Movements of vaccinia virus intracellular enveloped virions with GFP tagged to the F13L envelope protein

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Vaccinia virus produces several forms of infectious virions. Intracellular mature virions (IMV) assemble in areas close to the cell nucleus. Some IMV acquire an envelope from intracellular membranes derived from the trans-Golgi network, producing enveloped forms found in the cytosol (intracellular enveloped virus; IEV), on the cell surface (cell-associated enveloped virus) or free in the medium (extracellular enveloped virus; EEV). Blockage of IMV envelopment inhibits transport of virions to the cell surface, indicating that enveloped virus forms are required for virion movement from the Golgi area. To date, the induction of actin tails that propel IEV is the only well-characterized mechanism for enveloped virus transport. However, enveloped virus transport and release occur under conditions where actin tails are not formed. In order to study these events, recombinant vaccinia viruses were constructed with GFP fused to the most abundant protein in the EEV envelope, P37 (F13L). The P37–GFP fusion, like normal P37, accumulated in the Golgi area and was incorporated efficiently into enveloped virions. These recombinants allowed the monitoring of enveloped virus movements in vivo. In addition to a variety of relatively slow movements ( < 0.4 μm/s), faster, saltatory movements both towards and away from the Golgi area were observed. These movements were different from those dependent on actin tails and were inhibited by the microtubule-disrupting drug nocodazole, but not by the myosin inhibitor 2,3-butanedione monoxime. Video microscopy (5 frames per s) revealed that saltatory movements had speeds of up to, and occasionally more than, 3 μm/s. These results suggest that a second, microtubule-dependent mechanism exists for intracellular transport of enveloped vaccinia virions.

Introduction

Vaccinia virus, the prototype of the poxviruses, is a large DNA virus, replication of which takes place in the cytoplasm of the infected cell (Moss, 1996). Virus particles contain more than 100 different polypeptides and a genomic DNA molecule encoding more than 200 genes. It is perhaps not surprising that vaccinia virus morphogenesis is a complex process involving many steps, many of which are poorly understood. Virion assembly proceeds through different morphological forms that, in addition to having different protein components, may have diverse functions in intracellular virus trafficking, cell-to-cell transmission or individual-to-individual transmission.

Vaccinia virus intracellular mature virus (IMV) particles are assembled in specialized areas of the cytoplasm located close to the cell nucleus. A portion of the IMV are wrapped by vesicles derived from the trans-Golgi network (TGN), acquiring a double membrane, to form what are called intracellular enveloped viruses (IEV) (Hiller & Weber, 1985; Schmelz et al., 1994). The egress of virions to the extracellular space is attained by fusion of the IEV outer membrane with the plasma membrane. Virus particles released to the medium, termed extracellular enveloped virus (EEV), contain an additional membrane with respect to IMV.

A number of observations indicate that the process of acquisition of the virus envelope is required for the exit of virions from the cell and therefore for virus transmission. Mutations that impede virus envelopment result in a block in EEV formation and virus transmission (Blasco & Moss, 1991; Rodriguez & Smith, 1990). For example, deletion of the F13L gene, encoding the major protein of the EEV envelope, results in a virus that forms normal amounts of IMV, but dramatically reduced amounts of EEV (Blasco & Moss, 1991, 1992). It is important to note that, even under these circumstances, IMV are not able to reach the extracellular...
space, since no significant amounts of IMV were found in the medium. Also, treatment with N\textsubscript{2}-isonicotinoyl-N\textsubscript{2}-3-methyl-4-chlorobenzoylhydrazine (IMCBH), a drug that blocks virus envelopment, blocks virus release completely (Hiller et al., 1981; Kato et al., 1969; Payne & Kristenson, 1979).

One aspect that has received attention recently is the intracellular transport of virus structures assembled at late times during infection. Fully assembled IMV particles must be transported from the assembly areas into areas containing the wrapping vesicles and IEV must subsequently be transported towards the cell periphery, where they fuse with the plasma membrane. Mechanisms accounting for some of these movements have been described. Recent studies indicate that the microtubule cytoskeleton is involved in IMV assembly and is required for IEV formation (Ploubidou et al., 2000) and for the transport of IMV to the sites of wrapping (Sanderson et al., 2000).

The most prominent feature of IEV transport is the induction of actin tails by IEV particles, which give rise to engorged microvilli at the cell surface with virus particles at the tip. These were recognized some time ago by electron microscopy (Stokes, 1976) and immunofluorescence (Hiller et al., 1979) and are the only well-characterized mechanism for the transport of enveloped virions. Actin tails are formed by nucleation of actin close to the cytosolic side of the outer IEV membrane (Cudmore et al., 1995, 1996, 1997; Way, 1998), a process that is dependent on the phosphorylation of viral protein A36R (Frischknecht et al., 1999a, b).

