Entry of the vaccinia virus intracellular mature virion and its interactions with glycosaminoglycans

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Vaccinia virus (VACV) produces two distinct enveloped virions, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV), but the entry mechanism of neither virion is understood. Here, the binding and entry of IMV particles have been investigated. The cell receptors for IMV are unknown, but it was proposed that IMV can bind to glycosaminoglycans (GAGs) on the cell surface and three IMV surface proteins have been implicated in this. In this study, the effect of soluble GAGs on IMV infectivity was reinvestigated and it was demonstrated that GAGs affected IMV infectivity partially in some cells, but not at all in others. Therefore, binding of IMV to GAGs is cell type-specific and not essential for IMV entry. By using electron microscopy, it is demonstrated that IMV from strains Western Reserve and modified virus Ankara enter cells by fusion with the plasma membrane. After an IMV particle bound to the cell, the IMV membrane fused with the plasma membrane and released the virus core into the cytoplasm. IMV surface antigen became incorporated into the plasma membrane and was not left outside the cell, as claimed in previous studies. Continuity between the IMV membrane and the plasma membrane was confirmed by tilt-series analysis to orientate membranes perpendicularly to the beam of the electron microscope. This analysis shows unequivocally that IMV is surrounded by a single lipid membrane and enters by fusion at the cell surface.

INTRODUCTION

Vaccinia virus (VACV) is the most intensively studied poxvirus (Moss, 2001). The study of VACV entry into cells has been complicated by the presence of two morphologically distinct infectious virions, called the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). These virions are surrounded by different numbers of membranes (Appleyard et al., 1971), they have different proteins on their surface (Payne & Norrby, 1976; Payne, 1978) and they bind to different cell receptors (Vanderplaschen & Smith, 1997). The majority of IMV remain intracellular until cell lysis, but some become wrapped by additional membranes during a complex morphogenesis pathway to form intracellular enveloped virus (IEV). IEV are transported on microtubules to the cell surface, where they fuse with the plasma membrane to expose a virion on the cell surface. This virion may be retained on the cell surface as cell-associated enveloped virus (CEV) or released as EEV (reviewed by Smith et al., 2002). Despite being the more abundant and simpler form of VACV to work with, the process of IMV binding and entry is poorly understood.

Virus assembly occurs in cytoplasmic factories, where crescent-shaped membranes extend to form oval structures that become packaged with the viral DNA, enzymes and core proteins. The origin and the number of membrane layers present in the crescents are disputed. Early investigators proposed that IMV particles are surrounded by a single lipid bilayer that is synthesized de novo (Dales, 1963; Dales & Mosbach, 1968). Later, others claimed that IMV is surrounded by two membranes that are derived from the intermediate compartment (IC) between the endoplasmic reticulum and the Golgi apparatus (Sodeik et al., 1993). Another study concluded that the IMV membrane has a single lipid bilayer (5 nm in width) that lacks continuity with cellular membranes (Hollinshead et al., 1999). It was also proposed that the IMV contains a continuously folded membrane cisterna that is wrapped around the core and exterior, so that the virion has multiple membranes and is not enclosed in a continuous (sealed) lipid envelope (Griffiths et al., 2001). The number of membranes surrounding VACV particles is fundamental to virus entry. A single IMV membrane simplifies the re-entry process, but poses the question of how it is formed. A double membrane can be derived more easily from existing membranes, but creates a topological problem for virus entry.

