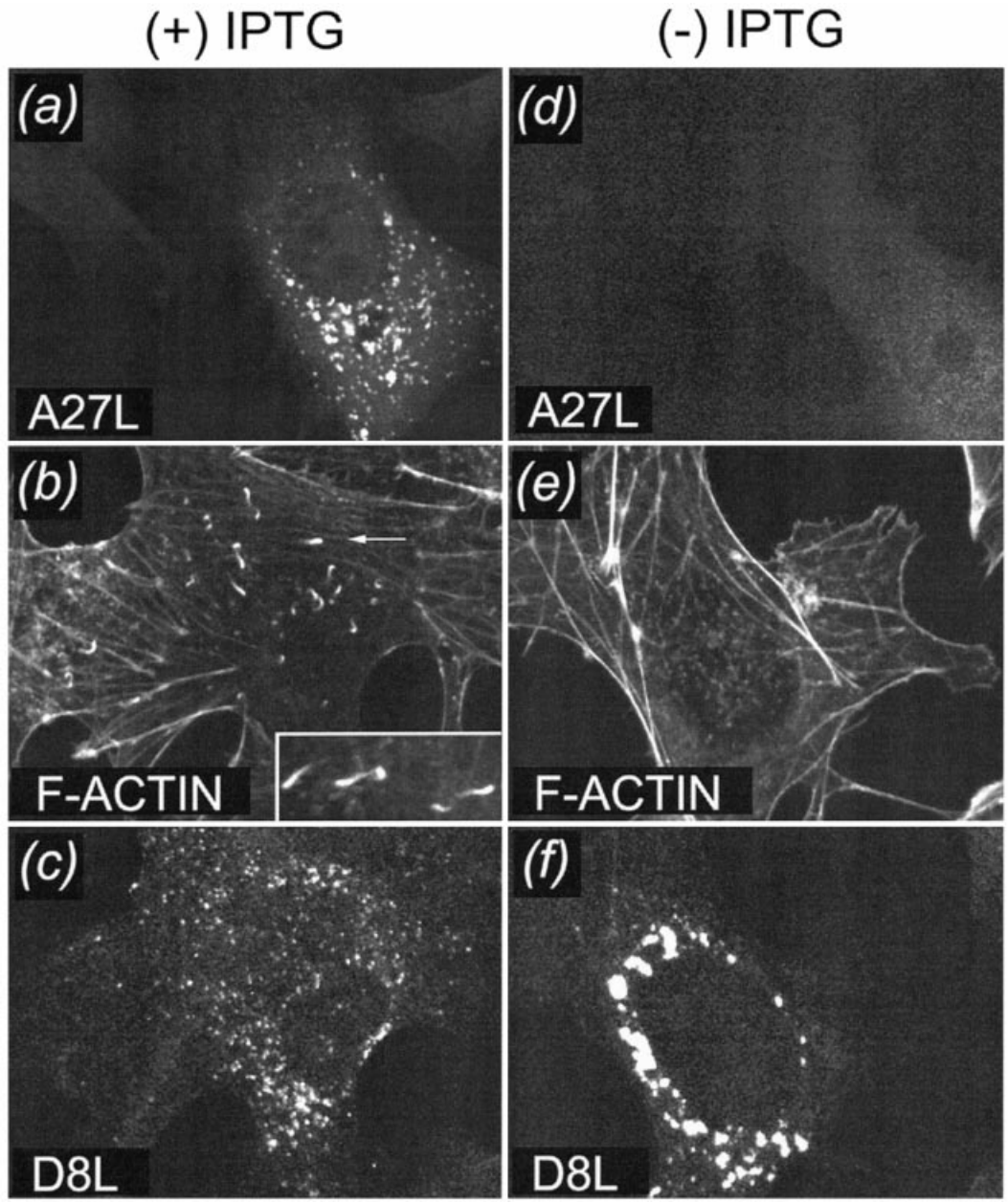

Fig. 1. Sub-cellular distribution of VV particles, F-actin and VV A27L. BS-C-1 cells were infected with VV WR32-7/Ind 14K at 1 p.f.u. per cell in the presence (*a–c*) or absence (*d–f*) of 10 mM IPTG for 12 h. Cells were either stained with MAb C3B to show the distribution of A27L (*a*, *d*) or co-stained with TRITC–phalloidin and MAb AB1.1 to show the distribution of F-actin (*b*, e) or virus particles (*c*, *f*) respectively. The insert in panel (*b*) shows an enlargement of two VV-associated actin tails.

1 cells were infected with the indicated virus at 0·1 p.f.u. per cell so that each productive infection should result from the entry of a single infectious particle. In each case viruses were adsorbed onto cells for 1 h at 4 °C and unbound viruses were removed by washing repeatedly with PBS (4 °C) before addition of MEM at 37 °C. At the indicated times, cells were fixed in 4% formaldehyde–PBS for 30 min at room temperature. After fixation, cells were processed for immunofluorescence using MAb AB1.1 to identify virus particles. For each virus, projected reconstructions of five infected cells were prepared and for each cell the number of cytoplasmic virions was counted manually from images compiled in Adobe Photoshop. For each time-point the standard deviation of the mean was calculated form data derived from five cells.

