

## The Vaccinia Virus A36R Protein Is a Type Ib Membrane Protein Present on Intracellular but Not Extracellular Enveloped Virus Particles

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Vaccinia virus gene A36R encodes a 45-kDa protein that is conserved in orthopoxviruses. A virus lacking the A36R protein formed a small plaque, was unable to induce the polymerization of actin tails, and was avirulent *in vivo*. Here we present a further characterization of the A36R protein by *in vitro* transcription and translation and analysis of infected cells by confocal microscopy and immunoelectron microscopy of cryosections using a monoclonal antibody raised against the C-terminal domain of the A36R protein. Translation of the A36R mRNA *in vitro* produced a protein of the same size whether or not the translation reaction was performed in the presence of canine pancreatic microsomes. However, the polypeptide synthesized in the presence of microsomes was associated integrally with the membrane and was sensitive to digestion by exogenous protease without permeabilization of the membrane with detergent, indicating that the majority of the protein is exposed on the outside of the vesicle. Consistent with this, immunofluorescent analysis of virus-infected cells demonstrated that the C-terminal domain of A36R was not exposed on the cell surface but was detected once the cell membrane was permeabilized. Immunoelectron microscopy of cryosections of infected cells showed that the protein was absent from IMV particles but present on intracellular enveloped virus (IEV) particles, predominantly on the cytosolic face of the IEV outer membrane. Where cell-associated enveloped virus (CEV) particles were attached to the cell surface, the A36R protein was detected only on the cytosolic surface of the plasma membrane where the virus particle remained attached to the cell and not elsewhere on the plasma membrane or on the CEV particle. A36R and actin copurified with EEV particles due to the association of fragments of cellular membranes with the EEV particles. Therefore, A36R represents the first example of a virus-encoded protein that is present on IEV but not CEV particles. © 2000 Academic Press

**Key Words:** vaccinia virus; membrane protein; extracellular enveloped virus; *in vitro* translation; immunoelectron microscopy.

### INTRODUCTION

Vaccinia virus (VV) is a large and complex virus that replicates in the cell cytoplasm and produces two forms of infectious virus termed intracellular mature virus (IMV) and extracellular enveloped virus (EEV) (Moss, 1996). IMV particles are formed within cytoplasmic factories and represent the majority of infectious progeny. These particles move from the virus factories in a process that is dependent upon microtubules and the A27L protein of the IMV surface (Sanderson *et al.*, 2000) to sites where the virion is wrapped by membranes (Ichihashi *et al.*, 1971) derived from the early endosomes (Tooze *et al.*, 1993) or trans-Golgi network (TGN) (Hirt *et al.*, 1986; Schmelz *et al.*, 1994). The resultant particles are called intracellular enveloped virus (IEV). The formation of these particles is necessary for the polymerization of actin tails that aid virus dissemination to surrounding cells (Cudmore *et al.*, 1995). At the cell surface, the outer membrane of IEV particle fuses with the plasma membrane to release EEV from the cell. Some enveloped virus may

remain attached, or reattach, to the cell surface where it is called cell-associated enveloped virus (CEV) (Blasco and Moss, 1992).

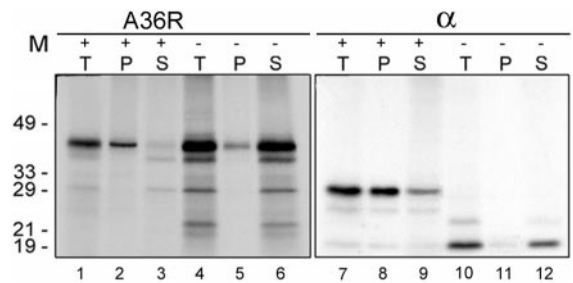
Six genes (F13L, A33R, A34R, A36R, A56R, and B5R) have been reported to encode proteins that are associated with EEV but not IMV particles (Hiller and Weber, 1985; Shida, 1986; Duncan and Smith, 1992; Engelstad *et al.*, 1992; Isaacs *et al.*, 1992; Parkinson and Smith, 1994; Roper *et al.*, 1996). These proteins have different roles in virus morphogenesis. Four, F13L (Blasco and Moss, 1991; Cudmore *et al.*, 1995), A33R (Roper *et al.*, 1998), A34R (Duncan and Smith, 1992; McIntosh and Smith, 1996; Wolffe *et al.*, 1997; Sanderson *et al.*, 1998), and B5R (Engelstad and Smith, 1993; Wolffe *et al.*, 1993; Sanderson *et al.*, 1998), are needed for efficient wrapping of IMV to IEV and consequently for efficient actin tail formation. A36R is not required for IEV formation but is required to make actin tails (Parkinson and Smith, 1994; Sanderson *et al.*, 1998; Wolffe *et al.*, 1998; Frischknecht *et al.*, 1999). The other gene, A56R, is not required for either of these processes (Sanderson *et al.*, 1998), but its loss causes the plaque phenotype to become syncytial (Ichihashi and Dales, 1971). In cases where actin tail formation is reduced or abolished, the plaque formed by the mutant

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virus is small. EEV formation is influenced in different ways by these gene products. Loss of F13L (Blasco and Moss, 1991), A36R (Parkinson and Smith, 1994), and B5R (Engelstad and Smith, 1993; Wolffe *et al.*, 1993) caused a 100-, 3- to 5-, and 10-fold reduction in EEV infectious titer, respectively. In contrast, viruses lacking A33R (Roper *et al.*, 1998) or A34R (McIntosh and Smith, 1996) produced 2- to 4- or 25-fold more EEV, although in the latter case the EEV had a 5-fold reduced specific infectivity.

The A36R gene product was identified as a 45-kDa protein that copurified with EEV during density gradient centrifugation and is conserved in orthopoxviruses (Parkinson and Smith, 1994). It possesses an N-terminal hydrophobic sequence and seven potential sites for addition of N-linked carbohydrate, but its size was unaltered after synthesis in the presence of tunicamycin, suggesting that these were unused (Parkinson and Smith, 1994). Based upon the sensitivity of the A36R protein in purified EEV to digestion with protease, it was proposed that the majority of the protein was exposed on the outside of the virion with a type II membrane topology (Parkinson and Smith, 1994). However, subsequently it became evident that EEV purified by sucrose or CsCl density gradient centrifugation has a damaged outer envelope that permits the entry of anti-IMV antibody (Vanderplasschen and Smith, 1997) and so presumably exogenous protease too. Thus the protease sensitivity of A36R in purified EEV did not prove type II membrane topology of the protein. Recently, the A36R protein was shown to have a type Ib topology in which the N-terminus was exposed in the lumen of the wrapping membranes and the outside of the virion, while the majority of the protein, including the C-terminus, was exposed in the cytosol (Röttger *et al.*, 1999). A prediction from this observation was that most of the A36R protein would be internal of the EEV outer envelope, and in accord with this, EEV particles were not stained with antibody raised against the C-terminal domain of A36R (Röttger *et al.*, 1999). However, an alternative interpretation of these data would be that A36R is not present in EEV. A36R was shown by immunoprecipitation to interact with the A33R and A34R EEV proteins (Röttger *et al.*, 1999).

