The formation and function of extracellular enveloped vaccinia virus

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Vaccinia virus produces four different types of virion from each infected cell called intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV). These virions have different abundance, structure, location and roles in the virus life-cycle. Here, the formation and function of these virions are considered with emphasis on the EEV form and its precursors, IEV and CEV. IMV is the most abundant form of virus and is retained in cells until lysis; it is a robust, stable virion and is well suited to transmit infection between hosts. IEV is formed by wrapping of IMV with intracellular membranes, and is an intermediate between IMV and CEV/EEV that enables efficient virus dissemination to the cell surface on microtubules. CEV induces the formation of actin tails that drive CEV particles away from the cell and is important for cell-to-cell spread. Lastly, EEV mediates the long-range dissemination of virus in cell culture and, probably, in vivo. Seven virus-encoded proteins have been identified that are components of IEV, and five of them are present in CEV or EEV. The roles of these proteins in virus morphogenesis and dissemination, and as targets for neutralizing antibody are reviewed. The production of several different virus particles in the VV replication cycle represents a coordinated strategy to exploit cell biology to promote virus spread and to aid virus evasion of antibody and complement.

Introduction

Vaccinia virus (VV) is a large DNA virus that replicates in the cytoplasm and is a member of the genus Orthopoxvirus of the family Poxviridae (Moss, 2001). Other orthopoxviruses include variola virus (VAR), the cause of smallpox, cowpox virus (CPV), monkeypox virus (MPV), ectromelia virus (ECT), camelpox virus (CMPV), raccoonpox virus and taterapoxvirus (Fenner et al., 1989). The genomes of VV (Goebel et al., 1990), VAR (Massung et al., 1994), MPV (Shchelkunov et al., 2001), CMPV (Afonso et al., 2002; Gubser & Smith, 2002), ECT (www.sanger.ac.uk), and CPV (AF482758; Shchelkunov et al., 1998) have been sequenced. These viruses are all morphologically indistinguishable and antigenically related, such that prior infection with any one provides some protection against each member of the genus (Fenner et al., 1989). This was the basis for CPV (Jenner, 1798) and later VV (Downie, 1939) being effective vaccines against smallpox. VV has been developed as an expression vector for foreign genes and as a live recombinant vaccine for infectious diseases and cancer (Mackett et al., 1982; Panicali & Paoletti, 1982).

An overview of morphogenesis

An overview of VV morphogenesis is shown in Fig. 1. Intracellular mature virus (IMV) particles are formed within cytoplasmic factories from non-infectious precursors called crescents and immature virus (IV). IMV represent the majority of infectious progeny and mostly remain within the cell until lysis. However, some IMV leave the factory on microtubules and become wrapped by a double layer of intracellular membrane derived from the early endosomes or trans-Golgi network (TGN) to form intracellular enveloped virus (IEV). IEV then move to the cell surface (again requiring microtubules) where the outer membrane fuses with the plasma membrane exposing an enveloped virion on the cell surface. Particles retained on the cell surface are termed cell-associated enveloped virus (CEV) and those released are called extracellular enveloped virus (EEV). CEV and EEV are physically indistinguishable and contain one fewer membrane than IEV and...
one more membrane than IMV. CEV induce the formation of actin tails that drive the virions away from the cell and are important for cell-to-cell spread. EEV mediate long-range dissemination of virus.

**Recognition of intracellular and extracellular virus**

Immunization against smallpox with live VV caused some vaccine-related complications that varied with the strain of VV used (Fenner *et al*., 1988). Although safer strains of virus, such as Lister and New York City Board of Health (Wyeth), were used during the latter part of the smallpox eradication campaign, the complications prompted inactivated (killed) virus vaccine preparations or more attenuated strains, such as LC16m8 (in Japan) or modified vaccinia virus Ankara (in Germany), to be considered as alternatives. Killed vaccines were prepared from virus released by disruption of infected cells, predominantly IMV, that was inactivated by ultra-violet light (Collier *et al*., 1955), formaldehyde (Amies, 1961) or heat (Madeley, 1968; Turner *et al*., 1970) and were largely ineffective in preventing orthopoxvirus infection in man (Kaplan, 1962; Kaplan *et al*., 1962, 1965) and in animal models (Madeley, 1968; Boulter *et al*., 1971; Turner & Squires, 1971; Appleyard & Andrews, 1974). For instance, 18 out of 20 infants immunized with killed vaccine developed high levels of antibody to VV but had typical primary ‘takes’ when subsequently inoculated with live vaccine (Kaplan *et al*., 1965). Similarly, rabbits immunized with killed IMV vaccine had high levels of antibody but still developed lesions at the site of intradermal challenge (Boulter, 1969; Boulter *et al*., 1971; Turner & Squires, 1971). Such antibody did not prevent secondary plaque formation by virus growing in cell monolayers and did not neutralize virus released into the supernatants of infected cells (Boulter, 1969; Appleyard *et al*., 1971; Turner & Squires, 1971; Appleyard & Andrews, 1974). Evidently, antibody resulting from immunization with inactivated IMV lacked some component(s) that was (were) essential for protecting cells from infection *in vitro* and *in vivo* and this led to the proposal that extracellular virus (EEV) differed antigenically from the virus retained in cells (Boulter, 1969). This proposal was supported by several observations: (i) antibody raised against inactivated intracellular virus failed to neutralize extracellular virus (Madeley, 1968; Boulter, 1969; Appleyard *et al*., 1971; Turner & Squires, 1971); (ii) the proteins immunoprecipitated with anti-IMV and anti-EEV antibody were different (Turner & Squires, 1971); and (iii) electron microscopy showed that EEV contained an additional lipid envelope (Appleyard *et al*., 1971; Ichihashi *et al*., 1971). Consistent with the extra lipid envelope, EEV had a lower buoyant density (1–23–1–24 g/ml) in caesium chloride density gradients than IMV (1–27–1–28 g/ml) (Boulter & Appleyard, 1973). Later, Payne (1978, 1979) identified 10 proteins in EEV that were absent from IMV. These early studies demonstrated the existence of antigenically and structurally distinct forms of infectious virions and showed that antibody to the EEV was important for protection against live virus challenge. Although antibody that neutralizes IMV can have benefit in protecting...
against challenge with IMV (Hooper et al., 2000; Ramírez et al., 2002), where the roles of antibody to IMV and EEV have been compared side-by-side, the anti-EEV antibody has much greater benefit, and in particular this benefit extends to after virus challenge, when the virus infection is spread by EEV against which the anti-IMV antibody is ineffective (Boulter et al., 1971; Boulter & Appleyard, 1973).