We present here a characterization of the dynamics of enveloped virus movement within the infected cell and provide evidence for mechanisms of IEV transport that are different from the induction of actin tails.

### Methods

**Cells, plasmids and virus.** BSC-1 and CV-1 cells were grown in minimal essential medium (MEM) supplemented with 5% foetal bovine serum (FBS). BHK-21 cells (ATCC CCL10) were grown in BHK medium containing 5% FBS, 3 g/ml tryptose phosphate broth and 0.01 M HEPEs. All cells were grown in a 5% CO\textsubscript{2} atmosphere at 37 °C. Plasmid pRB21 (Blasco & Moss, 1995) contains vaccinia virus gene F13L and flanking sequences and a synthetic early/late promoter placed downstream of the P37 coding sequence. vRB12 is a vaccinia virus mutant derived from the Western Reserve (WR) strain, in which 95% of the P37 stream of the P37 coding sequence is deleted (Blasco & Moss, 1992). Vaccinia virus infections were carried out in media containing 2% FBS and incubated at 37 °C in a 5% CO\textsubscript{2} atmosphere.

**Construction of W-rsGFP.** W-rsGFP, a vaccinia virus recombinant expressing an enhanced version of the GFP gene, rsGFP (Quantum Biotechnologies), was constructed by inserting the rsGFP gene into the vaccinia virus (WR strain) genome downstream of the F13L gene. Briefly, three DNA fragments containing the F13L gene, the rsGFP gene and recombination flanking sequences were amplified by PCR. Plasmid pRB21 was used as a template to amplify the F13L gene and left flanking sequence with oligonucleotides HF1510 (5’ GGACATGCTTATGTACGTAAGAAA 3’) and pRB21-1870 (5’ CATTATATATCCA-AAAAAAAATA 3’). The right flank of the vaccinia virus F13L gene was amplified from pRB21 by using the primers pRB21 1914 (5’ TAAATAAAAATTTTTATGGATCGG 3’) and HF3400 (5’ CGTTCTAAAGCTAGTCTATATATCTCCC 3’). The rsGFP gene was amplified by PCR from plasmid pQBI25 with oligonucleotides 5’ ATATGGCTAGCAAAGAGAGAAA 3’ and 5’ TTTTTAAAGGTTATCTACTTAGGTGACATTGTATATTC 3’. Overlapping ends were included in the oligonucleotides to facilitate the assembly of the PCR fragments. Insertion into the vaccinia virus genome (vRB12) was accomplished as described previously (Lorenzo & Blasco, 1998). W-rsGFP was isolated from progeny virus by three rounds of plaque purification on BSC-1 cells (Earl & Moss, 1991), during which plaques were screened for GFP fluorescence (Lorenzo & Blasco, 1998). The resulting virus, W-rsGFP, contained the F13L gene and the rsGFP gene downstream of a strong synthetic early/late promoter.

**Construction of W-P37g and I-P37g.** A vaccinia virus recombinant expressing the GFP gene fused to the C terminus of the F13L gene (W-P37g) was constructed as follows: two overlapping DNA fragments were amplified by PCR from W-rsGFP viral DNA. One of the fragments, containing the P37 coding sequence, was amplified by using the oligonucleotides HF1510 (5’ GGACATGCTTATGTACGTAAGAAA 3’) and P37/rsGFPPL (5’ CATTGTCTATATTTAAGCTTTTG- AATGGT 3’). The second fragment, including the rsGFP sequence, was amplified with primers P37/rsGFP (5’ GATGCAATTGCGAGCAAAAGGAAGAAA 3’) and HF3400 (5’ CGTTCTAAAGGTTATCTACTATATCCC 3’). This PCR fragment was then inserted into the P37 deletion mutant derived from IHD-J (vRB12) (Blasco & Moss, 1991). Virus I-P37g was isolated by selecting large virus plaques following purification on BSC-1 cells (Earl & Moss, 1991), during which plaques were screened for GFP fluorescence (Lorenzo & Blasco, 1998). The resulting virus, W-P37g, contained the F13L gene and the rsGFP gene fused to the P37 coding sequence and the chimeric gene is therefore under the control of the natural F13L promoter.