In this study, we have investigated the topology and location of the A36R protein in cellular membranes and virions by immunoelectron microscopy, confocal microscopy, and *in vitro* transcription and translation. These data show that the protein has a type Ib topology and is present on the membranes that wrap IMV particles to form IEV within infected cells. In addition, we report that the A36R protein is predominantly on the outer IEV membrane, and where CEV particles were in close contact with the plasma membrane, the A36R protein was detected only on the cytosolic face of the plasma membrane rather than on the CEV particle. This places A36R in a perfect location for the polarized formation of actin tails from the plasma membrane to drive CEV particles



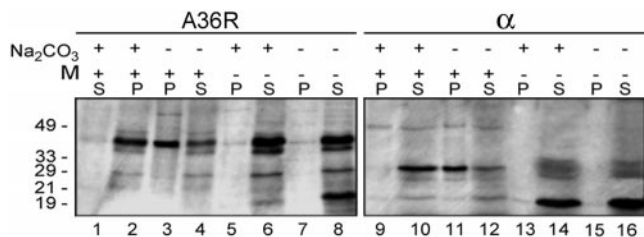
**FIG. 1.** The A36R protein is associated with microsomes. The VV WR A36R protein (lanes 1–6) or yeast  $\alpha$  factor (lanes 7–12) were transcribed and translated *in vitro* in the presence (+) or absence (–) of microsomal membranes as described under Materials and Methods. Reaction products were then separated in total (T), supernatant (S), or pellet (P) fractions. Samples were resolved by SDS–PAGE (12% gel) and detected by autoradiography of the dried gel. Molecular mass markers in kilodaltons are indicated.

away from the cell. We propose that A36R was detected by immunoblotting with EEV particles because of the tight association of some EEV particles with fragments of cell membranes that copurify with EEV particles.

## RESULTS

### *In vitro* transcription and translation of A36R

The membrane association of the A36R protein was investigated by cloning the A36R ORF into plasmid pGEM3Z downstream of the promoter recognized by bacteriophage T7 RNA polymerase. This plasmid was then transcribed *in vitro* using T7 RNA polymerase and the RNA was translated *in vitro* using a micrococcal nuclease-treated rabbit reticulocyte lysate in the presence or absence of canine pancreatic microsomes as described (Parkinson *et al.*, 1995) (Fig. 1). The size of the A36R protein made in the presence or absence of microsomes was the same (Fig. 1, compare lanes 1 and 4) suggesting a lack of posttranslational proteolysis or glycosylation of the protein. However, the behavior of the protein during subsequent fractionation by centrifugation differed depending on whether the protein had been translated in the presence or absence of microsomes. In the absence of microsomes (Fig. 1, lanes 4–6) the majority of the protein was in the supernatant (S) after centrifugation, whereas after translation with microsomes, the majority of the protein was present in the pellet (P) (Fig. 1, lane 2). For comparison, the luminal protein yeast  $\alpha$  factor was translated in parallel (Fig. 1, lanes 7–12). As expected, the size of the  $\alpha$  factor increased from 19 kDa (without microsomes) to 29 kDa (with microsomes), due to the glycosylation of the protein. As for A36R, the protein synthesized without microsomes was in the supernatant after centrifugation, whereas the protein pelleted during centrifugation if it was translated in the presence of microsomes (Fig. 1, lane 8). These data demonstrated an association of A36R with microsomal membranes.



**FIG. 2.** The A36R protein is an integral membrane protein. The A36R protein (lanes 1–8) or yeast  $\alpha$  factor (lanes 9–16) were transcribed and translated *in vitro* and then extracted with  $\text{Na}_2\text{CO}_3$ , pH 11.5, or with PBS as indicated. Samples were then separated into pellet (P) or supernatant (S) fractions as described in the legend Fig. 1 and all samples were resolved by SDS–PAGE (12% gel) and detected by autoradiography of the dried gel. Molecular mass markers in kilodaltons are indicated.

### The A36R protein is an integral membrane protein

The nature of the association of A36R with the microsomes was examined by extracting translation reactions with  $\text{Na}_2\text{CO}_3$  at pH 11.5 (Fig. 2), a method that enables integral membrane proteins to be distinguished from proteins associated with membranes peripherally or in the vesicle lumen. When the A36R protein was translated in the presence of microsomes and then extracted with  $\text{Na}_2\text{CO}_3$ , it was associated predominantly with the pellet fraction (Fig. 2, lane 2), whereas if microsomes were omitted during translation, the protein was soluble after  $\text{Na}_2\text{CO}_3$  extraction (Fig. 2, lane 6). As shown in Fig. 1, the protein was soluble in PBS after translation without membranes, but was in the pellet if translated with membranes (Fig. 2, lanes 3, 4, 7, and 8). These data showed that A36R was an integral membrane protein. In contrast, when yeast  $\alpha$  factor was translated in the presence of microsomes (Fig. 2, lanes 9–12) and fractionated with  $\text{Na}_2\text{CO}_3$ , it was predominantly a soluble protein (Fig. 2, lane 10), consistent with the release of the protein into the lumen of the vesicle.

### The A36R protein synthesized in the presence of microsomes is sensitive to exogenous protease

To determine the membrane topology of the A36R protein, mRNA was translated in the presence or absence of microsomes and then subjected to digestion with trypsin in the presence or absence of Triton X-100 (Fig. 3, lanes 1–6). The 45-kDa A36R protein (Fig. 3, lane 1) was destroyed by incubation with trypsin even if the sample was not treated with detergent to disrupt the microsomal membrane (Fig. 3, lane 2). This indicated that the majority of the A36R protein was exposed to protease on the outside of the vesicle. In contrast, similar experiments with yeast  $\alpha$  factor (Fig. 3, lanes 7–9) showed that the protein was resistant to protease unless the membrane of the vesicle was first destroyed by addition of detergent (Fig. 3, lanes 7–9). This served as a control to show that the microsomal membranes were

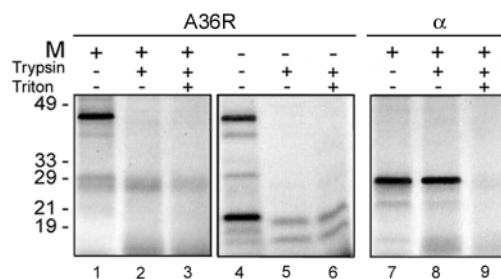
intact and impermeable to protease in the absence of detergent.