The cellular receptors and the viral attachment protein for
VACV are unknown. Cell-surface glycosaminoglycans (GAGs) are highly anionic, linear polysaccharide chains that are composed of sulphated disaccharide units (Kjellen & Lindahl, 1991). Three IMV envelope proteins were reported to bind to GAGs. The 14 kDa protein, encoded by gene A27L, is required for EEV formation (Rodriguez & Smith, 1990) and was implicated in cell fusion because mAbs to this protein inhibited cell fusion (Rodriguez et al., 1987; Gong et al., 1990; Vázquez et al., 1998; Vázquez & Esteban, 1999). A27L also binds cell-surface heparan sulphate (HS) (Chung et al., 1998), possibly via its positively charged N-terminal 11 aa (Hsiao et al., 1998). Another IMV surface protein, D8L, competes with IMV for binding to cells and interacts with a different GAG, chondroitin sulphate (CS) (Hsiao et al., 1999). However, the same group showed previously that soluble CS did not inhibit IMV infectivity (Chung et al., 1998), so the significance of CS as the IMV receptor is uncertain. In addition, a double-mutant virus revealed that A27L- and D8L-negative virions were still infectious, suggesting that other viral protein(s) must exist to mediate virus–cell interactions (Hsiao et al., 1999). The H3L protein was also implicated in IMV binding because soluble H3L protein bound to HS on cells and competed with the binding of VACV (Lin et al., 2000).

The first study on VACV entry investigated the entry of IMV into L cells by using electron microscopy (EM) and proposed that IMV enter cells by phagocytosis, because virions were observed inside intracellular vesicles (Dales & Siminovitch, 1961). Later, other researchers reported that IMV fused with the plasma membrane and images were presented showing the IMV membrane in continuity with the cell membrane (Armstrong et al., 1973; Chang & Metz, 1976). A similar observation was reported earlier with an insect poxvirus (Granados, 1973). The use of lysosomotropic drugs that prevent acidification of endosomes yielded contradictory conclusions from different studies. Some groups found that these drugs inhibited IMV entry (Dales, 1963; Dales & Kajioka, 1964; Payne & Norrby, 1978), whereas others found that they had no effect (Janceczko et al., 1987; Doms et al., 1990; Chillakuru et al., 1991; Vanderplaschen et al., 1998). Another entry mechanism was proposed by those who believe that the IMV particle is surrounded by multiple membranes: they suggested that the IMV is unwrapped to discard all its membranes outside the cell and the core then somehow crosses the plasma membrane (Krijnse Locker et al., 2000; Sodeik & Krijnse-Locker, 2002). This proposal is inconsistent with the repeated observation that virus cores are recognized by anti-core antibodies only after they have entered the cell, and not at the cell surface (Vanderplaschen et al., 1998; Krijnse Locker et al., 2000; Carter et al., 2003; Senkevich et al., 2004a). Krijnse Locker et al. (2000) also reported that IMV binding to HeLa cells induces the formation of actin protrusions that are essential for the entry of cores, but this has been contradicted (Senkevich et al., 2004a). Recently, the A28L protein was shown to be essential for entry of IMV and CEV virions (Senkevich et al., 2004a, b).

In this study, we have reinvestigated the role of GAGs in IMV binding and show that the effect of soluble GAGs on IMV infectivity is cell type-specific, suggesting that IMV can enter cells in a GAG-independent manner. In addition, IMV entry was re-investigated by electron and confocal microscopy and we demonstrate that IMV enters by fusion at the cell surface. After fusion, the viral membrane was in direct continuity with the plasma membrane and flattened into the plane of the cell surface. A naked core was released into the cytosol. Tilt-series analysis showed unequivocally that IMV has a single membrane.

METHODS

Reagents. Low-molecular-mass heparin (M_r 4000–6000) (HP), heparan sulphate (HS), chondroitin sulphate A (CSA), chondroitin sulphate B (dermatan sulphate) (CSB), chondroitin sulphate C (CSC) and poly-L-lysine (M_r 4000–15 000) (PL) were purchased from Sigma. High-molecular-mass dextran sulphate (M_r 500 000) (DS-HMM) and low-molecular mass dextran sulphate (M_r 5000) (DS) were obtained from BDH and Fluka, respectively. High-molecular-mass heparin (M_r 15 000) (HP-HMM), oversulphated HP (M_r 13 000) (HP-OverS) and de-sulphated HP (M_r 12 000) (HP-DeS) were purchased from Neoparin.