**Construction of W-gA33R.** For the construction of the recombinant virus W-gA33R, DNA from vaccinia virus WR was used as a template to amplify the A33R coding sequence with oligonucleotides A33Hila (5’ TAAATAGCCTTTATATTCTGATGACAC 3’; HindIII site underlined) and A33Hib (5’ GCCATTCTAAGCTTAAATCTGAC 3’; HindIII site underlined). The PCR product was digested with HindIII and cloned into plasmid pRSBrGFP, which had been digested previously with HindIII, resulting in pRB-gA33R. This plasmid was then used to insert the fused gene into virus vRB12, using procedures described previously (Blasco & Moss, 1995). The recombinant virus isolated, W-gA33R, contains, in addition to the natural A33R gene, the rsGFP gene fused to the A33R coding sequence, downstream of a strong synthetic early/late promoter.

**Construction of W-B5Rg.** For the construction of the recombinant vaccinia virus W-B5Rg, the C terminus of the B5R gene was amplified by PCR from vaccinia virus WR genomic DNA using primers BSerH (5’ CTACGGAAGCTTITGATCCAGTGGATCA 3’; HindIII site underlined) and B5R-N (5’ TTTAACAGCTACATTTGACGTCAGAC 3’; Nhel site underlined). The PCR product was digested with HindIII and Nhel and inserted into the HindIII/Nhel site upstream of the rsGFP gene.
in plasmid pFus (B. Perdiguerio and R. Blasco, unpublished), which contains the rsGFP gene and the *pac* gene (puromycin acetyltransferase), a selectable marker in vaccinia virus that confers puromycin resistance (Sanchez-Puig & Blasco, 2000). Primer B5R-N eliminated the stop codon at the end of the B5R gene and provided in-frame fusion with the rsGFP gene. The resulting plasmid (pFus-B5R) was used to transfect cells infected with vaccinia virus WR to construct virus recombinant W-B5Rg following procedures described previously (Earl & Moss, 1991; Sanchez-Puig & Blasco, 2000). This recombinant virus contained the rsGFP gene fused to the B5R coding sequence and the chimeric gene is under the control of the natural B5R promoter. Since W-B5Rg is the result of a single recombination event, and could produce the parental WR virus by intramolecular recombination, puromycin selection was applied during amplification of the virus stocks.

Western blotting. Western blots of infected cell lysates were carried out using BSC-1 cells grown in 6-well plates and harvested 24 h after infection. The cells were pelleted and lysed in 50 µl lysis buffer as described previously (Herrera et al., 1998). Proteins were electrophoresed in 12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes by electrophoresing. After transfer, the membranes were incubated overnight at 4 °C in blocking buffer (PBS containing 0–0.05% Tween 20 and 5% non-fat dry milk). The membranes were then incubated with monoclonal antibody anti-P37 (diluted 1:50) or monoclonal antibody anti-GFP (diluted 1:5000) (Clontech) for 1 h at room temperature in PBS supplemented with 0–0.05% Tween 20 and 1% non-fat dry milk. The membranes were then incubated with monoclonal antibody anti-P37 (diluted 1:50) or monoclonal antibody anti-GFP (diluted 1:5000) (Clontech) for 1 h at room temperature in PBS. After extensive washing with PBS-0.05% Tween 20, the membranes were incubated for 1 h at room temperature with rat or mouse anti-IgG antibody (diluted 1:3000) conjugated with horseradish per-
oxidase (Amersham) in PBS–0·05% Tween 20 containing 1% non-fat dry milk. After washing, membranes were incubated for 1 min with a 1:1 mixture of solution A [2·5 mM luminol (Sigma), 0·4 mM p-coumaric acid (Sigma), 100 mM Tris–HCl, pH 8·5] and solution B (0·018% H₂O₂, 100 mM Tris–HCl, pH 8·5) and exposed to X-ray film.

Fluorescence microscopy. BHK-21 cells grown to 70% confluence on round coverslips were infected with viruses at a multiplicity of 5 p.f.u. per cell. At 7 h post-infection (p.i.), the medium was removed and the cells were washed twice with PBS and fixed by the addition of ice-cold 4% paraformaldehyde for 12 min at room temperature. All subsequent incubations were carried out at room temperature. When permeabilization was desired, the fixed cells were incubated in PBS containing 0·1% Triton X-100 for 15 min. After washing with PBS, cells were incubated for 5 min with PBS containing 0·1 M glycine and then with primary antibodies diluted in PBS–20% foetal calf serum for 30 min. Rat monoclonal antibodies 15B6 (anti-P37) and 19C2 (anti-B5R) were made available by G. Hiller (Boehringer Mannheim, Germany). Rabbit polyclonal anti-A33R antiserum was provided by M. Way (European Molecular Biology Laboratory). After washing for 5 min in PBS, the cells were incubated for 30 min with rabbit anti-mouse IgG conjugated with TRITC (Dako) diluted 1:200. Some preparations were incubated with 0·02 mg/ml TRITC-conjugated wheat germ agglutinin (WGA) (Sigma), 2 μg/ml bisbenzimide Hoechst or 1 unit/ml Alexa594–phalloidin (Molecular Probes) for 30 min at room temperature.