An important conclusion from these studies, and one pertinent to the current development of second generation smallpox vaccines, was that tests to measure neutralizing antibody that are relevant to immunological protection should utilize EEV rather than IMV (Boulter & Appleyard, 1973; Appleyard & Andrews, 1974). The EEV neutralization test is difficult because of the presence of contaminating IMV in EEV preparations and the fragility of the EEV outer envelope (Boulter & Appleyard, 1973). However, Appleyard et al. (1971) described two methods for measuring antibody to EEV: (i) the anti-comet test; and (ii) the modified neutralization test using EEV pretreated with antibody against inactivated IMV. Some strains of VV [such as rabbitpox and International Health Department (IHD)-J] release high levels of EEV and if these viruses are allowed to grow on cell monolayers they give rise to characteristic comet-shaped plaques in which the head of the comet represents the primary plaque and the comet tails represents secondary plaques caused by unidirectional spread of EEV by convection currents (Law et al., 2002). The formation of comets is inhibited by antibody to EEV but not IMV.

**IMV formation**

Investigations of VV morphogenesis by electron microscopy reported that the initial stages of virion formation take place in cytoplasmic sites called virus factories from which cellular organelles are largely excluded (Dales & Siminovich, 1961). Within these factories the first visible viral structures were crescents composed of lipid and virus-encoded protein. These crescents grow to form ovals, called IV, which then mature by proteolytic cleavage of several capsid proteins (Moss & Rosenblum, 1973) and condensation of the virus core to form IMV.

The nature and origin of the crescents are disputed. Early investigators proposed that these were formed from a single lipid bilayer that was synthesized de novo and lacked continuity with cellular membranes (Dales, 1963; Dales & Mosbach, 1968). Subsequently, it was proposed that the crescent was composed of a pair of tightly apposed membranes that were derived from and were continuous with cell membranes of the intermediate compartment (IC) between the endoplasmic reticulum (ER) and the Golgi stack (Sodeik et al., 1993). Another study reported no continuity between virus and cellular membranes and only a single lipid bilayer (Hollishead et al., 1999). Recently, additional reports claimed IMV has two (Risco et al., 2002) or more membranes (Griffiths et al., 2001). The de novo model of membrane biosynthesis contradicts dogma stating that membranes grow from existing membranes. However, a single membrane around the outside of IMV simplifies the virus re-entry mechanism (see below). In contrast, the double membrane model fits with our knowledge of cell biology, but creates a topological difficulty during virus re-entry: namely, how the multiple membranes surrounding the virus are shed to release the core into the cytosol. The issue is fundamental to aspects of virus morphogenesis and re-entry and additional study is needed. This review considers events after IMV formation and builds on an earlier review (Smith & Vanderplasschen, 1998).

**Egress of IMV from factories**

After their formation IMV particles may move to sites where they become wrapped by a double layer of membrane to form an IEV. Alternatively, IMV may remain in the cell until cell lysis or, with some orthopoxviruses, become occluded in A-type inclusion (ATI) bodies (see below). Late in infection some IMV bud through the plasma membrane (Ichihashi et al., 1971; Tsutsui, 1983).

The movement of IMV particles from the factory to the wrapping membranes requires microtubules and the A27L protein (Sanderson et al., 2000), which is present on the IMV surface and is a target for antibodies that neutralize IMV infectivity (Rodriguez et al., 1985). Repression of A27L gene expression caused a deficiency in IEV formation, a small plaque size and 20-fold reduced EEV production (Rodriguez & Smith, 1990). The A27L protein is required for both transport and wrapping since loss of A27L prevented IMV transport and an Ala-25 to Asp substitution prevented transport but wrapping was inhibited (Sanderson et al., 2000). This multi-functional protein also forms a complex with two other IMV proteins (A17L and A14L) (Rodriguez et al., 1993) and promotes cell-to-cell fusion (Rodriguez et al., 1987).

In another study virus particles were found to accumulate near the microtubule organizing centre (MTOC) and this accumulation was prevented by disruption of microtubules by nocodazole or by expressing dominant negative mutants of p50/dynamin, which disrupts the function of dynein–dynactin (Ploubidou et al., 2000). These observations supported the requirement for microtubules for IMV transport. Later during infection the MTOC was disrupted (Ploubidou et al., 2000).

Late during infection, a greater proportion of IMV particles remains unwrapped and may either stay in the cytosol until cell lysis, bud through the plasma membrane or become occluded in ATIs. ATIs are proteinaceous bodies that appear late in infection (Ichihashi et al., 1971) and are composed predominantly of a single polypeptide (160 kDa in cowpox virus) (Patel et al., 1986). The majority of orthopoxviruses, including VV, do not make ATIs because the gene encoding the 160 kDa protein is disrupted. However, several strains of cowpox virus and raccoonpox virus make ATIs (Ichihashi et al., 1971; Patel et al., 1991).
et al., 1986). The ATI enhances IMV stability after cell death and aids virus transmission between hosts.

Proteins of IEV, CEV and EEV

Before considering the next stages of morphogenesis the virus-encoded proteins that are associated with the IEV, CEV and EEV, but not IMV particles, are described. The properties and membrane topology of these proteins are illustrated in Fig. 2 and their locations on virions are illustrated in Fig. 3. The phenotypes of virus mutants lacking these proteins individually are shown in Table 1 and the stages at which morphogenesis is affected are shown in Fig. 4.

Payne (1978, 1979) identified ten proteins (210K, 110K, 89K, 42K, 37K, plus five proteins from 20K to 23K) that were present in purified EEV (strain IHD-J) and absent from IMV. All

Fig. 2. Schematic representation of VV IEV proteins. The outer membrane of the IEV particle is shown as a lipid bilayer (red). Protein domains above the line are within the lumen of the wrapping membranes whereas domains beneath the membrane are within the cytosol. Where the topology of the protein is known the N and C termini are indicated. The predicted length of the polypeptide in amino acid residues (aa) and the apparent size in reduced SDS–PAGE are shown. A34R has a single C-type lectin-like domain, F13L has similarity to phospholipase D, A56R has a single Ig domain and B5R has four SCR domains. How F12L is associated with membranes is unknown. References: 1Roper et al. (1996); 2Payne (1992); 3Wolffe et al. (2001); 4Grosenbach et al. (2000); 5Duncan & Smith (1992); 6Ro$\text{\textdollar}$ttger et al. (1999); 7Payne (1979); 8Shida & Dales (1981); 9Brown et al. (1991b); 10Shida (1986a); 11Jin et al. (1989); 12Takahashi-Nishimaki et al. (1991); 13Engelstad et al. (1992); 14Isaacs et al. (1992); 15Martinez-Pomares et al. (1993); 16Hirt et al. (1986); 17Hiller et al. (1981a); 18Koonin et al. (1996); 19Ponting & Kerr (1996); 20Hiller & Weber (1985); 21Child & Hruby (1992); 22Parkinson & Smith (1994); 23van Eijl et al. (2000); 24Frischknecht et al. (1999b); 25Zhang et al. (2000); 26van Eijl et al. (2002).
were glycosylated except 37K. In contrast, IMV contains only a single glycopeptide of 40 kDa (Holowczak, 1970; Garon & Moss, 1971). Monoclonal antibodies (mAbs) to several of these EEV proteins were reported (Shida, 1986a; Payne, 1992; Schmelz et al., 1994) and the genes encoding most of these proteins have been identified. Gene A56R encodes the 89K virus haemagglutinin (HA) (Shida, 1986a), B5R encodes the 42K protein (Engelstad et al., 1992; Isaacs et al., 1992), F13L encodes the 37K protein (Hirt et al., 1986), and genes A33R (Roper et al., 1996) and A34R (Duncan & Smith, 1992) encode the collection of smaller proteins with various degrees of glycosylation. Genes encoding the 210K and 110K proteins have not been identified and these may represent complexes (Payne, 1992) (Fig. 2). The proteins encoded by F13L, A33R, A34R, A56R and B5R are present in IEV, CEV and EEV particles (Fig. 3), although approximately one-third of EEV particles lack A56R (Krauss et al., 2002).