Collectively, these data showed that the A36R protein was associated integrally with membranes and that it had type Ib topology with the majority of the protein after the N-terminal, hydrophobic, membrane-spanning sequence exposed in the cytosol.

### The A36R protein is not exposed on the surface of infected cells

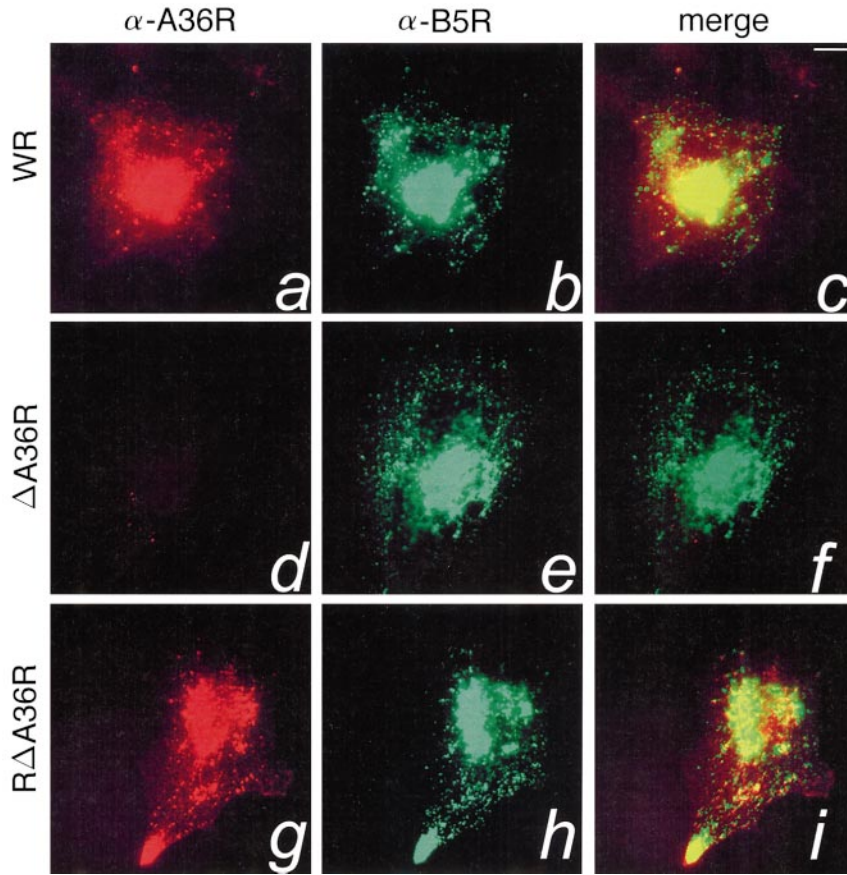
The polyclonal antibody against A36R used previously (Parkinson and Smith, 1994) did not work well for immunofluorescence or immunoprecipitation and therefore a monoclonal antibody (MAb) to the protein was produced to characterize the A36R protein in cells. Mice were immunized with A36R protein that had been expressed and purified from *Escherichia coli* as described (Parkinson and Smith, 1994) and a MAb was isolated as described under Materials and Methods. The specificity of MAb 6.3 for A36R was demonstrated by immunofluorescence (Fig. 4). BS-C-1 cells were infected with VV Western Reserve (WR), a recombinant VV lacking the A36R gene ( $\Delta$ A36R), or a revertant virus, RA36R, in which the A36R gene was reinserted into  $\Delta$ A36R (Parkinson and Smith, 1994), and at 12 h postinfection (p.i.) the cells were fixed and processed for indirect immunofluorescence. Rat MAb 19C2 (Schmelz *et al.*, 1994) recognized the B5R protein in cells infected by all viruses (Figs. 4b, 4e, and 4h), whereas mouse MAb 6.3 recognized cells infected with WR (Fig. 4a) and RA36R (Fig. 4g) but not  $\Delta$ A36R (Fig. 4d). The cell shown in Figs. 4d–4f was definitely infected because it stains with MAb 19C2 (Fig. 4e).

To examine the location of A36R in virus-infected cells, BS-C-1 cells were infected with VV strain WR and then were stained with MAb 6.3 or 19C2 (Fig. 5). If the cells were fixed and permeabilized before addition of the

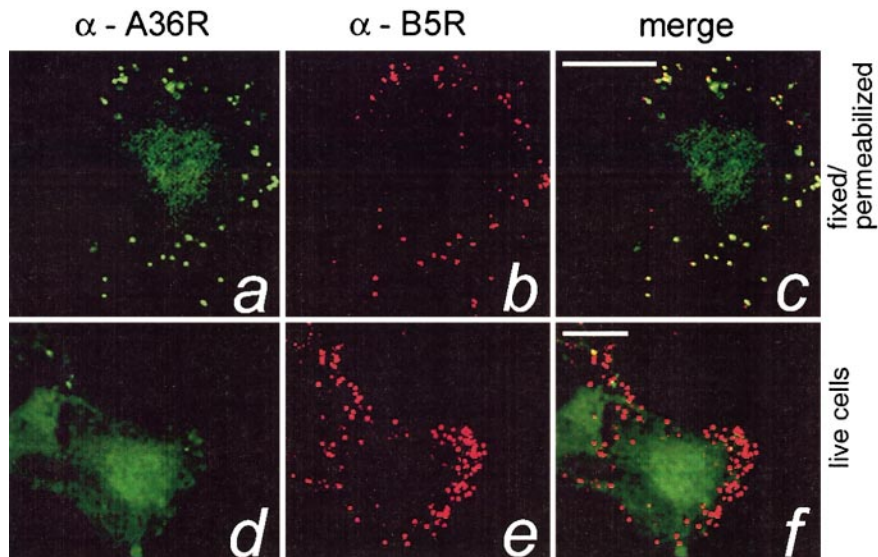


**FIG. 3.** The A36R protein is exposed on the outside of the microsomal membrane. The A36R protein (lanes 1–6) or yeast  $\alpha$  factor (lanes 7–9) were transcribed and translated *in vitro* in the presence (+) or absence (–) of microsomes as described in the legend to Fig. 1. The samples were then separated into three equal fractions and incubated for 10 min on ice with equal volumes of PBS (lanes 1, 4, and 7), PBS containing trypsin (0.2 mg/ml) (lanes 2, 5, and 8), or PBS containing trypsin and 0.2% Triton X-100 (lanes 3, 6, and 9). Samples were then resolved by SDS–PAGE (12% gel) and detected by autoradiography of the dried gel. Molecular mass markers in kilodaltons are indicated.