Results

Distribution of IMV particles when A27L expression is repressed

BS-C-1 cells were infected with the recombinant VV WR32-7/Ind 14K (1 p.f.u. per cell) in the presence or absence of

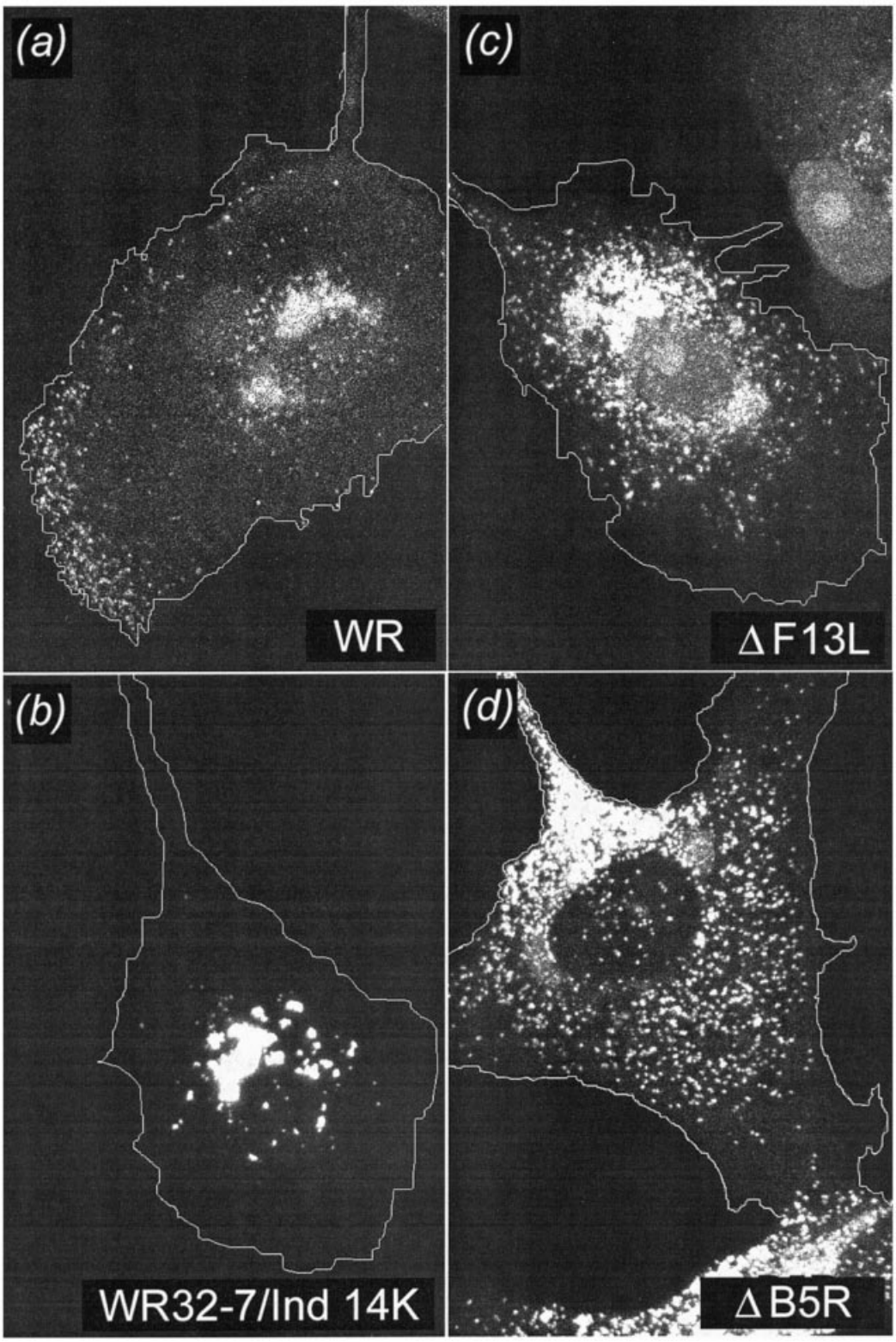

Fig. 2. Sub-cellular distribution of virus particles following infection of BS-C-1 cells with either WR (*a*), WR32-7/Ind 14K (−IPTG) (*b*), vΔF13L (*c*) or vΔB5R (*d*) viruses. In each case cells were infected at 1 p.f.u. per cell for 12 h before being fixed and stained with MAb AB1.1 to show the distribution of VV particles.

10 mM IPTG. At 12 h post-infection (p.i.) cells were fixed and stained for immunofluorescence using MAbs specific for the A27L (MAb C3) or the VV D8L (MAb AB1.1) protein. In addition, infected cells were stained with TRITC-labelled phalloidin to show the sub-cellular distribution of F-actin. In the presence of IPTG, virus particles containing A27L or D8L were observed as dispersed foci (Fig. 1*a*, *c*) and VV-induced actin tails were present within infected cells (Fig. 1*b*). In contrast, in the absence of IPTG no A27L or actin tails were detected (Fig. 1*d*, *e*) and virus particles appeared clustered close to the nucleus of the cell (Fig. 1*f*). To determine if the apparent clustering of IMV particles was real or due to redistribution of D8L, VV particles were also visualized using 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) to stain virus DNA. These data (not shown), together with ultrastructural evidence (see Fig. 4), confirmed that IMV particles cluster at the cytoplasmic interface of virus factories when A27L expression is repressed.