In addition, genes A36R (Parkinson & Smith, 1994; van Eijl et al., 2000) and F12L (Zhang et al., 2000; van Eijl et al., 2002) encode proteins that are present on IEV. Although some A36R and F12L proteins co-purify with EEV preparations, immuno-electron microscopy showed that they are absent from CEV and EEV envelopes (van Eijl et al., 2000, 2002). These proteins facilitate egress of IEV on microtubules (F12L) or CEV by actin polymerization (A36R) and therefore are termed transport proteins.

An interesting feature of the proteins encoded by these genes is that A33R, A36R, A56R, B5R and F13L proteins are palmitoylated (Grosenbach et al., 2000). Expression of most of these proteins individually by Semlik Forest virus vectors enabled the location of each protein to be studied in the absence of other VV proteins. The B5R, F13L and A34R proteins were present in intracellular vesicles, whereas the A33R and A56R proteins accumulated at the cell surface (Lorenzo et al., 2000).
Wrapping of IMV to make IEV

The origin of the membranes used to wrap IMV to form IEV is controversial. Some studies reported that the wrapping membranes were derived from early endosomes because fluid phase markers were incorporated into the lumen between the outer membranes of IEV particles (Tooze et al., 1993; van Eijl et al., 2002). Others reported that the membranes were from the Golgi (Ichihashi et al., 1971; Hiller & Weber, 1985) or TGN (Schmelz et al., 1994). There is increased traffic between these compartments late during VV infection (Tooze et al., 1993) and so these organelles might not be easily distinguished. Possibly both are used.

Early during infection the majority of IMV particles are wrapped to form IEV, whereas later during infection IMV predominates (Ulaeto et al., 1996), possibly due to depletion of wrapping membranes. The interaction of IMV with the wrapping membranes involves the cytosolic face of the wrapping membrane and the surface of IMV. The A27L protein on the IMV surface is implicated in this interaction (Sanderson et al., 1997; Grosenbach et al., 1998, 1999) and regulates vesicular budding (Bednarek et al., 1997). Mammalian PLD1 is expressed in the Golgi membranes (Hiller et al., 1981a), that prevents targeting of the F13L protein to the wrapping membranes (Hiller et al., 1981a). Passage of VV in the presence of IMCBH resulted in generation of drug-resistant virus proteins localizes in the Golgi (Lorenzo et al., 1997). A similar role has been suggested for F13L based on the observation that F13L expressed without other VV proteins localizes in the Golgi membranes and regulates vesicular budding (Bednarek et al., 1996; Colley et al., 1997). A similar role has been suggested for F13L based on the observation that F13L expressed without other VV proteins localizes in the Golgi membranes and regulates vesicular budding (Bednarek et al., 1996; Colley et al., 1997).

Analysis of virus mutants lacking individual genes has shown that the F13L (Blasco & Moss, 1991) and B5R (Engelestad & Smith, 1993; Wolff et al., 1993) proteins are each required for efficient wrapping, whereas F12L, A33R, A34R, A36R and A56R are not (Table 1). Without A34R there is an increased production of EEV yet fewer IEV are seen (Duncan & Smith, 1992; Wolff et al., 1997; Law et al., 2002).

Wrapping of IMV is inhibited by a drug, N4-s-sonicotinoyl-N2,3-methyl-4-chlorobenzoylhydrazine (IMCBH) (Kato et al., 1969; Payne & Kristensson, 1979; Hiller et al., 1981a), that prevents targeting of the F13L protein to the wrapping membranes. Passage of VV in the presence of IMCBH resulted in generation of drug-resistant virus containing an Asp to Tyr mutation within the F13L protein (Schmutz et al., 1991). The F13L protein is modified by acylation (palmitic and oleic acid) (Hiller & Weber, 1985; Child & Hruby, 1992; Payne, 1992). Mutation of Cys-185 and Cys-186 to serine prevented palmitoylation leaving the F13L protein soluble in the cytoplasm and preventing wrapping (Groshenbach et al., 1997; Groshenbach & Hruby, 1998; Groshenbach et al., 2000).

An interesting feature of the F13L protein is its limited amino acid similarity to phospholipase D (PLD) (Koonin, 1996; Ponting & Kerr, 1996). Although no PLD activity was detected in cells expressing F13L, mutagenesis of a motif conserved in PLDs disrupted F13L function and only tiny plaques were formed (Sung et al., 1997). Others reported that F13L is a broad specificity lipase with phospholipase C, phospholipase A and triacylglycerol lipase activity (Baek et al., 1997). Mammalian PLD1 is expressed in the Golgi membranes and regulates vesicular budding (Bednarek et al., 1996; Colley et al., 1997). A similar role has been suggested for F13L based on the observation that F13L expressed without other VV proteins localizes in the Golgi (Lorenzo et al., 2000) or post-Golgi vesicles (Husain & Moss, 2001) and that F13L causes redistribution of B5R from the TGN to endosomal membranes unless the conserved PLD motif is mutated (Husain & Moss, 2001). Consistent with a requirement for PLD activity in VV morphogenesis, the PLD inhibitor butanol-1 inhibited VV morphogenesis but expression of cellular PLD could not substitute for loss of the F13L protein (Husain & Moss, 2002).