**FIG. 4.** MAb 6.3 is specific for the VV WR A36R protein. BS-C-1 cells were infected with the indicated viruses at 1 PFU/cell and at 12 h p.i. were processed for indirect immunofluorescent microscopy as described under Materials and Methods. Panels a, d, and g were stained with MAb 6.3; (b, e, and h) stained with MAb 19C2 against the VV B5R protein. Panels c, f, and i show the merged images of a and b, d and e, and g and h, respectively. Bar, 10  $\mu$ m.



**FIG. 5.** The A36R protein is not exposed on the cell surface. BS-C-1 cells were infected with VV WR at 1 PFU/cell and then processed for immunofluorescent microscopy as described under Materials and Methods. In a–c the cells were stained with MABs 6.3 or 19C2 after fixation of the cells with paraformaldehyde and permeabilization with saponin, whereas in d–f the cells were stained with these antibodies prior to fixation or permeabilization (live cells). Bound antibodies were detected with secondary conjugates as indicated under Materials and Methods and cells were examined by confocal microscopy. (c and f) The merged images shown in a and b and d and e, respectively.

MAbs, both MAbs reacted with dispersed virus particles (Figs. 5a and 5b) and merging these two images showed the staining with these different antibodies was coincident (Fig. 5c). However, if the MAbs were added to live cells before fixation and permeabilization (Figs. 5d–5f), the A36R-positive structures were not evident. This demonstrated that the C-terminal domain of the A36R protein, to which the MAb is directed, was not exposed on the surface of cells or CEV particles on the cell surface.

### Immunoelectron microscopy

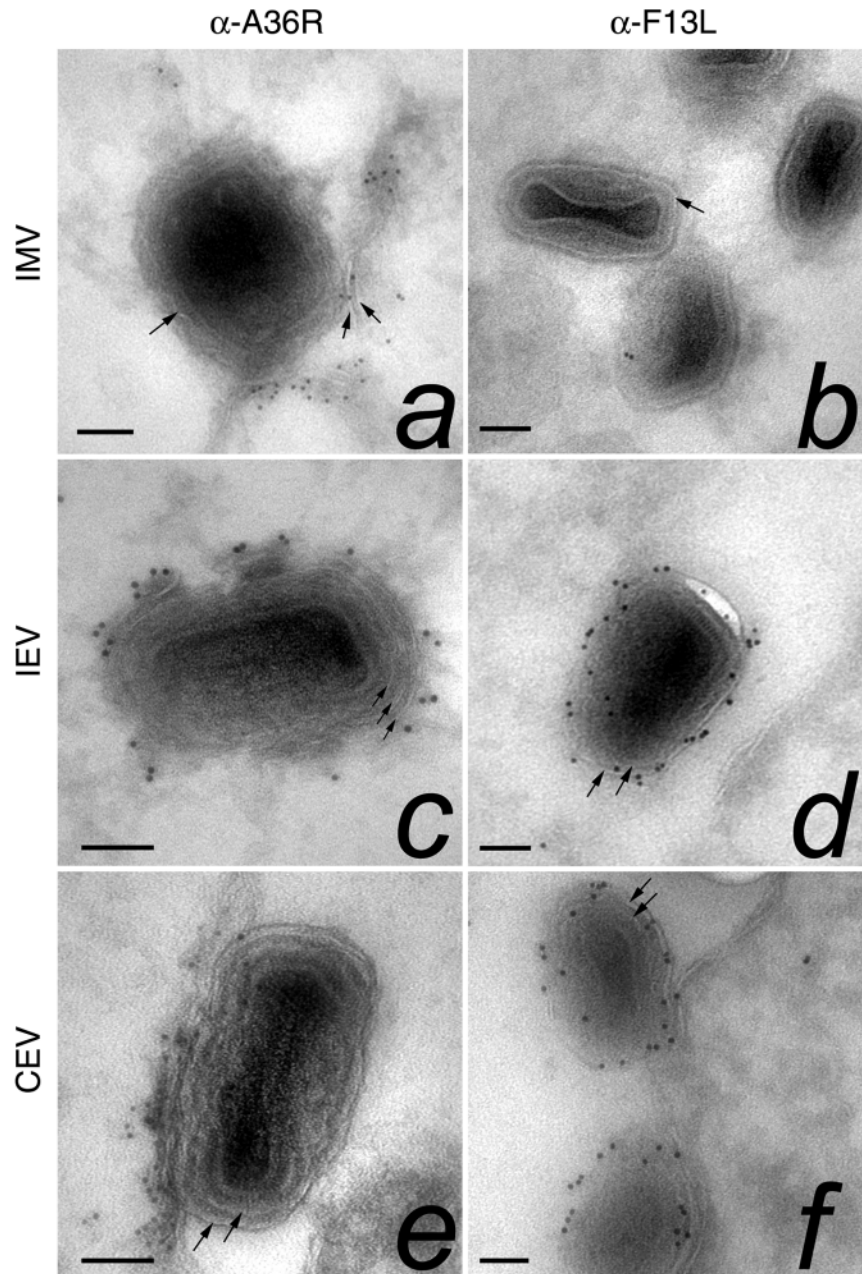
The location of the A36R protein in cells and virions was examined in more detail by immunoelectron microscopy. HeLa cells were infected with VV strain WR and processed for cryoelectron microscopy at 9 h p.i. as described under Materials and Methods. Cryosections of infected cells were stained with MAbs against the A36R (or F13L) proteins and detected by staining with protein A–gold particles. In sections stained with the anti-A36R MAb, isolated IMV particles were unstained, but membranes wrapping IMV particles to become IEV (Fig. 5a), or complete IEV particles (Fig. 5c) were stained with numerous gold particles. Careful examination of the distribution of these gold particles in several IEV particles (e.g., Fig. 5c) indicated that the great majority (85%) were located on the cytosolic face of the outer wrapping membrane rather than on the inner membrane or between the membranes. Examination of CEV particles on the cell surface revealed an unexpected distribution of A36R. Numerous gold particles were seen on the internal face of the plasma membrane where the CEV particle was in contact with the cell, but few if any gold particles were seen on the outer membrane of CEV or between the CEV outer membrane and the cell surface (Fig. 5e). As a control, cells were also stained with MAb 15B6 (Schmelz *et al.*, 1994) that is directed against the F13L gene product. Like A36R, the F13L antigen was not associated with IMV particles (Fig. 5b), but the outer membranes of IEV particles contained many gold particles (Fig. 5d). In addition, CEV particles at the cell surface stained strongly for the F13L protein, but extensive labeling at the interface of the CEV and plasma membrane was not observed (Fig. 5f). Thus the A36R and F13L proteins have different distributions at the cell surface where the cell is in contact with CEV particles, and whereas F13L is associated with CEV particles, A36R is not.