Comparative sub-cellular distribution of IMV particles following infection with viruses that are defective in IEV formation

To establish if IMV clustering was simply a consequence of inefficient IEV formation, BS-C-1 cells were infected with either WR, vΔF13L, vΔB5R or WR32-7/Ind 14K (in the absence of IPTG). At 12 h p.i. cells were fixed and virus particles were visualized using MAb AB1.1. In WR-infected cells virus particles were observed in and around virus factories and at the periphery of the cell (Fig. 2*a*). The distribution of VV particles in cells infected with vΔB5R or vΔF13L differed from that observed for WR as virus particles were dispersed within the cytoplasm but did not extend to the cell periphery (Fig. 2*c*, *d*). Although virus particles consistently were less dispersed in cells infected with vΔF13L than WR or vΔB5R, neither mutant virus exhibited the same restriction in IMV transport as observed when A27L expression was repressed (Fig. 2*b*). As such, it is highly unlikely that the observed defect in the intracellular transport of IMV is a consequence of reduced IEV formation. Infection of CEF cells with either WR virus, vΔF13L, vΔB5R or WR32-7/Ind 14K (in the absence of IPTG) produced comparable results to those described for BS-C-1 cells (data not shown).

Kinetic analyses of the dissemination of VV particles from virus factories to the cytoplasm

To establish if the lack of virus particles within the cytoplasm of cells infected with WR32-7/Ind 14K (−IPTG) was due to variation in the time at which virions were transported, BS-C-1 cells were infected with WR, vΔF13L or WR32-7/Ind 14K (in the presence or absence of IPTG) and the number of disseminated cytoplasmic virus particles was determined at different times p.i. In each case, cells were infected at 0·1 p.f.u. per cell to ensure that progeny virions were derived from a single infectious particle. At either 6, 18 or 24 h p.i. cells were fixed and stained with MAb AB1.1 to visualize virus particles. Fig. 3 shows that by 12 h p.i. approximately 500 virus particles could be detected in or on WR-infected cells and this number rose slightly to around 550 particles per cell by 24 h p.i. The number of virus particles detected after infection with vΔF13L or WR32-7/Ind 14K (+IPTG) was at all times reduced compared to WR-infected cells, but higher then after infection with WR32-7/Ind 14K (−IPTG) where the number of disseminated virus particles per cell reached an average of only 26 at 24 h p.i. These data demonstrate that the observed clustering of IMV particles following WR32-7/Ind 14K infection is not due to temporal changes in the rate of export of IMV particles.

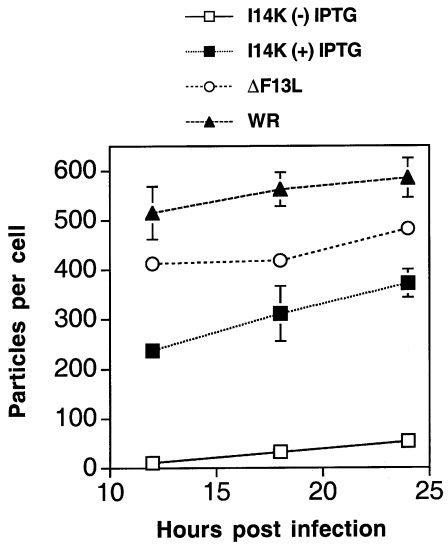

Fig. 3. Kinetic analyses of VV particles present in the cytoplasm of BS-C-1 cells infected with either WR, vΔF13L or WR32-7/Ind 14K (I14K) in the presence (+) or absence (−) of 10 mM IPTG. In each case cells were infected at 0·1 p.f.u. per cell. At the indicated time-points, cells were fixed and stained with MAb AB1.1 to show the distribution of VV particles. The number of VV particles within the cytoplasm was determined by confocal microscopy as described in Methods. Error bars represent the standard error of the mean where $n = 5$.

Localization of IMV particles relative to virus factories and wrapping membranes

During the process of virus morphogenesis IMV particles must move from virus factories to an area of the cytoplasm where they are wrapped by membranes to form IEV. To establish the sub-cellular location of accumulated IMV particles relative to wrapping membranes and virus factories, BS-C-1 cells were infected with WR32-7/Ind 14K in the presence or absence of IPTG for 12 h before being fixed and processed for fluorescent microscopy. When A27L expression was repressed clusters of IMV particles remained segregated from membranes containing the VV B5R protein (Fig. 4*a*). In contrast, when A27L expression was induced (+IPTG) virus particles infiltrated areas of the cell containing B5R-positive membranes and numerous D8L/B5R double-positive, enveloped particles