### Table 1. Phenotypes of virus mutants lacking IEV, CEV or EEV proteins

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plaque size</th>
<th>IMV</th>
<th>IEV</th>
<th>CEV</th>
<th>EEV</th>
<th>Virus-tipped actin tails</th>
<th>Virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td>vΔF12L</td>
<td>Tiny</td>
<td>Normal</td>
<td>Yes</td>
<td>No</td>
<td>7-fold</td>
<td>Very few</td>
<td>Attenuated</td>
</tr>
<tr>
<td>vΔF13L</td>
<td>Tiny</td>
<td>Normal</td>
<td>Very few</td>
<td>Few</td>
<td>10-fold</td>
<td>Very few</td>
<td>Attenuated</td>
</tr>
<tr>
<td>vΔA33R</td>
<td>Tiny</td>
<td>Normal</td>
<td>Yes</td>
<td>Yes</td>
<td>3-fold</td>
<td>No</td>
<td>?</td>
</tr>
<tr>
<td>vΔA34R</td>
<td>Small</td>
<td>Normal</td>
<td>Yes</td>
<td>Reduced</td>
<td>25-fold, 5-fold</td>
<td>specific infectivity</td>
<td>No</td>
</tr>
<tr>
<td>vΔA36R</td>
<td>Small</td>
<td>Normal</td>
<td>Yes</td>
<td>Yes</td>
<td>5-fold</td>
<td>No</td>
<td>Attenuated</td>
</tr>
<tr>
<td>vΔA56R</td>
<td>Syncytial, normal size</td>
<td>Normal</td>
<td>Yes</td>
<td>Yes</td>
<td>Normal</td>
<td>Yes</td>
<td>Normal, intranasal</td>
</tr>
<tr>
<td>vΔB5R</td>
<td>Small</td>
<td>Normal</td>
<td>Few</td>
<td>Few</td>
<td>5- to 10-fold</td>
<td>Very few</td>
<td>Attenuated</td>
</tr>
</tbody>
</table>

vΔF12L, vΔF13L, vΔA33R, vΔA34R, vΔA36R and vΔA56R have the majority of their polypeptide chains in the cytosol and so are better placed to interact with IMV. Interestingly, proteins F12L and A33R are associated pre-dominantly with the outer IEV membrane such that after fusion of IEV with the plasma membrane they are absent from CEV (van Eijl et al., 2000, 2002). How they are excluded from the outer IEV membrane is unclear. One possibility is that during the progressive wrapping of IMV, A36R and F12L become displaced due to the bulk of their polypeptide chain being between the IMV surface and the wrapping membrane.

Analysis of virus mutants lacking individual genes has shown that the F13L (Blasco & Moss, 1991) and B5R (Engelestad & Smith, 1993; Wolff et al., 1993) proteins are each required for efficient wrapping, whereas F12L, A33R, A34R, A36R and A56R are not (Table 1). Without A34R there is an increased production of EEV yet fewer IEV are seen (Duncan & Smith, 1992; Wolff et al., 1997; Law et al., 2002).
The other protein required for wrapping of IMV is B5R. This has four short consensus repeats (SCR) that are characteristic of regulators of complement activation (Takahashi-Nishimaki et al., 1991; Engelstad et al., 1992) (Fig. 2). The N-terminal signal peptide is proteolytically removed (Isaacs et al., 1992) and some of the protein is also cleaved near the transmembrane domain to produce a secreted 35 kDa protein of unknown function (Martinez-Pomares et al., 1993). The B5R protein is acylated (Payne, 1992) by addition of palmitic acid at Cys-301, and possibly a second unidentified site (Grosenbach et al., 2000), and forms higher molecular mass complexes in the absence of reducing agent (Engelstad et al., 1992; Payne, 1992). The B5R protein affects virus host-range in some cell types (Takahashi-Nishimaki et al., 1991; Martinez-Pomares et al., 1993).

Virus mutants lacking B5R are very inefficient at wrapping IMV to IEV, have 5- to 10-fold lower levels of EEV, form a small plaque and are attenuated in vivo (Takahashi-Nishimaki et al., 1991; Engelstad & Smith, 1993; Martinez-Pomares et al., 1993; Wolfe et al., 1993) (Table 1). The signals necessary for correct targeting of B5R to the Golgi membranes reside in the transmembrane/cytoplasmic tail since fusion of these domains to other proteins such as human immunodeficiency virus gp120 (Katz et al., 1997), green fluorescent protein (GFP) (Ward & Moss, 2000; Hollinshead et al., 2001; Rodger & Smith, 2002) or vesicular stomatitis virus G protein (Ward & Moss, 2000) directed these chimaeras to IEV and EEV. Deletion of one or more SCR domains impaired wrapping of IMV and caused a small plaque phenotype, but EEV production was enhanced 10- to 50-fold (Herrera et al., 1998; Mathew et al., 1998; Rodger & Smith, 2002). Loss of the cytoplasmic tail did not affect wrapping or reduce plaque size (Lorenzo et al., 1998; Mathew et al., 2001), but the protein was less rapidly transported through the exocytic pathway (Mathew et al., 2001). Another study reported reduced accumulation of the C-terminally truncated protein in the Golgi membranes and concluded that the cytoplasmic tail had a role in retrieving B5R from the plasma membrane (Ward & Moss, 2000). Addition of an ER retrieval sequence to the C terminus of B5R caused the relocation of B5R to the ER and a reduced plaque size, but did not prevent IEV and EEV formation (Mathew et al., 1999).

**Transport of IEV to the cell surface**

The IEV particle is an intermediate between IMV and CEV and functions to (i) transfer virus to the cell periphery and (ii) cloak the virus particle released from the cell with a membrane that shields IMV from antibody and complement, to which it is very sensitive.

The movement of a large virion (250 × 350 nm) through the cytoplasm by diffusion is a slow and inefficient process (Sodeik, 2000) and so to expedite egress, VV uses cellular transport pathways. Two mechanisms have been proposed. One was that IEV particles induce polymerization of actin to drive these virions to the cell surface (Cudmore et al., 1995, 1996; Frischknecht et al., 1999b). This proposal was consistent with the prior observations that VV-infected cells produce numerous actin bundles (diameter 0.3 µm) resembling filopodia or specialized microvilli with enveloped virions at their tip (Stokes, 1976; Hiller et al., 1979, 1981b; Krempien et al., 1981; Blasco et al., 1991). These structures were seen only late during infection and required VV particle formation (Hiller et al., 1979). However, the proposal by Cudmore et al. (1995) was problematic. First, cytochalasin D prevented actin tail formation but CEV particles were still found on the cell surface (Payne & Kristensson, 1982). Second, there are several virus mutants that are unable to produce actin tails (Table 1) but which still form CEV and EEV and in some cases with enhanced EEV levels. Third, the drug PP1, which inhibits tyrosine phosphorylation [which is necessary for actin tail formation (Frischknecht et al., 1999a, b)], did not prevent CEV formation (Hollinshead et al., 2001). Evidently, transport to the surface is not reliant on actin polymerization. Lastly, the actin tails are found on one side only of the virus particle. This is reminiscent of intracellular bacteria such as *Listeria* and *Shigella* that also induce actin polymerization, but in those cases a bacterial protein located at one end only of the bacterium directs actin polymerization to that site only (Goldberg & Theriot, 1995; Smith et al., 1995). Yet with VV, the A36R protein, which is required for actin tail polymerization (see below), is distributed evenly over the IEV surface (van Eijl et al., 2000); so how is the polymerization polar?