Cells and virus. BS-C-1, BSC-40, RK13 and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). PtK2 cells were grown in low-glucose DMEM (Gibco) with 10% FBS. BHK-21 cells were grown in Glasgow minimum essential medium (Sigma) with 5% tryptose phosphate broth (Sigma) and 10% FBS. Virus infections were performed in DMEM 2.5% FBS.

VACV strain Western Reserve (WR) IMV was purified from infected RK13 cells by sucrose density-gradient centrifugation as described previously (Law & Smith, 2004). IMV from modified virus Ankara (MVA) was purified from infected chick-embryo fibroblasts in a similar way. Fresh EEV was obtained from the supernatant of BHK-21 cells infected at 3 p.f.u. per cell for 24 h. The supernatants were harvested and clarified by centrifugation for 10 min at 650 g and stored on ice until required (Law & Smith, 2004).

Electron microscopy (EM). Purified IMV was either bound passively (500 p.f.u. per cell) or spinoculated (325 p.f.u. per cell for WR and 225 p.f.u. per cell for MVA) at 650 g for 1 h at 4°C onto chilled PtK2 cells. MVA IMV was also spinoculated onto HeLa cells (31 p.f.u. per cell). Cells were washed with ice-cold PBS and samples were either fixed for virus-binding analysis or incubated at 37°C for 10 min to permit virus entry. Samples were fixed for 10 min on ice with 4% paraformaldehyde (PFA) in 250 mM Hepes, followed by 8% PFA at room temperature for 50 min and quenched for 20 min with 20 mM glycine in PBS.

Pre-embedding immunogold labelling was performed on all samples as described previously (Hollinshead et al., 1999). The IMV surface was labelled with mouse mAb AB1.1 (anti-D8L, diluted 1:100) for 1 h (Parkinson & Smith, 1994), followed by rabbit anti-mouse IgG (diluted 1:50). All samples were incubated with protein A conjugated to 6 nm gold particles (diluted 1:100) for 1 h before processing for conventional EM. Samples were viewed by using an FEI Tecnai G2 electron microscope with a Soft Imaging System Megaview III CCD camera. Images were collected at 1376 x 1032 x 16 pixels by using AnalySIS version Docu software. This microscope was capable of tilting the specimens in the goniometer and was used for all tilt series.
Confluent microscopy. Purified IMV and fresh EEV produced from vA5L-EGFP-N-infected cells (Carter et al., 2003) were spinoculated or bound passively to cells to obtain approximately 200 particles per cell. After binding, samples were either fixed (Rodger & Smith, 2002) or overlaid with DMEM/2·5 % FBS and incubated at 37°C for 8 h before fixing. Samples were also incubated at 37°C for 10 min, fixed and processed for immunofluorescence by using a rabbit antibody to green fluorescent protein (GFP) (Molecular Probes) (diluted 1:100), followed by tetramethylrhodamine isothiocyanate-conjugated donkey anti-rabbit antibody to detect uncoated viral cores. Samples were viewed on a Zeiss 510 Meta confocal microscope using Zeiss LSM software.

Effect of soluble GAGs on IMV infectivity. All soluble GAGs were diluted to 50 μg ml⁻¹ in DMEM/2·5 % FBS and mixed with an equal volume of purified IMV (300 p.f.u.) for 1 h at 37°C. The mixture was added to BS-C-1 cells for 90 min at 37°C. To repeat the protocol of Chung et al. (1998), GAGs and virus were incubated at 4°C for 30 min and then added to different cells for 30 min at 37°C. After the inoculum was removed, the cells were overlaid with semi-solid overlay (1 × DMEM, 2·5 % FBS, 1·5 % carboxymethyl cellulose), incubated at 37°C until plaques developed and stained with 0·1 % crystal violet in 15 % ethanol.