### Some released EEV particles are associated with A36R and polymerized actin

Previous immunoblot analyses had shown that A36R was associated with EEV particles that had been purified by density gradient centrifugation (Parkinson and Smith, 1994; Galmiche *et al.*, 1999; Röttger *et al.*, 1999). This result is inconsistent with the immunoelectron microscopy presented in Fig. 6. The distribution of A36R on the

plasma membrane suggested that A36R might associate with EEV particles indirectly via fragments of plasma membrane that remain attached to EEV particles as CEV particles are released from the cell. To address this, cells were infected with VV WR and stained with MAbs against A36R or B5R, costained with rhodamine–phalloidin and DAPI, and processed for immunofluorescent confocal microscopy (Fig. 7). A36R antigen was seen on particulate structures that were also stained by DAPI suggesting that they were virus particles (data not shown) and that in some cases were protruding from the cell surface and in other cases were well separated from the cell (Fig. 7a). Costaining with phalloidin (Fig. 7b) and merging of the images (Fig. 7c) showed that the A36R-positive particles (virions) protruding from the cell surface colocalized with actin. In addition, some of the DAPI-positive particles that were separated from the cell were positive for both actin and A36R. Similarly, B5R-positive particles were present at the cell surface and separated from the cell. In the latter case, some of the particles colocalized with polymerized actin. The images shown are reconstructions of a series of optical sections so that if the virions were still attached to the cell via actin tails these connections would have been evident. The lack of such connections is consistent with the virions being EEV particles and indicates that some of these virions are associated with both A36R and actin.

The presence of actin in EEV particles was also examined by immunoblotting (Fig. 8). EEV were purified from cells infected with WR or IHD-J and extracts of these virions were analyzed by immunoblotting in parallel with extracts of infected cells. A36R and actin were found in WR EEV but not on IHD-J EEV, although in some preparation of IHD-J EEV, small amounts were detected after much longer exposure (data not shown). However, in all cases the amount of A36R found in IHD-J EEV was very much lower than that present in WR EEV. Probing the filters with MAbs against F13L and D8L (virion proteins) confirmed that equal amounts of IHD-J EEV had been loaded onto the filter. All these antigens were detected in virus-infected cells. The presence of actin and A36R in WR EEV but not IHD-J EEV might be explained by the fact that IHD-J-enveloped virions predominantly are released from the cell surface rather than being retained as CEV, whereas WR-enveloped virions are mostly retained as CEV (Blasco and Moss, 1992). Perhaps the stronger interaction of WR-enveloped virions with the plasma membrane causes fragments of plasma membrane and associated actin tails to be torn from the cell surface (or the surface of protruding virus-tipped microvilli) during virus release. For IHD-J EEV, the natural release of enveloped virions might prevent this to a large degree.



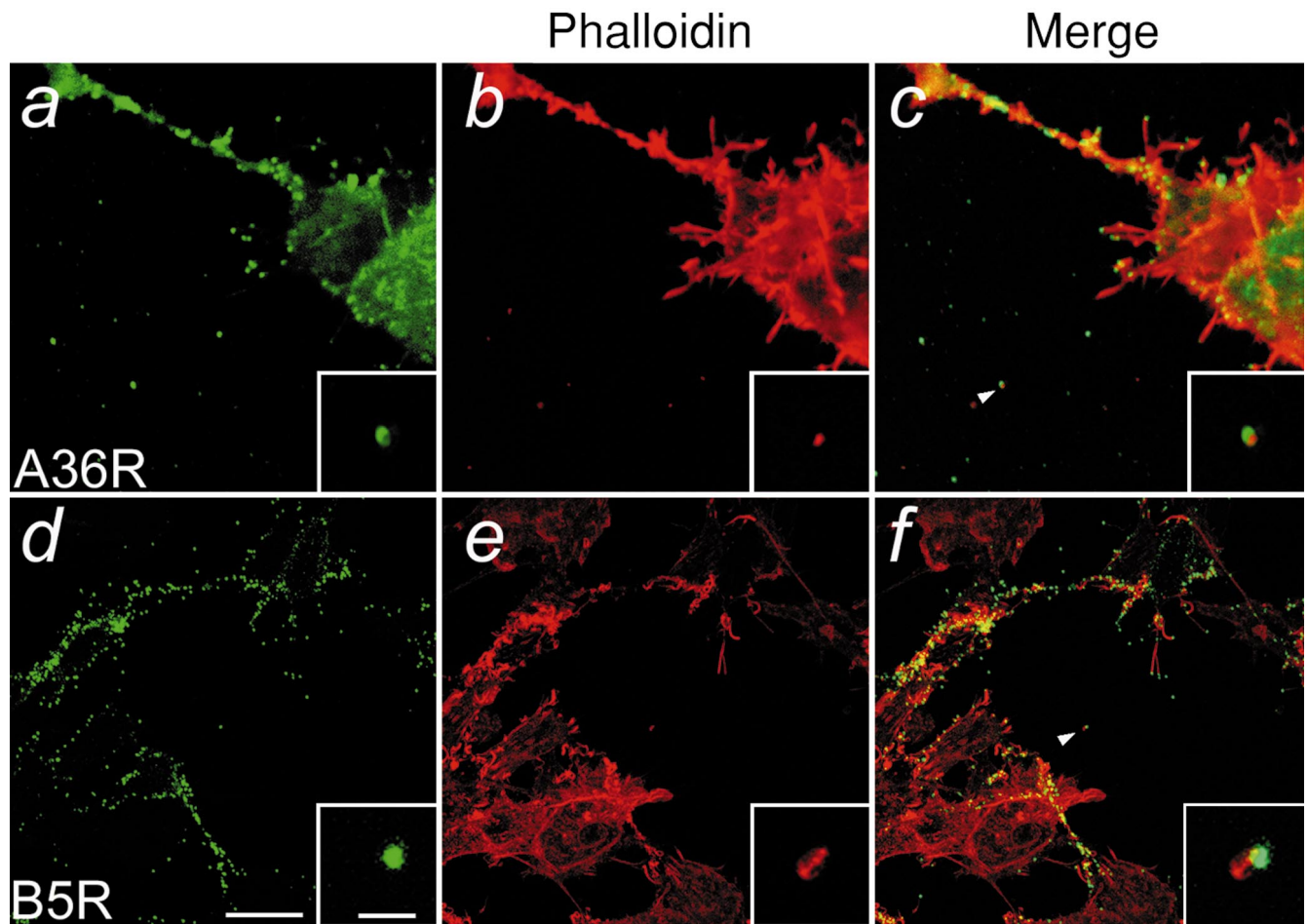
**FIG. 6.** Immunoelectron microscopy of infected cells. HeLa cells were infected with VV strain WR and processed for immunoelectron microscopy of cryosections at 9 h p.i. In a, c, and e the sections were stained with MAb 6.3 against the A36R protein, and in b, d, and f the sections were stained with MAb 15B6 against the F13L protein. For the detection of bound MAb 6.3, 6-nm gold particles were used, while for MAb 15B6 9-nm gold particles were used. (a and d) An IMV particle that in (a) is becoming wrapped with membranes containing the A36R protein; (b and e) IEV particles; and (c and f) CEV particles on the cell surface. Arrows point to the single IMV membrane of IMV particles, the three membranes of IEV particles, and the two membranes of CEV particles. Bars, 100 nm.