The second model proposes that IEV move to the cell surface on microtubules, and actin tails form at the cell surface beneath CEV particles. The proposal that actin tails form only at the cell surface was based on the observation that the A36R protein was on IEV and beneath CEV on the cytosolic face of the plasma membrane, but was absent from CEV and EEV (van Eijl et al., 2000). This location is ideal to induce actin tail formation to drive the particle away from the cell. Subsequently, several groups utilizing GFP-labelled virions reported that IEV movement to the cell surface requires microtubules (Geada et al., 2001; Hollinshead et al., 2001; Riedtendorf et al., 2001; Ward & Moss, 2001). IEV were found to move along defined pathways rather than randomly in the cytosol, their movement was inhibited reversibly by nocodazole, and they moved in a stop–start manner with an average speed of 60 µm/min characteristic of microtubular transport but 20-fold greater than VV movement on actin tails (2-8 µm/min) (Cudmore et al., 1995).

These observations demonstrate that microtubules are used at two stages during VV egress: first, for transport of IMV from the virus factories toward the MTOC, and second, for transport of IEV from the MTOC to the cell surface (Fig. 1). Evidence that disruption of dynein-dynactin (Ploubidou et al., 2000) inhibited IMV movement, while disruption of kinesin inhibited IEV movement (Riedtendorf et al., 2001), and the presence of different proteins on the surface of IMV and
IEV is consistent with this. For IMV, the A27L protein is implicated directly or indirectly in microtubular movement, but which IEV proteins are involved? IEV proteins F12L, F13L and A36R are candidates because they are predominantly cytosolic (Fig. 3). Of these, F13L is required for IEV formation (Blasco & Moss, 1991) whereas F12L and A36R are not. In the absence of A36R, IEV are transported to the cell surface and CEV are visible by confocal and electron microscopy (Sanderson et al., 1998a; Wolffe et al., 1998; van Eijl et al., 2000; Hollinshead et al., 2001), although another study reported that A36R is needed for IEV movement on microtubules (Rieddorf et al., 2001). The third protein, F12L, seems a better candidate for microtubular movement.

The F12L protein is a 65–70 kDa protein that is conserved in chordopoxviruses (Zhang et al., 2000). Immunoememicroscopy using an epitope-tagged F12L revealed the protein is located on the IEV surface and is absent from CEV and EEV. In this respect it resembles A36R, but one difference is the absence of F12L beneath CEV at the cell surface (van Eijl et al., 2002). A mutant lacking the F12L protein made IEV, but IEV were not transported to the cell surface, EEV levels were reduced, the plaque size was small and the virus was highly attenuated (Zhang et al., 2000; van Eijl et al., 2002). Disruption of the corresponding gene in fowlpox virus also caused a small plaque phenotype and decreased EEV production (Ogawa et al., 1993). The VV F12L deletion mutant is the only mutant reported to make IEV particles that are not transported and thus is a prime candidate for interactions with microtubules. The mode of interaction of F12L with the IEV is not understood.

**Actin tail formation**

Once IEV reach the cell surface the outer envelope fuses with the plasma membrane exposing CEV on the cell surface. Electron micrographs showed an array of electron-dense material on the cytosolic face of the plasma membrane beneath CEV (Ichihashi et al., 1971) and immunoelectron microscopy showed that the A36R protein becomes concentrated here and is absent from other parts of the plasma membrane and CEV (Fig. 3) (van Eijl et al., 2000). The mechanism(s) for this and how the A36R protein is located only on the outer IEV membrane, so that after fusion at the cell surface the protein is absent from CEV, are unknown. But A36R is a key protein for induction of actin tails and it is possible that the increased concentration of A36R beneath CEV is the trigger for its phosphorylation by Src-like kinases that initiates the cascade of interactions resulting in actin polymerization (Frischknecht et al., 1999b). The failure to polymerize actin on IEV particles might be due to insufficient A36R concentration or absence of other components.

The A36R protein was originally described as a component of the EEV surface based upon its co-purification with EEV and its sensitivity to digestion by exogenous trypsin (Parkinson & Smith, 1994). However, subsequent analysis showed that the protein was absent from CEV and EEV (van Eijl et al., 2000). The A36R protein has a type Ib membrane topology with the majority of the amino acids in the cytosol (Fig. 2) (Röttger et al., 1999; Grosenbach et al., 2000; van Eijl et al., 2000), explaining why the six potential sites for attachment of N-linked carbohydrate are unused (Parkinson & Smith, 1994). Although the A36R protein is expressed by all orthopoxviruses examined, the protein varies in length and sequence near the C terminus (Pulford et al., 2002) and in ectromelia virus strain MP1 the protein is significantly shorter (160 amino acids versus 220 in VV). Mutagenesis demonstrated that truncated versions of A36R can still induce actin tail formation, but phosphorylation of Tyr-112 [an amino acid conserved in all sequenced A36R proteins (Pulford et al., 2002)] is essential and can be inhibited by PP1 (Frischknecht et al., 1999b). After phosphorylation A36R interacts with Nck leading to recruitment of N-WASP to the site of actin assembly (Frischknecht et al., 1999b). The recruitment of A36R to IEV requires the A33R protein, which functions as a chaperone and with which A36R forms a non-covalent complex (Wolffe et al., 2001). Tyrosine phosphorylation of A36R is reduced in the absence of A34R or F13L and inhibited in the absence of A33R (Wolffe et al., 2001). The A36R protein is also phosphorylated on serine and threonine residues (Wolffe et al., 2001) and is acylated via Cys-25 (Grosenbach et al., 2000). Deletion of A36R causes a dramatic attenuation (Parkinson & Smith, 1994) comparable to that resulting from loss of F12L (Zhang et al., 2000).

A direct comparison of the plaque-size phenotype of all mutants listed in Table 1 (Law et al., 2002) highlighted the role for actin tails in cell-to-cell spread. These mutants include those lacking F12L (Zhang et al., 2000), F13L (Blasco & Moss, 1991; Cudmore et al., 1995), A33R (Roper et al., 1998), A34R (Duncan & Smith, 1992; McIntosh & Smith, 1996; Wolffe et al., 1997; Sanderson et al., 1998a), A36R (Parkinson & Smith, 1994; Sanderson et al., 1998a; Wolffe et al., 1998; Frischknecht et al., 1999b; Röttger et al., 1999) and B5R (Engelstad & Smith, 1993; Wolffe et al., 1993; Mathew et al., 1998; Sanderson et al., 1998a; Röttger et al., 1999). One report that a mutant lacking the SCR domains of B5R produced a normal size plaque but failed to produce actin tails (Herrera et al., 1998) did not fit with this model, but upon re-examination this mutant was found to form a small plaque (Rodger & Smith, 2002). The A56R protein is the only IEV/CEV/EEV protein not needed for efficient actin tail formation (Sanderson et al., 1998a).