RESULTS

Interaction of IMV with GAGs

HS and CS have been implicated in IMV binding (see Introduction). To reinvestigate the role of GAGs in IMV binding, we measured IMV infectivity in the presence of a variety of soluble GAGs, including HS, HP, CSA, CSB (also known as dermatan sulphate) and CSC. We also used a chemically sulphated, branched polysaccharide (DS). To investigate whether the size of the GAG is important, low- and high-molecular-mass varieties of HP and DS were tested. To determine the role of charge, over- and desulphated HP, as well as a polycationic polypeptide, PL, were tested in parallel.

Purified IMV was incubated with the above ionic polymers (25 μg ml⁻¹) for 1 h at 37°C and the mixture was then used to infect BS-C-1 cells for 90 min at 37°C. Fig. 1 shows that IMV infectivity was affected slightly by some soluble GAGs, but several others had very little effect. The greatest inhibition was achieved with oversulphated HP (HP-OverS) and DS-HMM, where infectivity was decreased by 44 and 22–24 %, respectively. We tested a range of concentrations of HP, CSA and CSB, from 0·1 to 25 μg ml⁻¹, but did not detect any inhibition (data not shown).

The finding that HP did not inhibit IMV infectivity contradicted a previous report that soluble HP inhibited IMV plaque formation by 35 % at 1 μg ml⁻¹ and reached a maximal inhibition of 60 % with 5–10 μg HP ml⁻¹ (Chung et al., 1998). However, those infectivity assays were performed by using BSC-40 cells and a different experimental protocol (see Methods). Therefore, we repeated our experiments using the same protocol as Chung et al. (1998) and a variety of cell lines, including BS-C-1, BSC-40, RK13, HeLa and PtK2 cells (Fig. 2).

Inhibition of IMV infectivity by HP was greater on BSC-40 cells (34 % at 25 μg ml⁻¹) than on BS-C-1 cells (no inhibition) and was 7, 27 and 10 % on RK13, HeLa and PtK2 cells, respectively. The greatest inhibition of IMV infectivity on all cell types was achieved with high-molecular-mass DS (DS-HMM), where between 42 and 65 % inhibition was observed. Interestingly, PL enhanced IMV infectivity on a number of cell lines; in particular, there was a 2·4-fold increase on RK13 cells. Overall, these data show that the inhibition of IMV infectivity by GAGs was modest and cell type-specific. This suggests that GAGs do not have an essential role in IMV binding.

Increasing the binding of VACV to cells

The study of virus entry by EM is difficult and time-consuming, unless there are reasonably high numbers of particles binding to the cell surface. Therefore, to facilitate the study of VACV binding and entry by EM, the number of virions bound to cells was enhanced by spinoculation (centrifugal force). Previously, spinoculation was used to enhance infection by human immunodeficiency virus type 1 (O’Doherty et al., 2000). To visualize bound virions, we used a recombinant virus in which the ASL core protein was fused to enhanced GFP (EGFP), vA5L-EGFP-N (Carter et al., 2003). The number of IMV bound per cell (n = 10) by passive absorption (Fig. 3a) was low (mean, 1·8), but spinoculation increased binding 118-fold (mean, 211·6)

Fig. 1. Effect of GAGs on IMV infectivity on BS-C-1 cells. Purified IMV was incubated at 37°C for 1 h with 25 μg ml⁻¹ of each GAG and control compounds. BS-C-1 cells were infected with the mixture at 37°C for 90 min. Data are expressed as infectivity remaining (%) in the presence of the test compound compared with that in its absence (−). The number of plaques obtained without test compound was between 100 and 250. The chart shows the mean ± SD of three independent experiments. HS, Heparan sulphate; HP-HMM, heparin (M, 15 000); HP-OverS, oversulphated HP; HP-DeS, de-sulphated HP; CSA, CSB and CSC, chondroitin sulphate A, B and C; DS, dextran sulphate derived from dextran (M, 5000); DS-HMM, DS (M, 500 000); PL, poly-L-lysine (M, 4000–15 000).

