An investigation of incorporation of cellular antigens into vaccinia virus particles

Oliver Krauss,† Ruth Hollinshead, Michael Hollinshead and Geoffrey L. Smith

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

Vaccinia virus (VV) infection produces several types of virus particle called intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV). Some cellular antigens are associated with EEV and these vary with the cell type used to grow the virus. To investigate if specific cell antigens are associated with VV particles, and to address the origin of membranes used to envelope IMV and IEV/CEV/EEV, we have studied whether cell antigens and foreign antigens expressed by recombinant VVs are incorporated into VV particles. Membrane proteins that are incorporated into the endoplasmic reticulum (ER), intermediate compartment (IC), cis-medial-Golgi, trans-Golgi network (TGN) or plasma membrane were not detected in purified IMV particles. In contrast, proteins present in the TGN or membrane compartments further downstream in the exocytic pathway co-purify with EEV particles when analysed by immunoblotting. Immunoelectron microscopy found only low levels of these proteins in IEV, CEV/EEV. The incorporation of foreign antigens into VV particles was not affected by loss of individual IEV or EEV-specific proteins or by redirection of B5R to the ER. These data suggest that (i) host cell antigens are excluded from the lipid envelope surrounding the IMV particle and (ii) membranes of the ER, IC and cis-medial-Golgi are not used to wrap IMV particles to form IEV. Lastly, the VV haemagglutinin was absent from one-third of IEV and CEV/EEV particles, whereas other EEV antigens were present in all these virions.

Introduction

Vaccinia virus (VV) is the most intensively studied poxvirus (Moss, 2001). It is a member of the Orthopoxvirus genus and is the vaccine used to eradicate smallpox (Fenner et al., 1988). Each cell infected with VV produces four different virus particles called intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV). These complex virions have different roles in the virus life-cycle and contain more than 100 polypeptides (Essani & Dales, 1979).

VV morphogenesis commences in cytoplasmic virus factories from which cellular organelles are largely excised. The first structures seen within these factories are crescent-shaped and are composed of lipid and virus-encoded protein. The nature and origin of the crescents has been disputed: early studies reported that they contained a single lipid bilayer and were synthesized de novo (Dales & Siminovitch, 1961; Dales & Mosbach, 1968). Subsequently, it was reported that the crescents contained a double lipid bilayer that was derived from and was continuous with membranes of the intermediate compartment (IC) between the endoplasmic reticulum (ER) and the trans-Golgi network (TGN) (Sodeik et al., 1993; Griffiths et al., 2001; Risco et al., 2002). Another study reported that the crescent lacked continuity with cell membranes and contained a single membrane (Hollinshead et al., 1999). Once formed, the crescent grows into an oval structure called immature virus (IV) that lacks infectivity. IVs mature into IMV particles by condensation associated with proteolytic cleavage of several core proteins. IMV is the first infectious form of virus and represents the majority of infectious progeny.

Many IMV particles remain in the cell until lysis; however, some are transported from the virus factory by microtubules (Sanderson et al., 2000) to sites where these particles are wrapped by two intracellular membranes to form intracellular
envelope virus (IEV) (Ichihashi et al., 1971). Using antibodies specific for TGN markers, one group reported that the wrapping membranes were derived from the TGN (Schmelz et al., 1994). Other studies using uptake of fluid phase markers provided evidence that the wrapping membranes were derived from the early endosomes (Töoze et al., 1993; van Eijl et al., 2002).

After their formation, IEV particles are transported to the cell surface on microtubules (Geada et al., 2001; Hollinshead et al., 2001; Rietdorf et al., 2001; Ward & Moss, 2001) and once at the plasma membrane the outer IEV membrane fuses with the plasma membrane to produce a CEV particle on the cell surface. CEV induce the polymerization of actin tails from beneath the virion (van Eijl et al., 2000; Hollinshead et al., 2001) to drive the CEV particle away from the cell either into adjacent cells or into the extracellular environment. When a CEV particle is released from the cell it is called EEV. CEV and EEV particles are morphologically indistinguishable and have one more lipid membrane than IMV and one less than IEV. With VV strain Western Reserve (WR) the majority of one more lipid membrane than IMV and one less than IEV. VV strain IHD-J greater amounts of one less than WR strain was used (van Eijl et al., 2000) but none of the virus-encoded EEV-specific proteins has been shown to interact directly with host proteins. The presence of host proteins can affect the biological properties of virus particles. With VV it was demonstrated that the presence of CD55 rendered EEV particles resistant to complement (Vanderplasschen et al., 1998) and with human immunodeficiency virus host proteins in the virus particle can affect virus neutralization by antibody (Arthur et al., 1992) or enhance virus infectivity (Fortin et al., 1997).

In this report we have investigated the incorporation of host cell proteins into IMV and EEV particles made by wild-type and seven mutant VVs by immunoblotting and immuno-electron microscopy. Using markers from a variety of intracellular membrane compartments we show that no cell proteins were incorporated into IMV particles, even if these proteins were targeted to the membrane compartment reported to form the IMV envelope and even if these proteins were overexpressed from recombinant VVs. However, cellular or foreign viral proteins did co-purify with EEV particles if these were located in the TGN or downstream compartments of the exocytic pathway. The implications of these findings are discussed.