Two other observations are noteworthy regarding VV-induced actin tail formation. First, VV gene A42R encodes a profilin-like protein (an actin-binding protein) but this is not required for formation of actin tails or for virus maturation and egress (Blasco et al., 1991). Second, VV infection induces cell migration and subsequent cellular projections up to 160 µm long that often are branched and require drastic rearrangement of actin cytoskeleton of the host cell (Sanderson et al., 1998b).
Cell migration required early virus gene expression only, whereas formation of projections required both early and late virus gene expression (Sanderson et al., 1998b).

Actin tails can continue to grow from the cell surface for considerable distances (Hiller et al., 1979) and facilitate virus penetration of surrounding cells. These tails can also re-enter the same cell (Hollinshead et al., 2001). Eventually, as the tail grows longer, it may be detached from the cell still containing the CEV at its tip. Alternatively, the CEV may be released to form EEV.

**Release of EEV**

Several factors influence the release of EEV. Payne (1979, 1980) showed that both the strain of virus and the host cell influence EEV release: thus the IHD-J strain produces more EEV than the WR or Lister strains and the greatest yields were from RK13 cells. The enhanced release of EEV by the IHD-J strain was due to a Lys-151 to Glu mutation in the A34R protein (Blasco et al., 1993) and the majority of VV strains and VAR have the WR genotype (McIntosh & Smith, 1996). Furthermore, deletion of A34R caused a 25-fold increase in EEV, although the specific infectivity of these EEV was reduced 5-fold (McIntosh & Smith, 1996) (Table 1). Other proteins affecting EEV formation or release are A33R, A36R and B5R. Loss of A33R caused a 3-fold increase in EEV (Roper et al., 1998), whereas deletion of A36R (Parkinson & Smith, 1994) and B5R (Engelstad & Smith, 1993; Wolfe et al., 1993) caused reductions of 5-fold and 5- to 10-fold, respectively. The reduction due to deletion of B5R is attributable to a defect in EEV formation, but other B5R mutations such as deletion of only one or more SCR domains caused a 10- to 50-fold increase in EEV (Herrera et al., 1998; Mathew et al., 1998; Rodger & Smith, 2002). Conversely, replacing the SCR domains with the extracellular domain of the VV A56R protein caused a 25-fold reduction in EEV formation (Mathew et al., 2001). Lastly, overexpression of F13L caused a reduction in yield of EEV (Schmutz & Wittek, 1995) showing that the correct level of F13L is important. Thus the retention or release of CEV is influenced by virus genetics and host cell phenotype.

**Why does VV retain CEV?**

Some viruses have mechanisms to enhance their release from the cell surface and prevent retention: for instance, influenza virus expresses neuraminidase, which removes the sialic acid receptors from infected cells and virions and thereby minimizes virion aggregation and enhances dispersal. So it is curious that VV retains significant amounts of CEV on the cell surface (Blasco & Moss, 1992). A plausible explanation is the requirement for CEV to induce actin tail formation. If enveloped virions were detached from the cell surface immediately, actin tails might not have time to form and drive the particles away from the cell. The retention of CEV enables actin tail formation and efficient cell-to-cell spread.

**Haemagglutination and haemadsorption**

Nagler (1942) reported that VV expressed an HA and the encoding gene (A56R) was identified 44 years later (Shida, 1986a). The HA is separable from intracellular virus (Chu, 1948) but is expressed on the surface of infected cells (Ichihashi & Dales, 1971; Blackman & Bubel, 1972) and EEV (Payne & Norrby, 1976). Not all VV strains express HA: for instance, the IHD-J strain is HA+ while the IHD-W is HA− (Ichihashi & Dales, 1971). IHD-W synthesizes a truncated HA protein with much less carbohydrate and which is not transported efficiently to the cell surface (Shida & Dales, 1982). Sequencing revealed a dinucleotide insertion at position +548 resulting in translation termination shortly thereafter (Brown et al., 1991a). The HA was identified as an 85–89 kDa glycoprotein (Fig. 2) by noting its absence from IHD-W EEV, the failure of antibody raised against IHD-W to precipitate this antigen, the binding of this antigen to rooster erythrocytes (Payne, 1979) and by purification (Shida & Dales, 1981). The HA is heavily glycosylated with N- and O-linked carbohydrate, and the latter is important for haemagglutination activity (Shida & Dales, 1981). Mutant HAs with altered cytoplasmic domains had different intracellular transport and location (Shida & Matsumoto, 1983; Shida, 1986b).

Comparisons of the HA sequences from different orthopoxviruses showed that the N-terminal Ig domain is more highly conserved than regions between the Ig domain and the transmembrane domain (Agudo et al., 1992; Cavallaro & Esposito, 1992). Replacement of the Ig domain with a single-chain antibody specific for the tumour-specific antigen ErbB2 enabled the fusion protein to be incorporated into the EEV envelope and for the EEV to bind to ErbB2 (Galmiche et al., 1997). Thus it may be possible to alter the tropism of EEV as a step towards specific anti-tumour therapy. Transcriptional and immunoblot analyses revealed that the HA is expressed from both early and late promoters but the majority of HA accumulates late (Brown et al., 1991b).

An unusual feature of the HA is that it functions to inhibit cell–cell fusion. This was demonstrated by comparison of the HA+ IHD-J and HA− IHD-W strains that are fusion (F)+ or F−, respectively (Ichihashi & Dales, 1971). Co-infection with both viruses prevented fusion (Ichihashi & Dales, 1971). In contrast, treatment of IHD-J-infected cells with HA-specific mAb prevented fusion (Seki et al., 1990). Analysis of 21 haemadsorption-negative mutants (Shida & Matsumoto, 1983) showed that 19 of these failed to express cell surface HA and were F−, and five HA-positive revertants were F+ (Seki et al., 1990). The other two mutants expressed HA at the cell surface, but had single amino acid substitutions that caused either loss of haemadsorption activity but retention of fusion inhibitory activity (Glu-121 to Lys), or loss of both activities (Cys-103 to Tyr) (Seki et al., 1990). Two other VV proteins also affect cell–cell fusion: the K2L serine protease inhibitor is a fusion-inhibition protein like HA (Law & Smith, 1992; Turner &
Moyer, 1992; Zhou et al., 1992); and the A27L IMV surface protein promotes fusion (Rodriguez et al., 1987).