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To investigate the entry of IMV by EM, purified WR IMV bound to cells without affecting cell viability or the ability of virus to infect these cells. Therefore, spinoculation increases the number of particles bound to cells without affecting cell viability or the ability of virus to infect these cells.

**IMV entry**

To investigate the entry of IMV by EM, purified WR IMV was spinoculated onto PtK2 cells and either kept on ice to show viral binding (Fig. 4a) or warmed to allow entry (Fig. 4b–f). After fixation, the IMV surface was labelled with an anti-D8L mAb, followed by secondary antibody and colloidal gold. Samples were processed for conventional transmission EM. Cells were embedded in epon and sections were cut parallel to the surface of the dish or vertically through the cells. Numerous IMV particles were seen bound to the plasma membrane via either their side or end, but there was no fusion or penetration at 4°C. At high magnification, the plasma membrane and IMV membrane were visible (Fig. 4b) and we found no evidence that spinoculation introduced artefacts, as suggested by Griffiths et al. (2001). Samples warmed to 37°C also showed examples of direct membrane fusion between the plasma membrane and the IMV membrane (Fig. 4c–f). The arrowheads in Fig. 4(c–e) point to direct continuity between the virus membrane and plasma membrane, the former being labelled with an anti-D8L mAb. After fusion, the IMV membrane flattened into the plane of the plasma membrane and the core was released into the cytosol (Fig. 4e, f). The IMV surface protein D8L was incorporated into the plasma membrane and was not seen on intracellular cores or membranes left outside the cell, showing that IMV particles enter cells by direct fusion at the cell surface.

**How many membranes surround IMV?**

The number of membranes surrounding VACV particles is disputed, but is critical to the entry process (see Introduction). The images showing direct fusion of the IMV membrane with the plasma membrane indicate that IMV has one membrane, because no other membrane is seen. To investigate this further, the IMV and plasma membranes were studied in more detail early after a virion had fused with the plasma membrane by tilting the samples in 5° increments from +50° to −30° in the microscope (Fig. 5). By this method, the membrane(s) became perpendicular to the electron beam and, therefore, in sharper focus. This showed clear continuity between the IMV membrane and the plasma membrane on the left side at tilt −10° and on
the right side at +50°. Arrows point to the membrane continuity at higher magnification (top right panel). In all of these images, extra membranes in IMV were not evident, either in the intact virion or at the cell surface during fusion, or surrounding the core after entry. These data show that IMV is surrounded by a single lipid membrane.

Fig. 3. Binding of A5L-EGFP-N IMV and EEV to cells by passive absorption or spinoculation. Freshly prepared EEV (4 p.f.u. per cell) or purified IMV (0.2 p.f.u. per cell) of vA5L-EGFP-N was spinoculated or bound passively onto PtK2 cells for 1 h at 4 °C. Samples were processed for confocal microscopy and bound particles were visualized by EGFP fluorescence. Images show IMV passive binding (a) and IMV spinoculation (b), EEV passive binding (c) and EEV spinoculation (d). After binding, some spinoculated IMV (g) or EEV (h) samples were incubated at 37 °C for 8 h and EGFP fluorescence was then visualized by confocal microscopy. To investigate the entry of spinoculated EEV, samples were kept on ice (e) or incubated at 37 °C for 10 min to allow entry (f), and were processed for immunofluorescence by using anti-GFP antibodies to label intracellular cores. The inset shows co-localization (yellow) of GFP (green) and anti-GFP antibodies (red). Cell outlines are indicated by broken lines. Bars, 10 μm (a–d, g, h); 5 μm (e, f).
Spinoculation does not affect the entry process