Time-lapse microscopy. Cells were grown on round coverslips that were mounted at the bottom of sealed ludin chambers (Life Imaging Services, Olten, Switzerland). The chamber was mounted in a metal case containing an internal electric resistor with electronic temperature control. All experiments were performed at 37 °C. Where applicable, drugs were included in the cell culture medium at the following final concentrations: 10 μg/ml IMCBH, 10 μM nocodazole, 10 mM 2,3-butanedione monoxime (BDM). Cells were observed with a Nikon Diaphot or Nikon Eclipse T300 inverted microscope equipped with fluorescence. Digital images were captured with a SBIG ST-7 cooled CCD camera at resolutions of 765 × 510 or 384 × 256 pixels. In general, digital images were acquired at 6 or 8 s intervals after 7 h p.i. For high-speed video microscopy, images were acquired with a Hamamatsu C5985 CCD camera and captured from the composite video signal to a Sony DCR-TR7000E digital video camera and subsequently transferred to a computer using an IEEE-1394 card. Finally, distances were measured on digital images by using the PC version of the HIH image software (Scion; http://www.scioncorp.com).

Results

Incorporation of GFP into the IEV/EEV envelope

IEV are the result of the wrapping of IMV with membranes derived from the TGN. Of the six virus proteins present in the wrapping membranes, we chose three (F13L, B5R and A33R) as candidates to direct a GFP tag for incorporation into the virus envelope. Viruses W-P37g, W-B5Rg and W-gA33R were constructed by fusing GFP to the C terminus of F13L, the C terminus of B5R or the N terminus of A33R. As the unmodified proteins have characteristic intracellular locations (Lorenzo et al., 2000), we compared the fluorescence patterns produced by the fusion proteins with immunofluorescence staining of the wild-type proteins (Fig. 1). Mock-infected cells or cells infected with vaccinia virus WR showed no significant fluorescence (not shown). Of the versions tested, the P37–GFP fusion best matched the normal distribution of the unfused protein. GFP–A33R deviated significantly from the intracellular distribution of normal A33R (Fig. 1C, D). On the other hand, B5R–GFP attained juxtanuclear localization, but also produced a diffuse staining that was not apparent in the case of normal B5R (Fig. 1E, F). In addition, the peripheral staining did not coincide with that of normal B5R, because B5R–GFP showed fewer, larger structures, that may represent membrane vesicles rather than individual virions. Thus, we chose P37–GFP as a good candidate to direct GFP to the virus.

Fig. 2. Western blots probed with anti-P37 or anti-GFP. BSC-1 cells were mock-infected or infected for 24 h with the viruses indicated at the top of each panel. Western blots were probed with antibodies to GFP (upper panel) or P37 (lower panel). Positions of protein molecular mass markers are indicated (in kDa). Arrowhead: P37–GFP fusion protein. IHD-J and WR are the parental vaccinia virus strains. vRB10 and vRB12 are P37-deletion mutants derived from IHD-J and WR, respectively. W-rsGFP is a recombinant vaccinia virus expressing unfused GFP from a synthetic early/late vaccinia promoter. I-P37g and W-P37g are recombinants expressing the P37–GFP fusion protein in the IHD-J or WR genetic background.
GFP-tagged enveloped vaccinia virus

Day 1, 2, 3
WR, W-P37g, IHD-J, I-P37g

Fig. 3. Characterization of recombinant viruses. (A) Plaque formation by recombinant viruses. BSC-1 cell monolayers infected with parental viruses WR and IHD-J or recombinant viruses W-P37g and I-P37g were incubated for the number of days indicated on the left, stained with crystal violet and photographed. (B) Extracellular virus production by recombinant viruses. BSC-1 cells were infected at an m.o.i. of 10 and harvested at 48 h.p.i. Virus titres in cell lysates (filled bars) and culture medium (shaded bars) were titrated by plaque assay in BSC-1 cells. (C) Induction of actin tails by W-P37g virus. BHK-21 cells infected with W-P37g were fixed at 7 h.p.i., permeabilized and stained with Alexa594-phalloidin. A confocal image showing GFP fluorescence (green) and F-actin staining (red) is shown.