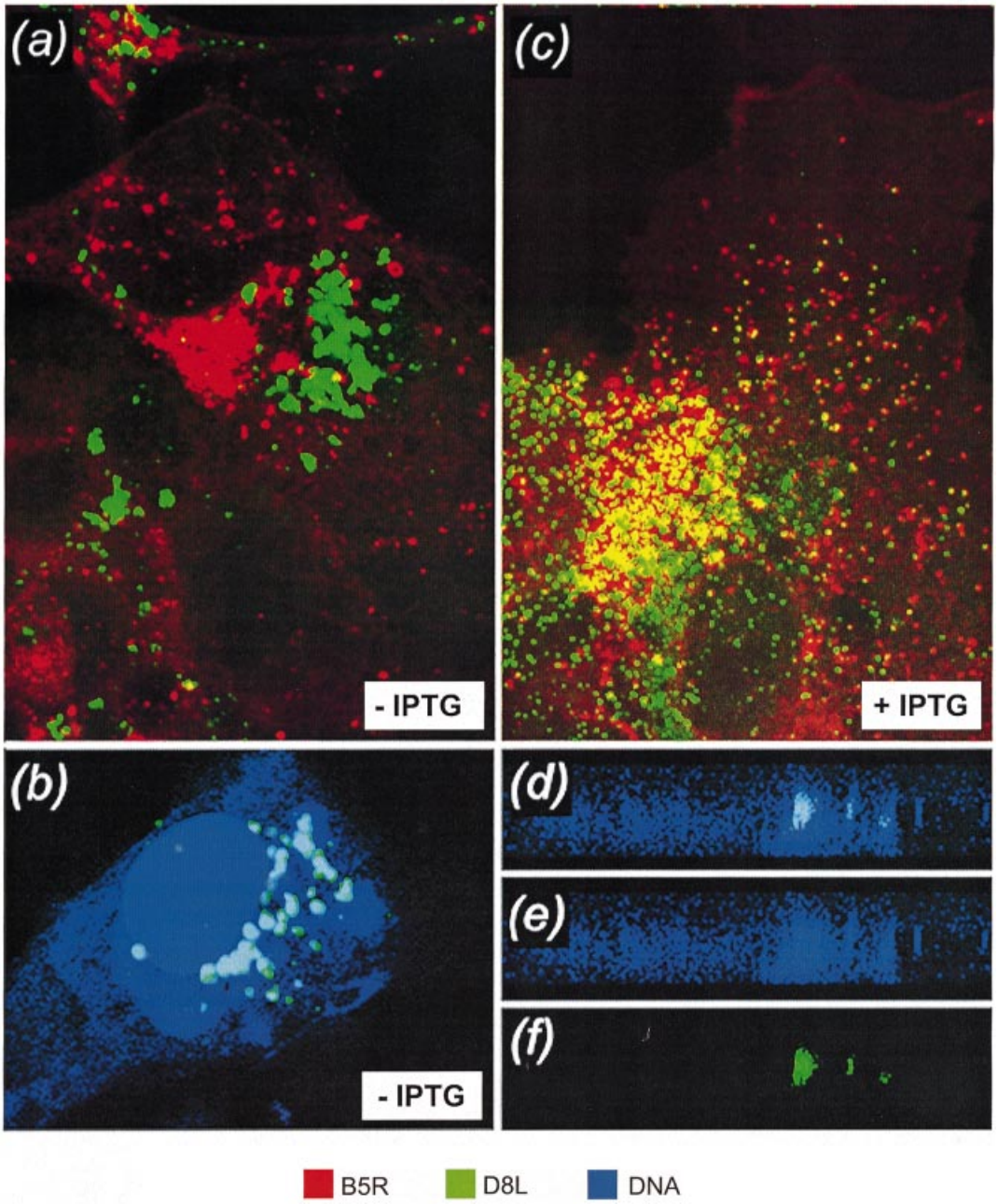

Fig. 4. Location of accumulated IMV particles relative to virus factories and B5R-containing cellular membranes. BS-C-1 cells were infected with VV WR32-7/Ind 14K at 1 p.f.u. per cell for 12 h in the presence (*c*) or absence (*a*, *b*, *d*–*f*) of IPTG. Cells were fixed and stained with MAb AB1.1 to detect virus particles and either MAb 19C2 to detect the VV B5R protein (*a*, *c*) or the DNA stain To-Pro (*b* and *d*–*f*). Bound MAb AB1.1 was detected using a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody while MAb 19C2 was visualized using a rhodamine B isothiocyanate (RITC)-conjugated donkey anti-rat antibody. The distribution of DNA is shown in blue. Panels (*a*)–(*c*) are projected reconstructions of whole cell images, while panels (*d*)–(*f*) show a single optical section taken vertically through an infected cell. Panel (*d*) shows a merged image of the distribution of DNA (blue) or D8L (green), as shown separately in panels (*e*) and (*f*), respectively.

were detected (Fig. 4*c*). These data suggest that when A27L expression is repressed, IEV particle formation is inhibited due to the inability of IMV particles to move to the correct subcellular location for IEV assembly. Comparison of the location of clustered IMV particles with virus factories visualized using the fluorescent DNA stain To-Pr (Molecular Probes) showed that in the absence of A27L expression IMV particles are retained close to virus factories (Fig. 4*b*). Also, optical sections taken vertically through an infected cell showed that IMV particles accumulate at the periphery of the virus factory (Fig.