To confirm that IMV particles enter cells by direct fusion and that the entry images observed were not a consequence of the spinoculation process, we also acquired images by using passively bound IMV (Fig. 6). These showed that the IMV membrane fuses with the plasma membrane to release the naked core inside the cell. Fig. 6 shows two examples of this fusion: Fig. 6(g) shows an IMV membrane in continuity with the plasma membrane, but the core has not yet been released into the cytosol, and Fig. 6(h) shows the IMV membrane in continuity with the plasma membrane and the released core. In the latter case, a second core is already present in the cell. The images in Fig. 6(a–f) show the particle in Fig. 6(g) after the specimen has been tilted in the EM (as described for Fig. 5) and again demonstrate the continuity between the single IMV membrane and the plasma membrane. Therefore, spinoculated and passively bound IMV particles enter into PtK₂ cells by direct fusion; this is in agreement with electron micrographs collected by other researchers (Armstrong et al., 1973; Chang & Metz, 1976).

Fig. 4. EM of IMV particles spinoculated onto PtK₂ cells. Purified WR IMV (325 p.f.u. ml⁻¹) was spinoculated onto PtK₂ cells to study the binding and entry of IMV and samples were either retained on ice or incubated at 37 °C for 10 min. The IMV surface antigen DBL was labelled with a DBL-specific mAb for immuno-EM (Methods). Images show IMV particles bound to cells at 4 (a) and 37 (b) °C, the IMV membrane fusing with the plasma membrane (c and d), the core entering into the cytosol (e) and the core leaving the site of entry (f). Bars, 200 nm (a); 50 nm (b); 100 nm (c–f).
Entry of MVA IMV

To investigate whether IMV from another strain of VACV enters cells by direct fusion, we spinoculated purified MVA IMV onto PtK₂ cells and then processed samples as described for Fig. 4. After binding of IMV to cells at 4 °C, no fusion was observed (data not shown). However, after warming to 37 °C, IMV fused with the plasma membrane (Fig. 7a), flattened into the cell surface and released the core into the cell (Fig. 7b, c) in a manner indistinguishable from that seen in VACV strain WR (Figs 4–6).

Lastly, we investigated the entry of IMV into a different cell type. Hitherto, we had used PtK₂ cells that had been selected because of their large, flat nature; therefore, a human cell line (HeLa) that has been used widely for studies of VACV entry was also studied (Fig. 7d). Sections were cut vertically through the cells and an image is presented showing an MVA IMV particle that has fused with the plasma membrane to release the virus core into the cytoplasm. The sequence of events was indistinguishable from the entry of IMV into PtK₂ cells. Therefore, the same entry events are observed irrespective of the strain of virus used (MVA and WR), the cell type used (PtK₂ and HeLa) and whether the virus was bound by spinoculation or by passive binding.

In summary, IMV enters cells by fusion of its single membrane with the plasma membrane and release of the core into the cytoplasm. After release of the core, the IMV membrane and plasma membrane remain in direct continuity and the viral membrane flattens towards the cell surface. All images show only a single membrane surrounding the IMV particle.

DISCUSSION

This study shows that IMV particles enter cells by fusion with the plasma membrane and that the virion is surrounded by a single lipid membrane. Incubation of IMV with different soluble GAGs had very little effect on the infectivity of the IMV and the degree of inhibition/enhancement was cell type-specific.

To study VACV entry by EM, we adapted a spinoculation protocol, which enhances the binding of virions by gentle centrifugal force. Spinoculation was shown to be a suitable method for studying poxvirus entry: it required less virus, a shorter incubation time and did not affect cell viability or the ability of virions to enter and replicate. Most importantly, it does not require virus at high concentration and is especially valuable for studying EEV that is produced.
Fig. 6. EM of IMV particles bound passively onto PtK₂ cells. Purified WR IMV (500 p.f.u. ml⁻¹) was bound passively onto PtK₂ cells and processed as described in the legend to Fig. 4. (a–f) The specimen shown in panel (g) has been tilted through different angles in the EM (as described in the legend to Fig. 5). (g, h) Images show IMV particles fusing with the plasma membrane to release the core into the cell. Bars, 100 nm.
at low concentration. We present electron micrographs showing that IMV enters by fusion at the plasma membrane and data were indistinguishable for spinoculation and passive binding of virions. Griffiths et al. (2001) argued that the data of Armstrong et al. (1973), Chang & Metz (1976) and Granados (1973) were unconvincing, because spinoculation might flatten the virus membrane on the cell surface. In our study, spinoculation did not introduce flattened membrane on the cell surface at conditions that only allow virus binding (Fig. 4a–b) and images were indistinguishable when either passive binding or spinoculation was used.