## DISCUSSION

The membrane topology of the A36R protein and its association with membranes of infected cells and virus particles have been examined. *In vitro* transcription and translation demonstrated that the A36R protein was an integral membrane protein with the majority of the protein on the outside of the vesicle and therefore in the cytosol of the infected cell. A MAb was generated

against the A36R protein and used to examine the location of the protein in infected cells and virus particles. A36R was not expressed on the cell surface and was absent from IMV and CEV particles but was present on the IEV particles and on the cytosolic face of the plasma membrane where a CEV particle was in direct contact with the cell. A36R and actin were detected associated with WR but not IHD-J EEV.



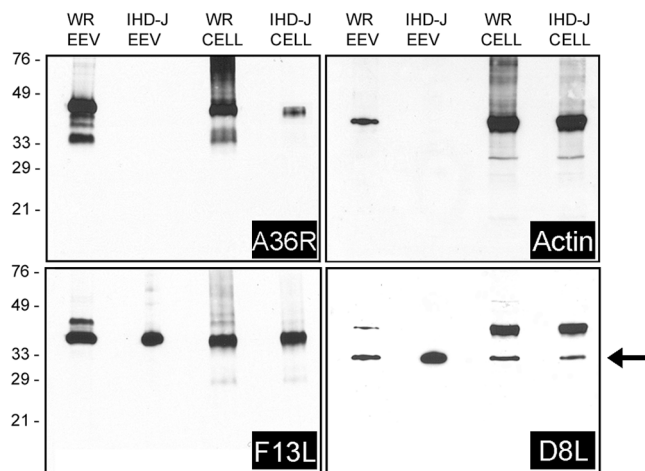


**FIG. 7.** The A36R protein and actin are associated with some EEV particles. RK<sub>13</sub> cells grown on glass coverslips were infected with VV strain WR at 1 PFU/cell. Cells to be stained with MAb 6.3 (a–c) were harvested at 13 h p.i., whereas cells to be stained with MAb 19C2 (d–f) were harvested at 9 h p.i. After washing  $\times 3$  with ice-cold PBS, the cells were fixed by incubation in paraformaldehyde as described under Materials and Methods. Cells were then washed in PBS (as above) and permeabilized by addition of 0.5% Triton X-100 in PBS for 2 min at RT. Cells were then washed in PBS and stained with MAb 6.3 or 19C2 as described in the legend to Fig. 4. F-actin was stained with TRITC-conjugated phalloidin (Sigma). Coverslips were mounted in Mowiol containing DAPI. Images were recorded using a Bio-Rad MRC 1024 confocal laser scanning microscope and processed using Adobe Photoshop software. Insets show enlarged particles indicated by arrows. Bars, 10  $\mu\text{m}$  in main panels and 1  $\mu\text{m}$  in insets.

The conclusion that the A36R protein is an integral membrane protein with the bulk of the protein in the cytosol and not on the cell surface (type Ib membrane topology) is in agreement with the recent work of Röttger *et al.* (1999). Using microinjection of antibody raised to the C-terminal domain of the A36R protein, Röttger *et al.* showed that the A36R protein was in the cytosol and not within lumen of the wrapping membranes. The observation that the A36R protein has seven potential sites for addition of N-linked carbohydrate, and yet none of these are used (Parkinson and Smith, 1994), is also consistent with the type Ib topology of the A36R protein. These motifs would not become accessible to the glycosylation enzymes within the lumen of the endoplasmic reticulum.

Here we have used confocal and immunoelectron microscopy to investigate the distribution of A36R protein in cells and virions. Immunoelectron microscopy showed that the A36R protein is not associated with IMV particles

but is associated with the cellular membranes used to wrap IMV particles to form IEV. Analysis of multiple IEV particles suggested that the majority of gold particles was located on the cytosolic face of the outer wrapping membrane rather than between the IMV particle and the inner wrapping membrane. If A36R was present on only the outer IEV membrane, it would be lost from IEV during fusion of this membrane with the plasma membrane. This unequal distribution of A36R between the inner and outer wrapping membrane is consistent with images of CEV particles in which only the cytosolic face of the plasma membrane was stained by A36R, and gold particles were not detected elsewhere on the plasma membrane nor on the CEV particle itself. The nonrandom distribution of the A36R on IEV particles and its absence from CEV might be explained by the inaccessibility of the epitope recognized by MAb 6.3 due to its tight association with another virus or cell protein. While this cannot