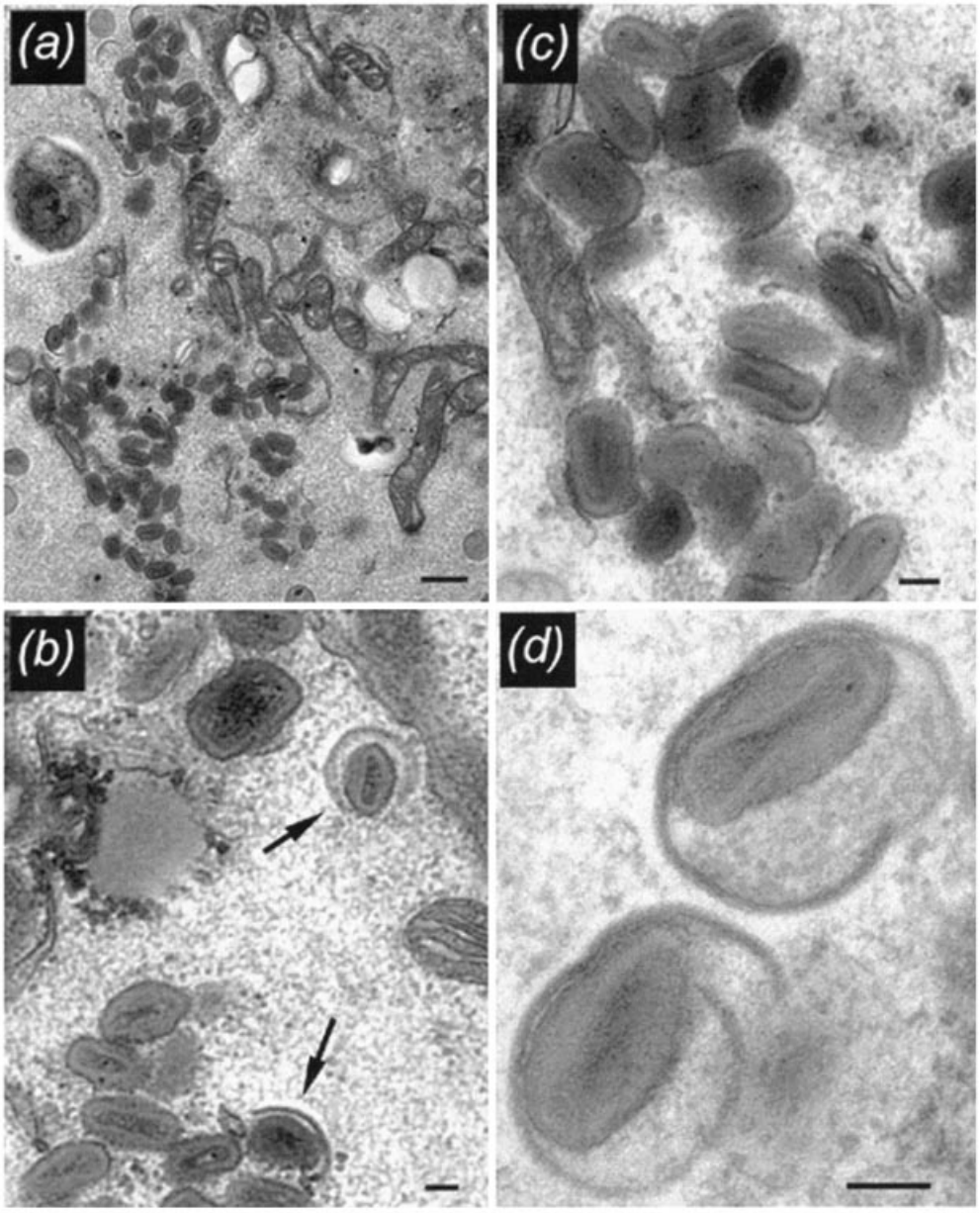

Fig. 5. Electron micrographs showing the sub-cellular location and morphology of accumulated IMV particles. BS-C-1 cells were infected with VV WR32-7/Ind 14K at 1 p.f.u. per cell for 12 h in the absence of IPTG. Panels (*a*) and (*c*) show the location of accumulated IMV particles relative to the virus factory and cytoplasmic organelles. Panels (*b*) and (*d*) show examples of aberrant IMV envelopment that occur when the expression of A27L is repressed. Size bars, 100 nm.

4*d–f*), suggesting that the defect in IMV particle transport occurs at the point at which IMV particles enter the cytoplasm. To assess this hypothesis further, BS-C-1 cells were infected with WR32-7/Ind 14K in the absence of IPTG for 12 h and samples were processed for electron microscopy. Sections taken through virus factories revealed peripheral clusters of morphologically mature IMV particles (Fig. 5*a*) in close proximity to granular areas of cytoplasm and cellular organelles (Fig. 5*c*). IMV particles undergoing aberrant secondary envelopment within virus factories were also observed frequently (Fig. 5*b*, *d*). These data confirm that export of IMV from virus factories is impaired in the absence of A27L, and suggest that recruitment of A27L to the surface of IMV particles may prevent deleterious interaction with other virus membranes within factories.

Expression of VV A27L results in microtubule-dependent dispersal of accumulated IMV particles

To confirm that the accumulation of IMV particles at the periphery of virus factories was due only to the lack of A27L