Characterization of W-P37g and I-P37g

Recombinant viruses W-P37g and I-P37g are expected to produce a protein of 67 kDa resulting from the fusion of P37 (F13L gene product) and rsGFP. Western blot analysis of W-P37g-infected cells demonstrated the presence of a protein with the expected molecular mass that reacted with antibodies to P37 and GFP (Fig. 2). Despite the natural P37 promoter driving expression of the recombinant protein, a slight decrease was noted in the amount of protein with respect to the amount of normal P37. P37 protein is required for virus envelopment, since the absence of P37 results in a block in IEV and EEV formation and, as a consequence, a block of virus cell-to-cell movement and long-range virus spread. Since P37 protein is replaced by the P37–GFP fusion protein in the W-P37g and I-P37g recombinant viruses, the phenotypes of the recombinant viruses should reflect the ability of the P37–GFP fusion protein to provide normal P37 function. Thus, recombinant viruses were compared with the respective parental strains for EEV formation and plaque phenotype, which are good indicators of P37 functionality (Fig. 3). Similarly sized virus plaques were formed by the WR-based parental and recombinant viruses, indicating that the fusion protein was fully functional. In addition, normal amounts of EEV were produced by W-P37g when compared to the parental WR strain. However, a small effect was noted in the case of the IHD-J-derived recombinant virus. In a standard plaque assay performed under liquid overlay, where viruses that release large amounts of EEV give rise to plaques with a characteristic comet shape, a reduction in the ‘comet’-forming ability of I-P37g was noted with respect to IHD-J. Since the ‘comet tail’ is believed to be formed by small secondary plaques derived from EEV released by the primary plaque, this is an indication of a decrease in EEV formation.
release. The effect on comet formation correlated with a slight decrease in the virus titres present in the medium after high-multiplicity infections (Fig. 3B). This small effect could be due to the slightly smaller amount of P37 that resulted from fusing GFP to this critical protein. We also checked the ability of the W-P37g recombinant virus to induce actin tails. Confocal microscopy of W-P37g-infected cells stained with phalloidin showed the presence of numerous actin tails with GFP-containing particles at their tips (Fig. 3 C). Overall, these results indicate that the P37–GFP fusion protein can functionally replace the normal P37 protein.

Visualization of the EEV outer envelope by fluorescence microscopy

To determine whether the fusion with rsGFP disrupted the normal targeting of P37 protein in infected cells, we studied the distribution of the P37–GFP fusion protein in infected cells. As discussed previously, cells infected with the recombinant virus expressing unfused GFP (W-rsGFP) produced fluorescence that was spread throughout the cell and was distributed both in the cytoplasm and the nucleus. Cells infected with W-P37g showed a distribution of P37–GFP similar to that of wild-type P37, showing intense juxtanuclear staining, suggesting a Golgi distribution, as well as a dispersed punctuate pattern that presumably corresponds to enveloped virions (see Fig. 1). To confirm that the juxtanuclear fluorescence localized to the Golgi region, we carried out labelling with TRITC-labelled WGA in W-P37g-infected cells (Fig. 4A–C). Clear co-localization in the Golgi region was evident, whereas other structures labelled by WGA, such as the nuclear membrane or the plasma membrane, did not show any significant GFP fluorescence. The peripheral punctate

Fig. 4. Intracellular localization of P37–GFP fusion protein. BHK-21 cells infected with vaccinia virus recombinant W-P37g were fixed at 7 h p.i. and stained with TRITC-conjugated WGA or Hoechst stain. (A)–(C) An infected cell viewed for GFP fluorescence (A) or TRITC–WGA fluorescence (B) and the merged image (C), showing GFP fluorescence in green and WGA staining in red. Note the co-localization of the bright juxtanuclear GFP staining with the Golgi complex. (D)–(F) General view of an infected cell for GFP fluorescence (D), DNA staining (E) and the merged image (F), showing GFP fluorescence in green and DNA staining in red. (G)–(H) GFP fluorescence (G) and DNA staining (H) in an area of the cell situated away from the cell nucleus (indicated by boxes in D and E), allowing the clear visualization of individual virions by Hoechst staining. Note the coincidence of GFP-labelled particles and DNA-containing structures, corresponding to enveloped virions. Arrows are included for comparison.
staining in W-P37g-infected cells consisted of bright spots in the image with a size (300 nm) attributable to that of single virus particles. To confirm that these were indeed virus particles, DNA was labelled with Hoechst stain (Fig. 4D–H). In most cells, virus factories were located close to the cell nucleus, around the Golgi complex. Individual virus particles were clearly detected by Hoechst staining in areas of the cell that were distant from the cell nucleus. In these areas, GFP, as well as DNA staining of virus particles, was evident (Fig. 4G, H). In addition to IEV labelling, the fusion protein was incorporated into the EEV envelope, since GFP fluorescence was detected on EEV particles released into the medium (data not shown). These results confirmed that P37–GFP attains the localization of normal P37 protein, providing a good fluorescent label for both envelope precursors (membranes of the wrapping compartment) and enveloped virions.