**FIG. 8.** A36R and actin are present in purified WR EEV. WR and IHD-J EEV were harvested from infected RK<sub>13</sub> cells and purified from by sucrose density gradient centrifugation as described (Parkinson and Smith, 1994). Samples (2  $\mu$ g) were resolved by SDS-PAGE (12% gel) alongside extracts from BS-C-1 cells that had been infected with WR or IHD-J at 10 PFU/cell for 24 h. After transfer of proteins to filters, blots were probed with MAb 6.3 [tissue culture supernatant diluted 1/5 in PBS containing 0.1% Tween 20 (Sigma) and 5% milk powder (PBS/T/M)] or with an anti-actin rabbit polyclonal antibody (Sigma, diluted 1/250 in PBS/T/M). Bound Ig was detected using secondary antibodies (Sigma) and ECL reagents (Amersham). Filters were then stripped and re-probed with MABs 15B6 (anti-F13L) (Hiller and Weber, 1985) or AB1.1 (anti-D8L) (Parkinson and Smith, 1994) diluted 1/500 or 1/1000 in PBS/T/M, respectively. Bound Ig was detected as before. Protein molecular weight markers are shown in kilodaltons.

be excluded, the MAb directed against F13L detected this antigen on CEV particles between the IMV surface and the outer CEV membrane. Assuming that A36R is inserted into the rough endoplasmic reticulum membrane during translation and is then processed through the export pathway to the TGN, the presence of A36R on only the outer IEV membrane and its absence from CEV particles indicates that a mechanism exists to create this unequal distribution. This might involve exclusion of A36R from between IMV and the inner wrapping membrane, as envelopment of the IMV particle progresses. For instance, interactions between the F13L protein on the cytosolic surface of the wrapping membranes and the A27L protein on the surface of IMV might progressively exclude A36R from this location. Notably, both A27L and F13L are required for wrapping to proceed, whereas A36R is not (Rodriguez and Smith, 1990; Blasco and Moss, 1991). Other EEV proteins, such as B5R, A33R, and A34R, might not be excluded since the bulk of each of these proteins is within the lumen of the wrapping membranes rather than in the cytosol. Alternatively, the selective recruitment of A36R to the outer IEV membrane might be aided by interactions of A36R with cytoplasmic components involved in the polymerization of actin (Frischknecht *et al.*, 1999). Whatever the mechanism, the exclusion of A36R from the inner membrane must have occurred prior to the completion of the wrapping pro-

cess, because subsequently the inner and outer membranes are no longer physically connected.

Originally, the A36R protein was found associated with EEV preparations that had been purified by density gradient centrifugation. However, this study indicates that A36R is not present in CEV, and therefore EEV, particles and was found by immunoblotting to be associated with EEV because of the copurification of fragments of cellular membranes with EEV. Consistent with this, we demonstrate here that some virus particles that have detached from the cell are associated with both A36R and polymerized actin. In other cases, detached virions stained for A36R and not actin because, presumably, the actin tail was no longer associated with the fragment of membrane. It is likely that many EEV particles are associated with neither A36R nor actin. Previously, Vanderplasschen *et al.* (1998) reported the copurification of several cellular proteins with EEV but not IMV preparations (Vanderplasschen *et al.*, 1998). Three of these proteins (CD46, CD55, and CD59) were shown by immunogold electron microscopy to be physically associated with EEV membranes. However, although the IHD-J strain of VV was used, it remains possible that the other proteins (CD71, CD81, and MHC class I antigen) were associated with plasma membrane fragments interacting with EEV particles. Immunoelectron microscopy is needed to investigate this.

The A36R protein represents the only example of a virus-encoded protein that is present on the IEV but not CEV (and EEV) particle. Therefore, the A36R protein is not required for virion infectivity, by promoting binding or reentry of virions into cells, but is required to promote the polymerization of actin and dissemination of virions to surrounding cells. Several observations support this conclusion: first, the  $\Delta$ A36R deletion mutant can form IEV and EEV particles, but not actin tails, and the plaque phenotype is small (Parkinson and Smith, 1994; Sanderson *et al.*, 1998; Wolffe *et al.*, 1998; Röttger *et al.*, 1999); second, the A36R protein is located on the cytosolic face of cell membranes in a position to promote actin tail formation (Röttger *et al.*, 1999; and this paper); and third, mutagenesis of the A36R protein shows that tyrosine phosphorylation of A36R is needed for actin polymerization (Frischknecht *et al.*, 1999). Concerning the site of actin tail polymerization, it was suggested previously that this occurs on IEV particles within the cell and the growing actin tail drives the IEV particle toward the cell surface (Cudmore *et al.*, 1995), although published data do not distinguish between intracellular and cell surface actin polymerization. We favor an alternative model in which actin tail formation takes place at the plasma membrane where a CEV particle is present. First, actin tails are not needed for the movement of IEV particles to the cell surface since viruses lacking A34R and A36R make IEV, CEV, and EEV but do not make actin



tails (Sanderson *et al.*, 1998). Second, if actin is polymerized on IEV particles, why does it take place on only one side of the virion in view of the fact that A36R is present over the entire virion? At the cell surface, however, A36R is present only immediately beneath the CEV particle in a position suitable to polymerize actin on only one side of the virion and to drive the CEV particle away from the cell. Notably, the plasma membrane on either side of the CEV particle is devoid of A36R, even though part of this membrane would previously have been the IEV outer membrane. This suggests an increased concentration of A36R immediately underneath the CEV particle after fusion of the IEV outer membrane with the plasma membrane and possibly an increased density of A36R is a trigger to promote actin polymerization. Third, the biochemical analysis of the cellular proteins recruited during vaccinia virus-induced actin tail polymerization concluded that this mimics receptor tyrosine kinase signaling (Frischknecht *et al.*, 1999), which occurs at the cell surface. Further experimentation is needed to address where actin tails are formed.

In summary, the A36R protein is shown to be a type Ib membrane protein that is present on IEV but not IMV or CEV particles.

## MATERIALS AND METHODS

### Cells and viruses

VV strain WR,  $\Delta$ A36R, and RA36R have been described (Parkinson and Smith, 1994). These viruses were grown and titrated in BS-C-1 cells as described (Mackett *et al.*, 1985). BS-C-1, HeLa, and RK<sub>13</sub> cells were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) (GibcoBRL).

### Plasmid construction

The VV WR A36R ORF was excised from plasmid pJEP6 (Parkinson, 1995) by digestion with *Bam*HI and ligated with pGEM3Z (Promega) that had been digested with the same enzyme, forming pHE4, so that the A36R ORF was downstream of the T7 RNA polymerase promoter.