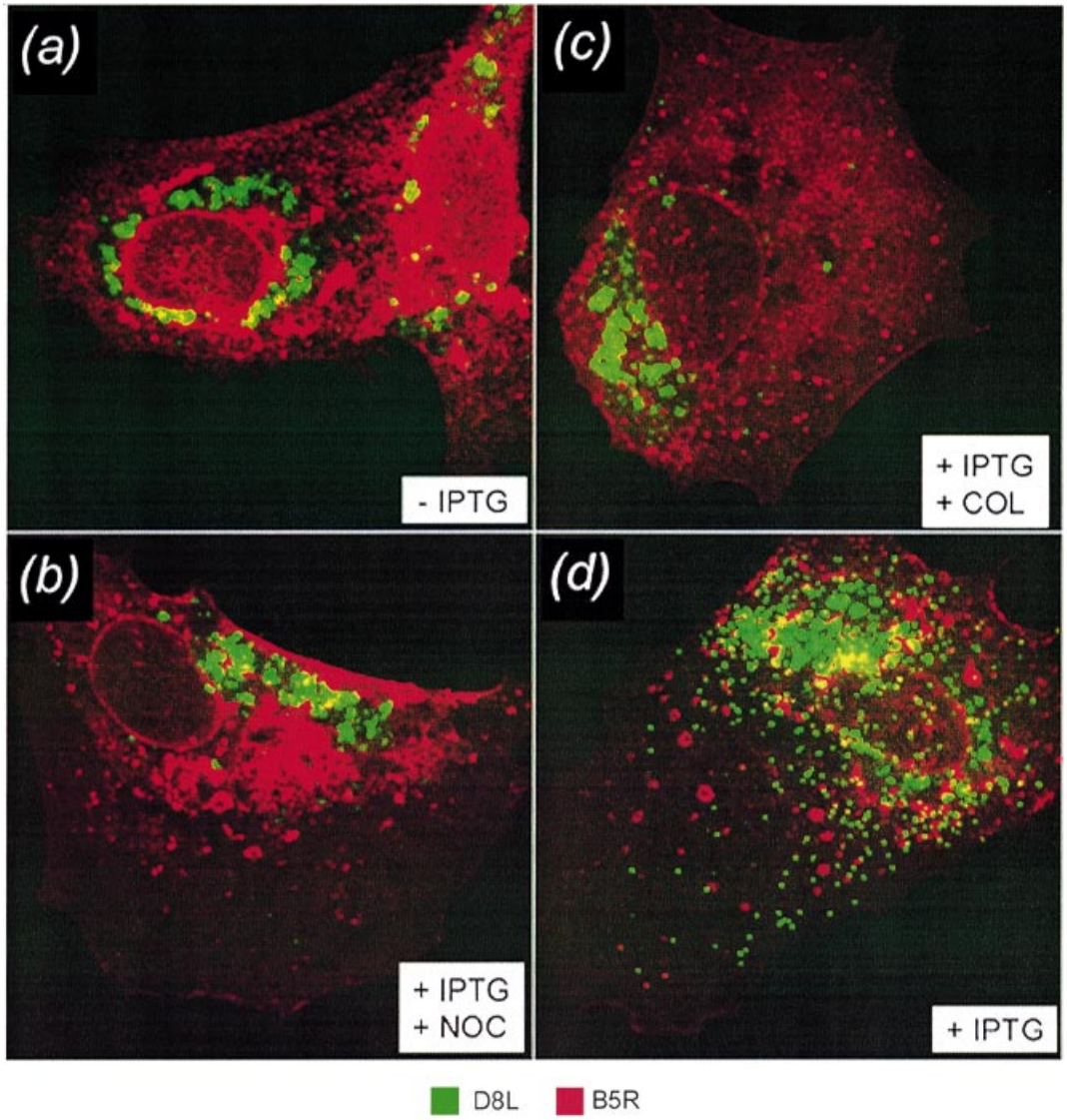

Fig. 6. Cytoplasmic dispersal of IMV particles is dependent upon both A27L expression and microtubules. BS-C-1 cells were infected with VV WR32-7/Ind 14K at 1 p.f.u. per cell. At 10 h p.i. rifampicin (40 μg/ml) was added to all cells to inhibit further IMV formation. At this time colchicine (100 μg/ml) or nocodazol (500 ng/ml) was added to cells shown in panels (*c*) and (*b*), respectively. At 11 h p.i. IPTG (10 mM) was added to cells shown in panels (*b*)–(*d*). All cells were fixed and processed for immunofluorescence at 16 h p.i. Virus particles were identified using MAb AB1.1 followed by a goat anti-mouse FITC-conjugated antibody, while B5R-containing membranes were detected using MAb 19C2 followed by a donkey anti-rat RITC-conjugated antibody.

expression, a rescue experiment was performed in which BS-C-1 cells were infected with WR32-7/Ind 14K for 10 h in the absence of IPTG. Before the addition of IPTG, cells were preincubated with 40 μg/ml rifampicin to prevent the formation of nascent crescents and IMV particles. Infected cells were then maintained in the presence or absence of IPTG for a further 3 h before being fixed and processed for fluorescent microscopy. Cells infected with WR32-7/Ind 14K and maintained in the absence of IPTG for 14 h exhibited characteristic perinuclear clustering of IMV particles with little co-localization with B5R-positive membranes (Fig. 6*a*). However, 3 h after induction of A27L expression at 11 h p.i. virus particles were detected throughout the cytoplasm and peripheral particles were co-stained with B5R and D8L showing that IEV (or CEV) particles were formed (Fig. 6*d*). Addition of drugs that disrupt microtubules, nocodazol (500 ng/ml) or colchicine (100 μg/ml), prior to the induction of A27L expression, did not affect the synthesis and steady state level of the 14K protein (data not shown), but prevented A27L-induced IMV transport and association with B5R-containing membranes (Fig. 6*b* and *c*, respectively). It is unlikely that the microtubule-inhibiting drugs prevented the association of the A27L protein with IMV particles, because the A27L protein does not appear to associate with microtubules in infected cells,