Deletion of the A56R gene does not affect virus morphogenesis, plaque size or EEV release, but the plaques are syncytial and the deletion mutant shows attenuation if injected intracranially into mice (Flexner et al., 1987) but not if administered intranasally (G. L. Smith, unpublished data). The function of the HA in the virus life-cycle is not understood; in particular it is curious to have a fusion-inhibition protein on the surface of EEV.

**Incorporation of cellular proteins into EEV**

Evidence for incorporation of cellular proteins into EEV has been presented. The profile of proteins associated with EEV varied with cell type, indicating involvement of host factors (Payne, 1978, 1979). However, as illustrated with the A36R and F12L proteins, the detection of proteins in preparations of EEV by immunoblotting does not prove these proteins are present in the virion (van Eijl et al., 2000, 2002). The association of cell-derived antigens is also influenced by the virus strain. Preparations of IHD-J EEV contained less cell actin and A36R protein than WR EEV (van Eijl et al., 2000) and, consistent with this, electron microscopy showed reduced membrane contamination (Krauss et al., 2002). A possible explanation for this is that IHD-J releases more EEV and retains less CEV than WR (Payne, 1979, 1980; Blasco & Moss, 1992). As actin polymerization drives CEV further from the cell, the chance of CEV and associated plasma membrane fragments being torn from the cell is increased.

Several host membrane proteins that are present in the TGN, early endosomes or plasma membrane fractions have been found in EEV preparations e.g. CD46, CD55, CD59, MHC class I and others (Vanderplasschen et al., 1998b; Krauss et al., 2002). Where investigated by electron microscopy these have also been found in IEV, CEV or EEV at low levels. Presumably these proteins are incorporated into the IEV outer membranes during wrapping. Biologically, the presence of CD55 protected EEV against destruction by homologous complement (Vanderplasschen et al., 1998b).

Host proteins from the ER, IC and early Golgi membranes were not found in EEV preparations suggesting these membranes are not utilized for EEV formation. Similarly, these antigens were not detected in IMV preparations (Krauss et al., 2002). This demonstrated that if membranes of the IC are utilized to form IMV particles there must be a mechanism to exclude host antigens from these membranes during morphogenesis.

**Protein–protein interactions**

In addition to interactions between A33R and A36R (Wolffe et al., 2001), and A36R with host proteins Nck (Frischknecht et al., 1999b) and Grb2 (Scaplehorn et al., 2002), interactions between A34R and B5R, A34R and A36R, and A36R and A33R were demonstrated by co-precipitation experiments (Röttger et al., 1999). Other interactions between IEV, EEV proteins are likely to exist, and the proteins mediating interaction of IMV and IEV with microtubules and IMV with the wrapping membranes remain to be elucidated.

**Mechanisms of virus spread**

VV has evolved several mechanisms for cell-to-cell spread in cell culture (Fig. 5). First, virus released from the cell may infect adjacent cells to increase plaque size or infect distant cells to form comets (Appleyard et al., 1971). Second, virus may spread by transfer from one cell to the next via regions of cell contact. This may be divided into processes that are dependent or independent on actin tails to propel CEV particles.

Investigations of the sensitivity of virus spread to inhibition by antibody showed that: (i) antibody to IMV was ineffective at inhibiting spread by any mechanism, indicating that spread is mediated by enveloped forms of virus; (ii) antibody to EEV inhibited spread mediated by mechanism 1, but had little effect on spread by mechanism 2 (Appleyard et al., 1971; Boulter et al., 1971; Boulter & Appleyard, 1973; Law et al., 2002). However, an analysis of mutants lacking IEV or EEV proteins showed that if the A33R protein was deleted the spread of virus by mechanism 2 was also inhibited by EEV antibody (Law et al., 2002). This implicates protein A33R, directly or indirectly, as mediating cell-to-cell spread in an antibody-resistant manner. Antibody to EEV induced the aggregation of CEV particles on the cell surface due to the inhibition of EEV release and this helps to control virus spread (Vanderplasschen et al., 1997).

**EEV interactions with antibody and complement**

EEV may represent only a fraction of a percent of total infectivity, but it is very important biologically and mediates the long-range virus dissemination in cell culture (the comet test) (Appleyard et al., 1971) and in vivo (Payne, 1980; Payne & Kristensson, 1985). It also has a higher specific infectivity (lower particle/p.f.u. ratio) (12.7 ± 6.3) than IMV (45 ± 11.1 for fresh IMV, and 64.6 ± 16.5 for purified IMV) (Vanderplasschen & Smith, 1997) and mutants with defects in EEV production are attenuated. The dissemination of EEV in vivo is aided by its resistance to destruction by complement and its relative resistance to neutralization by antibody compared to IMV. The resistance of EEV to complement is not due to the B5R protein, nor to the presence of VCP (VV complement protein) (Kotwal et al., 1990), but to the presence of complement control proteins in the outer envelope that are derived from the host cell (Vanderplasschen et al., 1998b). CD46, CD55 and CD59 were all detected in the EEV outer envelope by immunoblotting and immuno electron microscopy (Vanderplasschen et al., 1998b; Krauss et al., 2002), but CD55
(decay accelerating factor) was most important in resistance to complement (Vanderplasschen et al., 1998b).

There have been conflicting reports about the neutralization of EEV by antibody. Early reports indicated that neutralization of EEV by antibody was possible if antibody was derived from convalescent serum after a live infection (reviewed in Boulter & Appleyard, 1973). Later reports that EEV were not neutralized (Ichihashi, 1996; Vanderplasschen et al., 1997) have been refuted (Galmiche et al., 1999; Law & Smith, 2001) although higher concentrations of serum or purified antibody are needed to achieve the same degree of neutralization as obtained with IMV. A serum against purified EEV antigens or only against the extracellular domain of B5R each inhibited EEV infectivity (Galmiche et al., 1999). Further analysis indicated that SCR domain 1 of B5R was a target for this neutralizing antibody and that infectivity was reduced by inhibition of binding to cells and by virus aggregation (Law & Smith, 2001).

The B5R protein remains the only EEV antigen identified as a target for neutralizing antibody, although immunization of animals with A33R protein or recombinant DNA, or passive transfer of antibody to A33R protein, also induced protection (Ichihashi, 1996; Vanderplasschen et al., 1997) when inoculated intramuscularly, but protection was not demonstrated (Ichihashi et al., 1996; Vanderplasschen et al., 1997). However, this virus does not make the smallpox vaccine in Japan. LC16m8 was introduced during the latter years of the smallpox eradication campaign because of its increased safety compared to the parental Lister strain (Hashizume et al., 1985). However, this virus does not make the B5R protein and the reduced plaque size of LC16m8 was attributable to this defect (Takahashi-Nishimaki et al., 1991). Given that B5R is the only established target for EEV neutralizing antibody, this virus might have diminished potency as a smallpox vaccine.