### Methods

#### Cells and viruses.

HeLa, TK-143 and CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) in 10% (v/v) foetal bovine serum (FBS). The WR mutants ΔA3R (Roper et al., 1998), ΔA34R (McIntosh & Smith, 1996), ΔA56R (Sanderson et al., 1998), ΔB5R (Engelstad & Smith, 1993), B5R-K<sub>Δ</sub> and B5R-K<sub>Δ</sub> (Mathew et al., 1999) were described previously. The recombinant VVs expressing mouse hepatitis virus (MHV) M protein and infectious bronchitis virus (IBV) M protein (Klumperman et al., 1994) and influenza virus HA (Smith et al., 1987) were described before.

#### Construction of recombinant VVs.

Recombinant VVs expressing the Bunyamwera virus G1 and G2 proteins were constructed by cloning the genes for each protein into plasmid pSC11 (Chakrabarti et al., 1985). Each gene was amplified by PCR using plasmids pT7-5G2 or pT73G1 as template (Lappin et al., 1994). For amplification of the G1 gene oligonucleotides 5′-GATCCAGAGGGTACATAAA (forward primer) and 5′-GATCCAGAGGGTACATAAA (reverse primer) were used. The G2 gene was amplified using oligonucleotide 5′-GATAGTGAATCCGTCGTACATCAAA (forward primer) and oligonucleotide 5′-GATAGTGAATCCGTCGTACATCAAA (reverse primer) yielded PCR products for G1 and G2 were cut with Smal (underlined in each oligonucleotide) and cloned into...
pSC11 forming plasmids pSC11G1 and pSC11G2. These were sequenced to confirm the fidelity of the PCR products. These plasmids were transfected into VV WR-infected cells from which thymidine kinase-negative recombinant viruses were isolated by plaque assay on TK−143 cells as described previously (Chakrabarti et al., 1985).

| Virus purification. | EEV and IMV were purified from HeLa cells 48 h post-infection (p.i.) with 0.1 p.f.u. per cell. For EEV, the cell supernatant was clarified by centrifugation (10000 g, 20 min, 4 °C) and the virus in the supernatant was pelleted by ultracentrifugation (35000 g, 90 min, 4 °C). Pelleted virus was resuspended in 10 mM Tris–HCl, pH 9, and kept on ice until the next step of the purification. IMV was purified from Dounce-homogenized, infected cell extracts from which nuclei and cell debris were removed by centrifugation (10000 g, 10 min, 4 °C). Further steps of purification were identical for EEV and IMV. Both materials were sonicated and then sedimented (35000 g, 80 min, 4 °C) through a sucrose cushion (36%, w/v, in 10 mM Tris–HCl, pH 9). The IMV and EEV pellets obtained were purified further by sucrose velocity sedimentation as described (Doms et al., 1990). Virus bands were collected, and the virus was recovered by centrifugation.

| Antibodies. | Antibodies used against VV proteins were mouse monoclonal antibody (mAb) AB1.1 against the D8L protein (α-D8L) (Parkinson & Smith, 1994), rat mAb 15B6 against the F13L protein (α-F13L) (Hiller & Weber, 1985), rat mAb 19C2 against BSR (Schmelzle et al., 1994), mouse mAb 1H83 against A50R (Shida, 1986) and rabbit polyclonal antibodies against A33R (α-A33R) and A34R (α-A34R) (Röttger et al., 1999). Mouse mAbs against human proteins J4-48 (α-J4-48), BRIC 110 (α-CD55), MEM-43 (α-CD59), and JS64 (α-CD81) were all obtained from Serotec, P4C10 (α-CD71) was obtained from Immunotech, and BM-63 (α-CD81)-microglobulin) were obtained from Sigma. HTR-H68.4 (α-CD71) was a gift from S. White (White et al., 1992), and mAb HCA2 was against the major histocompatibility complex (MHC) class I antigen (α-MHC-I) (Stam et al., 1990). Murine mAb G1/296 (α-p53) (Schweizer et al., 1993), G1/93 (α-p53) (Schweizer et al., 1990) and G1/133 (α-giantin) (Linstedt & Hauri, 1993) were a gift from H.-P. Hauri. Rabbit polyclonal antibody N11 against human β-1,4-galactosyltransferase (α-GalTrans) (Watzele et al., 1991) was a gift from E. Berger and rabbit antisera to EEA-1 (α-EEA-1) (Mu et al., 1995) was a gift from H. Stenmark. The rabbit antisera against the MHV M protein was a gift from P. Rottier (Klumperman et al., 1994) and the mAb against the IBV M protein was a gift from D. Cavanagh (Mockett et al., 1984). Bunyamwera virus proteins G1 and G2 were detected using a polyclonal rabbit serum raised against the virus (Watret et al., 1985) and the influenza virus HA was detected with a polyclonal antiserum against influenza virus A/Cam/1/40 (Smith et al., 1987).