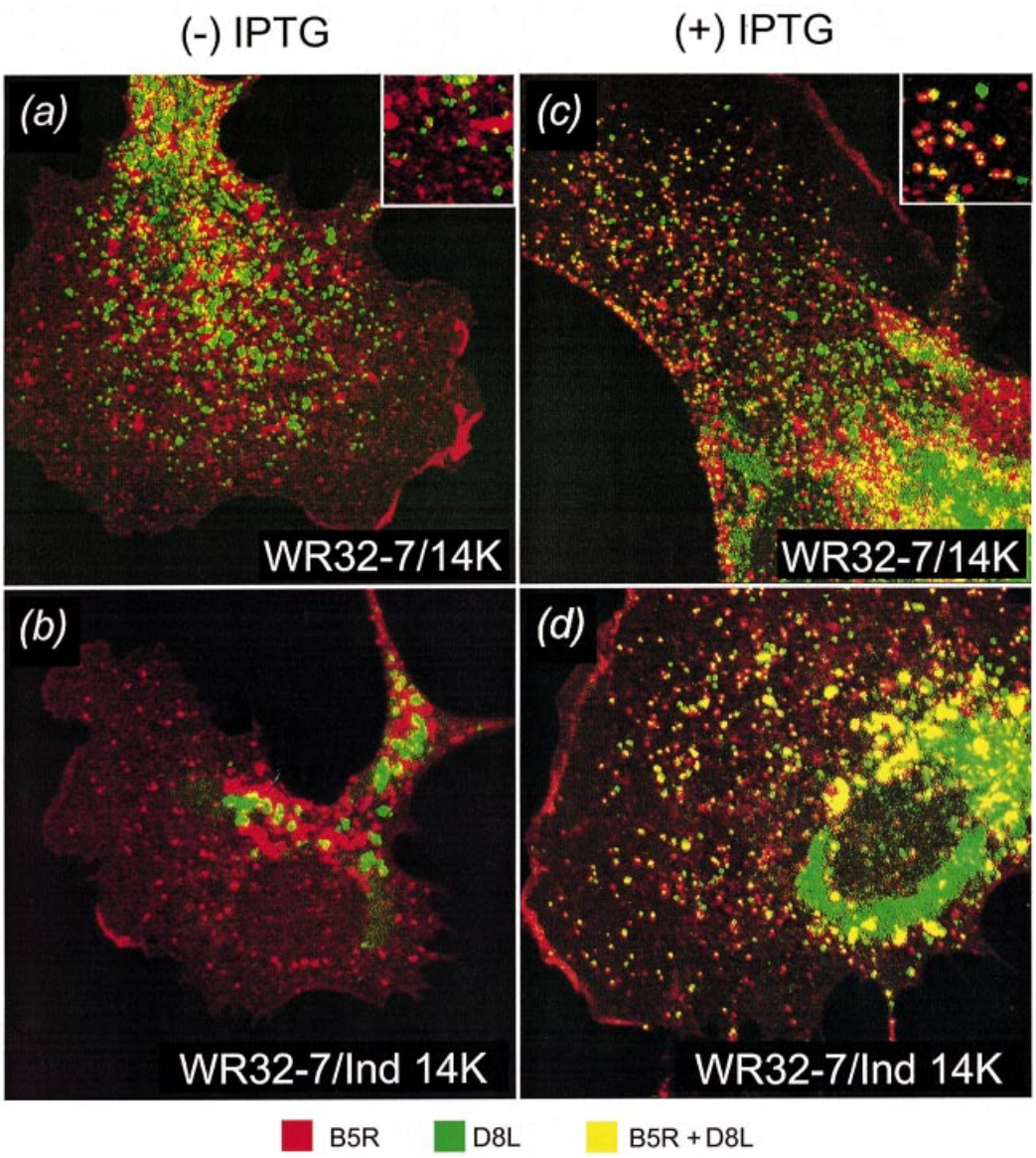

Fig. 7. Sub-cellular distribution of IMV particles following infection of BS-C-1 cells with VV WR32-7/14K or WR32-7/Ind 14K in the absence or presence of IPTG. BS-C-1 cells were infected with either VV WR32-7/14K (*a*, *c*) or WR32-7/Ind 14K (*b*, *d*) at 1 p.f.u. per cell for 12 h in the absence (*a* and *b*) or presence (*c* and *d*) of 10 mM IPTG. VV particles were detected using MAb AB1.1 followed by a goat anti-mouse FITC-conjugated antibody while B5R-containing membranes were detected using MAb 19C2 followed by a donkey anti-rat RITC-conjugated antibody. Inserts in panels (*a*) and (*c*) show enlarged areas of each cell to demonstrate the absence (*a*) or presence (*c*) of B5R/D8L (double-positive) enveloped virus.

but does associate with A17L protein on the IMV surface. These data demonstrate that both microtubules and A27L are required for the transport of IMV particles from virus factories to the site of IEV formation.

A27L is required for two distinct stages of IEV formation

As WR32-7/14K and WR32-7/Ind 14K are both known to be defective in IEV formation in the absence of IPTG, despite WR32-7/14K expressing a mutant 15·5 kDa version of A27L, we were interested to determine if each virus exhibited a similar defect in the cytoplasmic dissemination of IMV particles. To address this, BS-C-1 cells were infected with WR32-7/Ind 14K or WR32-7/14K in the presence or absence of IPTG (10 mM) for 12 h before being fixed and processed for fluorescent microscopy. In each case the distribution of virus particles was determined using MAb AB1.1 and donor IEV membranes were visualized using MAb C3. Fig. 7 shows that although both WR32-7/Ind 14K and WR32-7/14K fail to form double-stained (B5R/D8L), wrapped virus particles in the absence of IPTG, only WR32-7/Ind 14K exhibits a defect in

the cytoplasmic transport of IMV particles (Fig. 7*a*, *b*). In each case double-stained (B5R/D8L), wrapped virus particles were formed in the presence of IPTG (Fig. 7*c*, *d*) when expression of the WT A27L gene was induced. Although changes in the N terminus of the A27L gene inhibit normal plaque formation, a process that requires enveloped virus formation, this mutation does not prevent IMV migration (Fig. 7*a*). Therefore, it appears that the N-terminal part of A27L is required for targeting or envelopment of IMV particles while other parts of the protein are required for intracellular movement of IMV.