EEV binding and entry

A report that VV bound to the epidermal growth factor receptor (EGFR) did not describe which form of VV was considered to bind EGFR (Eppstein et al., 1985) and has been rebutted (Hügin & Hauser, 1994). Similarly, the proposal that poxviruses use chemokine receptors for binding (Lalani et al., 1999) has not been substantiated (Masters et al., 2001).

The study of EEV binding and entry has lagged behind that of IMV because of the low amounts of virus, the difficulty in obtaining EEV preparations that are free from IMV contamination and the fragility of the EEV outer envelope. For IMV, the A27L (Chung et al., 1998), D8L (Maa et al., 1990; Hsiao et al., 1999), and H3L (Lin et al., 2000) proteins have all been demonstrated to bind to cell surface glycosaminoglycans, and an IgM mAb to a cell surface antigen blocked the binding of IMV to the cell surface (Chang et al., 1995). For EEV, no specific virus protein has been demonstrated to bind to a cell molecule, although the A34R and B5R proteins may have a role due to the increased release of EEV when these proteins are mutated (Blasco et al., 1993; McIntosh & Smith, 1996; Herrera et al., 1998; Mathew et al., 1998) and the reduced specific infectivity of A34R-deficient EEV (McIntosh & Smith, 1996). Even the factor to which HA binds on rooster erythrocytes (the haemagglutination reaction) is unknown.

To study EEV binding and entry it is necessary to either use pure preparations of EEV or to distinguish IMV and EEV particles in mixed populations and measure each simultaneously. Although physical methods exist to separate IMV and EEV due to their different buoyant densities (Boulter & Appleyard, 1973), these processes result in damage to the EEV outer envelope so that an increased proportion of infectivity is neutralized by IMV-specific mAb (Ichihashi, 1996; Vanderplasschen & Smith, 1997). Such damaged virions might bind to cells via either EEV or IMV antigens. To overcome these difficulties, EEV binding was studied using fresh EEV preparations and confocal microscopy. The IMV and EEV particles were distinguished using mAbs specific for the IMV or EEV surface (Vanderplasschen & Smith, 1997, 1999). Using this methodology it was shown that: (i) IMV and EEV bind to different cell types with differing relative efficiency; (ii) treatment of cells with Pronase, trypsin or neuraminidase affected IMV and EEV binding differently; (iii) a mAb that blocked the binding of IMV to the cell surface (Chang et al., 1995).
1995) did not affect EEV binding; and (iv) IMV and EEV bound to distinct sites on the cell surface (Vanderplasschen & Smith, 1997). Evidently, IMV and EEV bind to different receptors.

The mechanism of EEV entry is not understood. A fundamental issue is the number of lipid bilayers that must be shed from the virion to enable the core to access the cytosol. If IMV has a single membrane then EEV has two, and if IMV has two or more membranes EEV has three or more. A single fusion event cannot enable the EEV core to enter the cytosol and the mechanism of EEV entry must result in loss of one more membrane than IMV. Early studies on VV entry (using IMV) reported that entry was via pinocytosis (Dales, 1965). Other workers reported fusion at the plasma membrane (Armstrong et al., 1973; Chang & Metz, 1976; Janeczko et al., 1987) and showed electron micrographs of the IMV surface membrane in continuity with the plasma membrane (Armstrong et al., 1973; Chang & Metz, 1976). In addition to thin section electron microscopy Chang & Metz (1976) detected virus antigen on the cell surface after virions had penetrated the cells, consistent with cell surface fusion.

Several methods to study EEV entry have been used including electron (Krijnse Locker et al., 2000) and confocal microscopy (Vanderplasschen et al., 1998a; Krijnse Locker et al., 2000) to follow the appearance of cores within the cytosol, loss of radioactively labelled virions from the cell surface (Payne & Norrby, 1978) and a lipid mixing assay based upon dilution of a fluorescent probe (Doms et al., 1990). While these studies all conclude that EEV enters more rapidly than IMV, despite having to shed an additional lipid membrane, there is discrepancy about whether the penetration is affected by pH, where fusion takes place and whether drugs that affect actin influence entry. When comparing the data from these different studies the method used to obtain the EEV preparation should be considered. EEV purified by centrifugation and with an additional labelling procedure may have an increased proportion of virions with damaged outer envelopes and thus might bind to cells via either IMV or EEV proteins. This type of preparation should be avoided. Using fresh EEV from the supernatant of infected cells and an IMV mAb to neutralize IMV, Ichihashi (1996) proposed a model for EEV entry that required a low-pH step. In this model EEV are taken up by pinocytosis into intracellular vesicles that become acidified. At reduced pH the outer EEV membrane is disrupted and the IMV particle released into the vesicle fuses with the vesicle membrane releasing the core into the cytosol. Support of this model, Vanderplasschen et al. (1998a) noted that drugs that raise the intracellular pH reduced the uptake of EEV but not IMV, and a low-pH shock caused rupture of the EEV outer membrane so that virion infectivity was neutralized by an anti-IMV mAb. On the other hand Doms et al. (1990) reported that the rate of fusion of IMV and EEV was not affected by pH. Another study reported that IMV, but not EEV, induce signalling and the formation of actin-containing cell surface protrusions (Krijnse Locker et al., 2000). Another model for IMV entry proposed that IMV enter cells without a membrane fusion event; IMV were suggested to ‘unfold’ outside the cell and cores were then somehow able to pass across the plasma membrane (Krijnse Locker et al., 2000; Griffiths et al., 2001; Sodeik & Krijnse Locker, 2002). This proposal is inconsistent with the images of IMV membrane in continuity with the plasma membrane (Armstrong et al., 1973; Chang & Metz, 1976). Additional studies are needed to determine the exact mechanisms of VV entry.

Summary

Each cell infected by VV produces four forms of virus that have different roles in the virus life-cycle. IMV is the most abundant form of virus and due to its physically robust nature is well suited to mediate transport between hosts, but is poorly suited to mediate spread within a host due to its sensitivity to complement and antibody. IEV serves as an intermediate between IMV and CEV/EEV to ensure the incorporation of the EEV-specific proteins, transport virions to the cell surface using microtubules, disguise the IMV particle with an additional membrane and host proteins to make it less sensitive to antibody and complement, and broaden the range of host receptors to which VV can bind. CEV is needed to induce the formation of actin tails from beneath virions at the cell surface and to facilitate the efficient cell-to-cell spread of virus. Lastly, EEV is released from the cell surface and mediates spread of infection within the host.

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