### *In vitro* transcription and translation

*In vitro* transcription and translation reactions were carried out as described previously (Parkinson *et al.*, 1995). Briefly, plasmid pJEP6 was digested with *Hinc*II and then transcribed by T7 RNA polymerase using the RiboMAX large-scale production system (Promega). One-tenth of the resultant RNA was translated in 25  $\mu$ l of a micrococcal nuclease-treated rabbit reticulocyte lysate (Amersham) in the presence of 20  $\mu$ Ci of [<sup>35</sup>S]methionine (*in vivo* cell labeling grade, Amersham) with or without canine pancreatic microsomes (Promega). Yeast  $\alpha$  factor

was transcribed and translated in parallel. Where indicated, the *in vitro* translation reactions were centrifuged (13,000 rpm, 30 min, 4°C in a Heraeus Biofuge) to pellet microsomes and associated proteins. The supernatant was then removed and the pellet was resuspended in 25  $\mu$ l of phosphate-buffered saline (PBS). Aliquots were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 12% gel and radiolabeled proteins were detected by autoradiography of the dried gel.

### Sodium carbonate extraction

Proteins that had been translated *in vitro* in the presence or absence of canine pancreatic microsomes were extracted with Na<sub>2</sub>CO<sub>3</sub>, pH 11.5, as follows. Ten microliter aliquots were mixed with an equal volume of 0.2 M Na<sub>2</sub>CO<sub>3</sub> or PBS and left on ice for 10 min. Samples were then centrifuged (13,000 rpm, 30 min, 4°C in a Heraeus Biofuge) and the supernatant (S) and pellet (P) fractions were collected. Pellets were resuspended in 20  $\mu$ l of protein loading buffer and then all samples were analyzed by SDS–PAGE (12% gel) and autoradiography.

### Trypsinization

*In vitro* translation reactions were divided equally into three microfuge tubes and these aliquots were mixed with equal volumes of PBS, PBS plus 0.2 mg/ml trypsin (Sigma), or PBS plus trypsin and 0.2% Triton X-100. Each sample was incubated on ice for 10 min and the reaction stopped by the addition of trypsin inhibitor (Sigma) to a final concentration of 1  $\mu$ g/ml. Samples were then analyzed by SDS–PAGE as above.

### Generation of monoclonal antibody against A36R

Balb/c mice were immunized with purified recombinant A36R protein, produced from *E. coli* as described previously (Parkinson and Smith, 1994), until they had mounted a strong A36R-specific antibody response that could be detected by immunofluorescence and immunoblotting. After a final boost with antigen, the spleens were removed and fused with mouse HAT-sensitive NS1/1 myeloma cells and the resultant hybridomas were seeded into 96-well plates and grown for 10 days. Supernatants were screened for A36R-specific antibody by indirect immunofluorescence on plaques formed by VV WR or  $\Delta$ A36R on monolayers of RK<sub>13</sub> cells. Positive clones were confirmed by immunoblotting using extracts from cells that had been infected with these viruses. After three cycles of cloning by limiting dilution, a clone that secreted an A36R-reactive antibody was selected and called MAb 6.3. The subclass of this antibody was identified as IgG1 using a immunoglobulin typing kit (Sigma).

## Immunofluorescence

BS-C-1 cells were grown on glass coverslips and were infected with VV WR,  $\Delta$ A36R, or RA36R at 1 PFU/cell and incubated in MEM containing 2.5% FBS. For staining of live cells, at 12 h p.i. the culture medium was replaced with MEM containing mouse MAb 6.3 against A36R (hybridoma culture supernatant diluted 1:10) and rat MAb 19C2 against B5R (Schmelz *et al.*, 1994) (hybridoma culture supernatant diluted 1:50) and incubation continued for 1 h at 37°C. Cells were washed three times in ice-cold PBS and then fixed by incubation in 4% paraformaldehyde, 250 mM HEPES, pH 7.4, for 10 min on ice, followed by 50 min on ice in 8% paraformaldehyde in the same buffer. Cells were then washed with PBS and quenched in 50 mM  $\text{NH}_4\text{Cl}$  for 5 min at room temperature (RT). After washing again in PBS, the cells were permeabilized by incubation in PBS, 0.1% saponin (Sigma), 10% FBS for 10 min at RT. After washing with PBS containing 10% FBS, the cells were incubated in PBS, 0.1% saponin, 10% FBS containing fluorescein b isothiocyanate (FITC)-conjugated goat anti-mouse IgG that had been preadsorbed against rat IgG (diluted 1:100) (Stratech Scientific, Luton, Bedfordshire, UK), and rhodamine-conjugated donkey anti-rat IgG that had been preabsorbed against mouse IgG (diluted 1:100) (Stratech Scientific) for 30 min at RT. Cells were washed twice in PBS and once in water and then mounted in Mowiol as described previously (Sanderson *et al.*, 1996).

To stain fixed and permeabilized cells with MAbs, cells were incubated in MEM containing 2.5% FBS until 12 h p.i., the medium was then changed to MEM containing 2.5% FBS, and incubation was continued for 1 h. Cells were then fixed, permeabilized, and incubated with MAbs 6.3 and 19C2 as above. After washing, the cells were stained by FITC- or TRITC-conjugated secondary antibodies as above and examined with a Bio-Rad MRC 1024 laser scanning confocal microscope. Images were collected at 60-fold magnification using COMOS software and processed using Adobe Photoshop.

## Electron microscopy

HeLa cells were infected with VV strain WR at 10 PFU/cell and at 9 h p.i. the cells were fixed in paraformaldehyde as described for immunofluorescence and frozen in 2.1 M sucrose. Frozen samples were sectioned on a Reichert Ultracut S microtome with FCS attachment, thawed, and placed on electron microscope grids. Sections were washed in PBS, incubated for 45 min at RT with mouse MAb 6.3 (undiluted hybridoma culture supernatant) or with rat MAb 15B6 (hybridoma culture supernatant diluted 1:10 in PBS containing 5% FBS), and then washed in PBS, 5% FBS. Bound Ig was detected by incubation with rabbit anti-mouse IgG (Sigma, diluted 1:50 in PBS, 5% FBS) for 30 min at RT, washed again in PBS, 5% FBS, and then incubated with protein A-gold

(6-nm gold particles diluted 1:100 in PBS, 5% FBS). After a final wash in PBS, the samples were embedded in uranyl acetate methyl cellulose and dried. Labeled sections were examined in an Omega 912 electron microscope (Zeiss, LEO Electron Microscope Ltd., Oberkochen, Germany) equipped with a Proscan cooled slow-scan charge-coupled device camera (1024  $\times$  1024 pixels) and a Dage-MTI Model SIT 66 low-light-level camera. All digital images were captured with the integrated Soft Imaging Software (SIS) image analysis package (Soft Imaging Software, GmbH, Münster, Germany) and processed with Adobe Photoshop software.

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