**IEV formation during infection**

One of the major advantages of GFP fusion proteins is that they make it possible to visualize the fusion protein within the cell without permeabilization or previous treatment with antibodies. Therefore, GFP fluorescence can be visualized easily in live cells. Expression of P37–GFP fusions allowed us to follow the appearance of envelope precursors and enveloped virions within infected cells. To obtain an overview of the process of enveloped virion formation, cells infected with W-P37g were photographed at 10 min intervals (Fig. 5). GFP fluorescence was detectable in the Golgi area at 4–5 h p.i. Punctate fluorescence representing enveloped virions started to appear at 5–6 h p.i. and became more evident between 5 and 6 h p.i. At later times, virions accumulated close to the cell surface and were particularly abundant in cell projections. In
Fig. 6. Enveloped virus movements. Examples of typical enveloped virion movements in W-P37g-infected cells are shown. Image of the cell showing the selected area are shown at the top. Series of 10 images taken at 6-8 s intervals are shown.
contrast, few IEV particles were present in the cytoplasm of I-P37g-infected cells (data not shown).

IEV movements

Time-lapse fluorescence microscopy was carried out to characterize the movements of enveloped virions in the infected cells. In W-P37g-infected cells, most of the virions that ended up close to the cell surface, accumulating in cell projections, were static. Some virions were located outside the cell body, presumably at the tips of enlarged microvilli. Many virus particles moved within the cytoplasm. Particles were seen moving toward the cell surface, but also in retrograde movements moving back towards the nucleus area. Virions could be seen both leaving the Golgi and moving into the Golgi. A close inspection of the image series taken at 6±8 s intervals revealed a variety of movements. Among these, some types of movements had recognizable patterns (Fig. 6). Video files are available as supplementary data in JGV Online (http://vir.sgmjournals.org). Firstly, some virions displayed continuous, slow, sinuous movements at up to 0.2 µm/s that were similar to those described for actin tails. More prominently, some virions performed faster, saltatory movements in approximately straight trajectories, both antegrade and retrograde. Under this frame-acquisition rate, these virions moved at maximum velocities of 0.3–1.0 µm/s for up to 35 s and then stopped. Over that time interval, virion ‘jumps’ were 3–25 µm in length, with a mean of approximately 10 µm. One striking feature observed was the apparent lack of directionality in the movement of virus particles. Both slow- and fast-moving particles moved towards and away from the cell periphery and, as a consequence, virions could be detected moving within the cells for long periods of time (> 20 min) without reaching the extracellular space.

Effects of nocodazole and BDM

The fast movements of vaccinia virus particles could be due to the action of microtubule- or microfilament-associated motors. To test this possibility, time-lapse microscopy was performed on cells treated with the microtubule-depolymerizing drug nocodazole or with the myosin inhibitor BDM (Higuchi & Takemori, 1989; McKillop et al., 1994). Since enveloped virions moved for long periods of time, a short treatment with nocodazole or BDM allowed us to test the effect of these drugs on pre-existing IEV. The effect of these drugs on infected cells was confirmed by tubulin staining (for nocodazole treatment) or changes in cell shape (for BDM treatment) (not shown). The faster virion movements in each sample were measured on series of digital images and the maximum speeds for these virions were measured (Fig. 7). Fast-moving virions were easily recognizable in time-lapse series of W-P37g-infected, untreated cells (mean maximum speed of 0.48 ± 0.17 µm/s, n = 75) or cells treated with BDM (mean maximum speed of 0.48 ± 0.23 µm/s, n = 75). In contrast, nocodazole treatment resulted in a clear decrease in fast movements (mean maximum speed of 0.23 ± 0.16 µm/s, n = 75), suggesting an involvement of microtubules in these movements. Incubation with both BDM and nocodazole did not result in further inhibition (mean maximum speed of 0.24 ± 0.23 µm/s, n = 75). Similar results were obtained in the case of I-P37g (see Fig. 7).

Fig. 7. Effects of BDM and nocodazole on virus movements. The maximum speeds of moving particles (n = 75) were measured in time-lapse series of images of cells infected with W-P37g (A) or I-P37g (B). At 7 h p.i., cells were treated with 10 mM BDM or 10 µM nocodazole (Noco) or with the two drugs (BDM+Noco). Cells were maintained in the presence of the drug for 1 h before observation by fluorescence microscopy. Mean speeds and the standard deviation for 75 moving particles from at least five experiments are shown.