After fusion, the IMV membrane and associated IMV surface antigen flattens into the plane of the plasma membrane and the naked core is released into the cytoplasm. By using immuno-EM with a mAb directed against the IMV surface protein D8L (an integral membrane protein), it was evident that this antigen remained in the plasma membrane. It was neither present on fragments of membrane left outside the cell, nor was it still associated with virus cores that have entered the cytosol.

These data agree fully with previous electron micrographs (Armstrong et al., 1973; Chang & Metz, 1976). These

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Fig. 7. EM of MVA IMV particles spinoculated onto PtK2 (a–c) and HeLa (d) cells. Purified MVA IMV was spinoculated onto PtK2 (325 p.f.u. per cell) or HeLa (31 p.f.u. per cell) cells. Samples were processed as described in the legend to Fig. 4. Images show IMV particles bound to or entering PtK2 (a–c) or HeLa (d) cells at 37 °C. Bars, 100 nm.
authors showed fusion between the plasma membrane and the IMV membrane, giving continuity between these membranes, and IMV antigen remaining in the plasma membrane after fusion had taken place. Others reported that the IMV particle is surrounded by multiple membranes (Sodeik et al., 1993; Griffiths et al., 2001; Risco et al., 2002) and that it enters by an unconventional mechanism involving uncoating of the core outside the cell, following transport of the core across the plasma membrane (Krijnse Locker et al., 2000; Griffiths et al., 2001; Sodeik & Krijnse-Locker, 2002). However, the claim that IMV particles are uncoated outside the cell is contradicted by multiple reports, including this study, which used anti-core antibodies and showed that virus cores were not present on the cell surface, but only after having entered the cell (Vanderplasschen et al., 1998; Krijnse Locker et al., 2000; Carter et al., 2003; Senkevich et al., 2004a). The fusion of IMV at the cell surface is also inconsistent with the proposal that IMV contains an interconnected network of membrane cisternae and tubules around the core and the IMV surface (Griffiths et al., 2001). A single fusion event of such a structure could not deliver an intact core into the cytosol. The fusion of IMV at the cell surface is also consistent with biochemical data that showed that IMV entry occurred at neutral pH and was not affected by drugs that raise the pH of intracellular vesicles (Janeczko et al., 1987; Doms et al., 1990; Vanderplasschen et al., 1998).

With regard to the number of membranes surrounding VACV particles, we show by tilt-series analysis that IMV particles have one membrane. Dales and co-workers reported that IMV has one membrane that is synthesized de novo (Dales, 1963; Dales & Mosbach, 1968), but this was challenged by a report that IMV has two membranes derived from the IC (Sodeik et al., 1993). These authors had difficulty in demonstrating clear images of two membranes and suggested this might be because ‘the membranes of this cisterna are so tightly apposed that it is often difficult to distinguish the two bilayers’. Hollinshead et al. (1999) investigated the number of membranes around IMV by using high-resolution EM, tilt-series analysis and serial sections, and questioned whether two membranes could be too closely apposed to be distinguished, as proposed by Sodeik et al. (1993). Measuring the thickness of membranes at tight cell junctions or in myelin sheaths, multiple membranes were always visible and each had a width of 5 nm, the same thickness as the crescent and IMV membrane from the same infected cell (Hollinshead et al., 1999). Subsequent work that claimed two membranes proposed that the outer membrane was not clearly visible because it was masked by a dense protein coat (Risco et al., 2002). However, earlier work by Grimley et al. (1970) showed that virus crescents that form after washout of rifampicin, an inhibitor of morphogenesis, have a single lipid membrane. Where the virus crescents have developed curvature, the outer surface is covered by a dense protein coat that might conceal a second membrane, but there were images where two crescents were linked and the connecting membrane lacking this protein coat unambiguously has a single membrane [see Fig. 9(c) of Grimley et al. (1970)]. Risco et al. (2002) also used freeze-fracture analysis to investigate the IMV surface structure and concluded that their images provided additional evidence for a second membrane. Our interpretation of the freeze-fracture images was that they provide support for a single membrane, because no image showed a fracture between two membranes around an IMV. The question of how the IMV membrane is formed remains unanswered and additional research is needed.