Discussion

The 14 kDa protein encoded by the A27L gene is involved in virus entry (Doms *et al*., 1990; Gong *et al*., 1990; Chung *et al*., 1998), plaque formation (Gong *et al*., 1989; Rodriguez & Smith, 1990*a*) and assembly of IEV (Rodriguez & Smith, 1990*b*). In this report we have used two closely related recombinant VVs, WR32-7/14K (Rodriguez & Smith, 1990 *a*) and WR32-7/Ind 14K (Rodriguez & Smith, 1990*b*), to characterize further the role of A27L in VV morphogenesis. Data presented show that WR32-7/14K and WR32-7/Ind 14K exhibit different defects in VV morphogenesis. When cells were infected with WR32-7/Ind 14K in the absence of IPTG, no A27L protein was expressed and there was a defect in the transport of IMV particles from virus factories. In contrast, when cells were infected with WR32-7/14K in the absence of IPTG, IMV particles appeared dispersed within the cytoplasm although IEV particles were not formed. Collectively, these results show that the A27L protein mediates both intracellular movement of IMV particles from virus factories and the correct targeting and/or envelopment of IMV by wrapping membranes. Also, these data show that the single amino acid substitution (Asp-25 for Ala) present in the 15·5 kDa version of the protein prevents correct targeting/envelopment but not IMV transport. A more detailed molecular dissection of the A27L gene is now needed to identify the amino acid sequences required for IMV movement.

Early work on the involvement of cytoskeletal components in VV assembly and dissemination concluded that both actin-containing microfilaments and microtubules were required for the formation of extracellular enveloped virus (Stokes, 1976; Hiller *et al*., 1979). Although the role of actin in movement of enveloped virus has been studied (Hiller *et al*., 1979, 1981; Hiller & Weber, 1982; Cudmore *et al*., 1996) and the requirement for several VV proteins defined (Wolffe *et al*., 1997, 1998; Mathew *et al*., 1998; Roper *et al*., 1998; Sanderson *et al*., 1998), the role of microtubules in VV assembly remains unclear. Neither the microtubule-dependent stage of VV assembly nor the VV proteins required were known. Data presented in this report show that movement of IMV particles from the periphery of virus factories to the site of IEV formation is microtubule-dependent. As such, this aspect of VV assembly is similar to that described for African swine fever virus (ASFV), which uses microtubules to facilitate intracellular movement of nascent virus particles (Carvalho *et al*., 1988; Alves de Matos & Carvalho, 1993).

Although our data show that both A27L and microtubules are required for the efficient cytoplasmic transport of IMV particles, the precise role of each in IMV movement remains unclear. One interpretation of these data would be that A27L mediates microtubule–IMV association directly. However, other explanations are possible. Firstly, the lack of A27L on the surface of IMV particles may prevent recruitment of other proteins (virus or host) that in turn mediate microtubule association or microtubule-dependent movement. Secondly, as a reduction in A27L expression induces inappropriate association between virus crescents and IMV particles (Fig. 5), it is possible that A27L may function to block deleterious interactions occurring between IMV particles or IMV particles and virus crescents. For example, A27L recruitment may block the interaction of A17L and A14L proteins in/on adjacent membranes of juxtaposed virus structures. Consequently, intracellular movement of IMV particles may be impeded by physical aggregation of particles when A27L expression is repressed. Induction of A27L expression at 11 h p.i. resulted in dispersal of IMV particles that were clustered at the periphery of virus factories. This suggests that any inter-particle interactions that occur are disrupted competitively in the presence of A27L.

As both A27L (p14) and F13L (p37) are required for IEV formation (Rodriguez & Smith, 1990*b*; Blasco & Moss, 1991) there has been speculation (Rodriguez & Smith, 1990*b*) that there may be direct interaction between A27L on the surface of IMV particles and F13L on the cytoplasmic surface of wrapping membranes (Hiller & Weber, 1985). Although no experimental evidence exists to support a direct A27L–F13L interaction, it is interesting to note that defects in the dissemination of IMV particles have also been reported when the F13L gene contains mutations which prevent palmitoylation (Grosenbach & Hruby, 1998) or when cells are infected with VV in the presence of the drug N^1-isonicotinoyl-N^2-3-methyl-4-chlorobenzoylhydrazine (IMCBH) (Hiller *et al*., 1981). Also, non-palmitoylated forms of F13L have been found in association with IMV particles (Grosenbach & Hruby, 1998). Given the similarities in the two phenotypes it is again tempting to speculate that accumulation of IMV particles results from incorrect A27L–F13L interaction. However, our data show that the sub-cellular dispersal of IMV particles was restricted more severely when cells were infected with WR32-7/Ind 14K (−IPTG) than when infected with vΔF13L or WR virus in the presence of IMCBH. This observation was also true in CEF cells where IMCBH-induced clustering of IMV particles had been observed previously (Hiller *et al*., 1981). Consequently, it is possible that defects resulting from the lack of A27L and F13L expression may be similar but not identical. Alternatively, the observed differences in IMV distribution in the absence of either A27L and F13L could reflect other genetic

variations in the genomes of WR and WR 32-7/Ind 14K viruses.

In conclusion, data presented show that the A27L protein is required to prevent deleterious interaction between IMV particles and virus membranes within virus factories, to facilitate efficient intracellular transport of IMV particles from virus factories to the site of IEV formation and for the targeting and/or envelopment of IMV particles during IEV formation. In addition, we have shown that movement of IMV particles from virus factories is dependent upon microtubules as reported for ASFV.

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