below, where the moving particle is indicated by the arrow. The graphs show the x,y coordinates, in µm, for the selected particle in the series of images. (A) Fast, saltatory movement of a virus particle. For this particle, the ‘jump’ was 8–5 µm, with a maximum speed of 0.8 µm/s. (B) Continuous, sinuous movement of a virus particle. Particle speeds between images ranged between 0.1 and 0.4 µm/s. Video files showing virion movements are available as supplementary data at JGV Online (http://vir.sgmjournals.org).
Fig. 8. Virion movements after IMCBH reversion. Cells infected with I-P37g were treated with 10 µg/ml IMCBH at 3 h p.i. At 20 h p.i., IMCBH was removed and fresh medium containing 10 µM nocodazole was added. One hour after removal of IMCBH, time-lapse microscopy was performed as described in Methods. Examples of fast, saltatory particle movement in the absence of nocodazole (A) and continuous particle movement in the presence of nocodazole (B) are shown. For further details, see legend to Fig. 6. Video files showing virion movements are available as supplementary data at JGV Online (http://vir.sgmjournals.org).
Since nocodazole inhibits the transport of IMV to the Golgi area (Sanderson et al., 2000), it could be argued that the lack of fast IEV movements was due to the block in IEV formation. However, under the conditions used, slow movements of IEV were not affected by the presence of nocodazole, indicating that IEV formed before the nocodazole block were still present in the cytoplasm (not shown). To rule out completely the possibility that the nocodazole block on IMV transport was responsible for its effect on IEV movements, additional experiments were performed in which cells infected with W-P37g or I-P37g were treated first with IMCBH, a drug that blocks IMV wrapping. After IMCBH washout, cells were treated with 10 µM nocodazole and analyzed by time-lapse fluorescence microscopy (Fig. 8). Video files are available as supplementary data in JGV Online (http://vir.sgmjournals.org). After IMCBH removal, fast movements similar to those observed in the normal infection were detected (mean maximum speed of 0.41 ± 0.16 µm/s, n = 25). These fast movements were absent if nocodazole was included after IMCBH removal (mean maximum speeds of 0.16 ± 0.09 µm/s, n = 25), confirming the involvement of microtubules at a step after IMV had been transported to the Golgi area.

**Video microscopy**

Particle speeds recorded by time-lapse microscopy render average values for the time between frames. Because of the long time intervals (6–8 s) used in the previous experiments, it was likely that virion speeds measured for the saltatory movements were underestimates of the maximum virion speeds. To clarify this, and to follow the movements within shorter periods, we carried out video microscopy experiments, using continuous, high-intensity illumination of the sample and 0.2 s exposures. This allowed us to record 5 frames per s for 15–60 s, after which the fluorescence of the sample was greatly diminished. These experiments showed that virions undergoing fast movements underwent frequent starts and stops, reaching speeds of up to 3 µm/s (see examples in Fig. 9). Occasionally, speeds exceeding 4 µm/s were recorded. As expected, virion speeds of fast-moving virions recorded at 0.2 s (mean 0.97 ± 0.73 µm/s, n = 594) were significantly higher than those recorded for longer time intervals. These results confirm that fast-moving virions had saltatory movements.

**Discussion**

We have used GFP directed to the vaccinia virus envelope to study kinetic aspects of virus trafficking during the later stages of the virus life-cycle. We isolated a recombinant virus in which the P37 gene, encoding the major protein in the EEV envelope, was replaced by a P37–GFP fusion gene. The recombinant virus expressing P37–GFP, unlike GFP fusions with B5R or A33R, produced a fluorescence pattern similar to that of the unfused protein. In infected cells, individual virions were easily detected by GFP fluorescence and therefore provided a means of tracking individual virions during infection. Interestingly, outside the Golgi area, few or no GFP-containing vesicles that differed from virus particles were present in the cytoplasm, indicating that exit of the P37–GFP protein from the Golgi was dependent on the wrapping process. The co-localization of GFP fluorescence with enveloped virions but not with similarly sized cytoplasmic structures allowed us to investigate the transport of enveloped virions within the cell.

In a recent report (Ward & Moss, 2001), GFP fused to the cytoplasmic tail of protein B5R was used to label the virus envelope. We found that a similar GFP–B5R fusion had a distribution that deviated from that of the wild-type B5R protein, although virus-like structures were clearly labelled (Fig. 1). One interpretation of our results is that fusion of the cytosolic domain of B5R with GFP may to some degree affect the intracellular distribution of the fusion protein or its incorporation into the EEV envelope. In any event, it seems likely that both the B5R and F13L GFP fusions are useful for visualizing enveloped virions. The results shown here, obtained with the F13L–GFP fusion, are in good agreement with those described for the B5R–GFP-expressing virus.