A recent study of VACV entry reported that IMV binding induces the formation of long actin/ezrin-containing protrusions, to which it binds and may enter. These long, ‘finger-like’ protrusions were not observed in uninfected cells or cells infected by EEV (Krijnse Locker et al., 2000). Senkevich et al. (2004b) reinvestigated this and reported no increase in protrusions following IMV binding. We also investigated this by using the exact methods employed in both previous studies and found no increase in surface protrusions following binding of IMV or EEV (data not shown).

IMV was reported to bind to cell-surface HS and CS and three membrane proteins, A27L, D8L and H3L, were implicated in this binding. Repetition of soluble GAG competition experiments by using BS-C-1 cells showed that IMV infectivity was not inhibited significantly by the most common GAGs; in particular, IMV infectivity was only inhibited by 2% by HP. To determine whether this difference was due to the different cell type used or the experimental protocol, the experiments were repeated by using the protocol of Chung et al. (1998) and BSC-40 cells. Under these conditions, we observed a partial (34%) inhibition of infectivity by using 25 μg HP ml⁻¹, but the degree of inhibition did not even reach the levels that were reported with 5 μg HP ml⁻¹ by Chung et al. (1998). Perhaps these differences reflect the different preparations of HP with varying size and charge. We also showed that HP with a higher negative charge (HP-OverS) was more effective at inhibiting IMV infectivity.

The infectivity of IMV on different cell lines was inhibited to varying degrees by GAGs and, in most cell lines tested, GAGs had very little effect. Only DS caused a considerable decrease in infectivity on all cell types and this probably reflected its high molecular mass, charge and the branched polysaccharide structure. Although HS did not reduce IMV infectivity greatly, HP inhibited IMV infectivity more in BSC-40 and HeLa cells than in others. It is possible that IMV utilizes a combination of cell-surface GAGs or negatively charged molecules for binding to different cell types. Overall, the degree of inhibition in the cell types tested was modest and no single GAG inhibited VACV infection completely. This is not in agreement with other researchers, who suggest that GAGs are important for IMV binding. Therefore, we propose that other receptors must
be involved and it is likely that IMV can utilize many different or a combination of receptors for binding to cells.

Finally, the enhancement of IMV infectivity by PL is an interesting observation. It was reported that cations can reduce the repulsion between the viral and cell membranes and thereby enhance virus infectivity (Chillakuru et al., 1991). PL may enhance the infectivity, particularly for RK13 cells, by the same mechanism. In contrast, polyanions may increase the repulsion, leading to a reduction of virus infectivity rather than a specific blockage, as discussed above.

In conclusion, we demonstrate that IMV enter cells by membrane fusion. The IMV membrane fuses directly with the plasma membrane to release a core into the cytoplasm; this membrane and its associated antigens then flatten into the plane of the plasma membrane. The role of GAGs in IMV binding revealed that soluble HP, HS and varieties of CS only affect IMV infectivity modestly. The degree of inhibition was found to be cell type-specific.

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