The only well-described IEV transport mechanism is the induction of actin tails that propel the virus out of the cell and into neighbouring cells. However, some virus mutants that do not induce actin tails are able to produce virus that reaches the extracellular space (Herrera et al., 1998; Mathew et al., 1998; Wolffe et al., 1997), implying that alternative mechanisms for IEV transport must exist. In this report, we have shown IEV movements that suggest the existence of at least one additional mechanism. In particular, fast, saltatory movements were demonstrated, that are clearly distinguishable from those driven by actin tails. That these fast movements could be dependent on microtubules is supported by the following observations: (i) virion speeds were 5- to 50-fold higher than those of virions propelled by actin tails (Cudmore et al., 1995) and were compatible with those provided by microtubule-dependent organelle movements (Lippincott-Schwartz et al., 2000), (ii) movements of virions were saltatory and followed approximately straight trajectories, suggesting that they were moving along pre-existing longitudinal structures and (iii) the microtubule-depolymerizing agent nocodazole, but not the myosin inhibitor BDM, blocked these movements.

Our results, however, do not rule out a potential role for myosin motors in IEV transport. It has been clearly established that the actin and microtubule cytoskeletons cooperate in organelle transport in a variety of situations (Goode et al., 2000; Rogers & Gelfand, 2000). For example, secretory vesicles move by both microtubule- and microfilament-based motors. It is possible that the slower IEV movements that persist in the
The fast IEV movements observed displayed speeds of up to 3 µm/s. In addition, a variety of slower movements were recorded that may be accounted for by actin tails and additional mechanisms. It is notable that even the slow movements described here are at least 100-fold faster than the calculated rate of IEV diffusion (Sodeik, 2000), reinforcing the idea of the existence of active transport mechanisms for virus particles.

The fast IEV movements detected in infected cells are reminiscent of normal Golgi-to-plasma membrane vesicular transport (Lippincott-Schwartz et al., 2000). Post-Golgi carriers are generated as tubules that extend from the Golgi complex (Hirschberg et al., 1998). Vesicle transport from the TGN to the plasma membrane is dependent on microtubules and has been reported to have maximum speeds of 0.2–0.4 µm/s (Kreitzer et al., 2000), 0.7 µm/s (Toomre et al., 1999), 1 µm/s (Wacker et al., 1997) or 2–7 µm/s (Hirschberg et al., 1998). We have observed several features, such as tubules extending from the Golgi and virions reversing direction, that are also seen in the generation of normal post-Golgi carriers (Hirschberg et al., 1998; Kreitzer et al., 2000; Wacker et al., 1997). Whether IEV behave functionally like normal cellular post-Golgi carriers remains an open question. It is conceivable that virus infection...
could modify microtubule-dependent transport mechanisms to facilitate IEV transport. Interestingly, several virus-encoded proteins are associated with microtubules during infection (Ploubidou et al., 2000), raising the possibility that these or other virus-encoded proteins could be involved in the modulation of microtubule-dependent IEV transport.

Ploubidou et al. (2000) have recently reported a study of the effect of vaccinia virus infection on the microtubule network. These authors note that, late during infection (5–12 h p.i.), the microtubule cytoskeleton is disorganized, giving rise to several types of aberrant organizations in which the microtubules are randomly oriented or form bundles or rings around the cell nucleus. It is unclear how modifications of the microtubule network may affect virus movements. We have consistently observed that fast virion movements do not occur only in the Golgi-to-plasma membrane direction, or only in the reverse direction, but occur in all directions. It is likely that the apparent lack of defined directionality of these movements may be related to the disorganization of the centrosome at late times of infection. In any event, our results support the notion that non-continuous, recurrent and reverse virion movements make IEV transport a surprisingly inefficient process.

At present, it is not known whether the different mechanisms for the transport of IEV fulfill different functions in the spread of virus in the tissue. For instance, transport of virions by actin tails correlates with plaque size but not with EEV formation, suggesting that the primary function of these virions is the direct cell-to-cell transmission of virus (Cudmore et al., 1995; Sanderson et al., 1998). Conversely, IEV transport by a different mechanism, such as the one depicted here, could produce enveloped virions free in the extracellular medium that would be optimal for long-range virus spread. Alternatively, different mechanisms could be involved in consecutive steps in the transport of enveloped virions from the Golgi area to the cell periphery, to the proximity of the plasma membrane and finally into the extracellular space.

Maria M. Geada and Inmaculada Galindo contributed equally to this work. This work was supported by contracts CT98-0456 and CT98-0225 from the European Commission and grant PB98-0046 from Dirección General de Investigación Científica y Técnica, Spain. We thank Stuart Isaacs for critical reading of the manuscript. Maria Lorenzo was the recipient of a fellowship from the Instituto Nacional de Investigaciones Agrarias, Spain.

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Received 27 April 2001; Accepted 9 July 2001
Published ahead of print (26 July 2001) in JGV Direct as DOI 10.1099/vir.0.17824-0