| Immunoblotting. | Extracts from cells or purified VV particles were prepared as described (Parkinson & Smith, 1994), and the Bunyamwera G2 protein samples were prepared as described (Nakatike & Elliott, 1993). After SDS-PAGE, proteins were transferred to nitrocellulose membranes and identified with specific antibodies and a chemiluminescent detection system (Amer sham) (Parkinson & Smith, 1994). The primary antibodies were used at the following dilution or concentration: mAb AB1.1 (1:2000), mAb J4-48 (0.5 µg/µl), mAb BRIC 110 (5 µg/µl), mAb MEM-43 (0.5 µg/µl), mAb JS64 (5 µg/µl), mAb HTR-H68.4 (10 µg/µl), mAb HCA2 (10 µg/µl), mAb P4C10 (1:1000), mAb 58K-9 (1:2000), mAb BM-63 (1:1000), mAb G1/296 (1:1000), mAb G1/93 (1:1000), mAb G1/133 (1:1000), rabbit polyclonal N11 (1:1000), rabbit antisera to EEA-1 (1:1000), rabbit antisera against the MHV M (1:1000), mAb against the IBV M protein (1:100), polyclonal rabbit antisera against Bunyamwera virus (1:1000) and the polyclonal antisera against influenza virus A/Cam/1/46 (1:1000). Bound Ig was detected by incubation with goat anti-rabbit, anti-rat or anti-mouse horseradish peroxidase conjugate (1:2000) (Sigma). Membranes were re-probed with a second or third antibody after they were stripped by incubation in 100 mM 2-mercaptoethanol, 2% SDS, 0.25 M Tris–HCl pH 6.7 (50 °C, 30 min with gentle agitation) and then washed in PBS containing 0.1% Tween 20.

| Pre-embedding immunogold labelling and preparation of cells for electron microscopy. | Cells were fixed in 250 mM HEPES buffer pH 7.4 containing 4% paraformaldehyde (w/v) for 10 min on ice and 50 min at 20 °C. After washing with PBS and then PBS containing 10% heat-inactivated PBS (PBSF), cells were incubated successively with primary Ab (at 100–500-fold higher concentration than that used for immunoblotting), rabbit anti-mouse IgG (20 µg/ml in PBSF) or anti-rabbit Ig (Cappel) and finally with protein A–gold particles as described (Slot & Geuze, 1985). All incubations were for 1 h at room temperature and were separated by extensive washing with PBS. Finally, the samples were fixed for 30 min in 200 mM cacodylate containing 0.5% glutaraldehyde and prepared for electron microscopy as described (Vanderplasschen et al., 1997).

| Cryo-electron microscopy. | EEV preparations and virus-infected cells were processed for cryo-electron microscopy as described previously (van Eijl et al., 2002).

Results

The presence of cellular proteins in EEV preparations is not dependent on specific EEV proteins

In a previous study it was demonstrated that several host proteins were associated with EEV samples that had been purified by density gradient centrifugation and the presence of some of these (the complement control proteins CD46, CD55 and CD59) on the EEV envelope was confirmed by immunoelectron microscopy (Vanderplasschen et al., 1998). The presence of CD55 rendered EEV resistant to destruction by complement, whereas IMV preparations lacked these host complement proteins and were sensitive to complement.

To extend these observations we have investigated whether other host proteins are present in EEV or IMV and whether the incorporation of these proteins is influenced by specific EEV or IEV proteins. EEV and IMV were purified after infection of HeLa cells with either VV strain WR or derivative mutants lacking the A33R, A34R, A36R, A50R or B5R proteins and were analysed by immunoblotting (Fig. 1). Two control mAbs were used in this and all subsequent immunoblots. First, to show that equal amounts of IMV and EEV had been loaded, the amount of the VV D8L protein was analysed with α-D8L and found to be equivalent. Second, the presence of the F13L protein was analysed with α-F13L. This protein is specific for EEV and its absence from IMV confirmed the purity of all IMV preparations. Next, the incorporation of CD46, CD55 and CD59, MHC-I, CD71 and CD81 was investigated. All were detected with uninfected cell lysates, wild-type EEV and EEV prepared from each mutant, but were absent from all IMV preparations. Evidently, the A33R, A34R, A36R, A50R and B5R proteins are not necessary for the presence of these cell antigens in EEV preparations.
**Fig. 1.** Immunoblot analysis of VV IMV and EEV preparations. IMV and EEV were purified from HeLa cells infected with VV strain WR or with the indicated mutant VVs. Virus protein (3 µg) and extracts from 2 × 10⁴ uninfected HeLa cells were resolved by SDS–PAGE and probed with the indicated Abs as described in Methods.

**Fig. 2.** Immunoblot analysis of VV IMV and EEV preparations. Samples were prepared and processed as in Fig. 1 using the indicated Abs.
The incorporation of other cell proteins that are markers for specific cellular membrane compartments was investigated next. Markers selected were from the ER (p63), IC (p53), the Golgi complex (giantin and 58 kDa protein), TGN (GalTrans), early endosomes (EEA-1) and two cell surface markers (CD29 and β2-microglobulin). The levels of p53, giantin, p58 and EEA-1 were below detectable levels under the conditions used, whereas the p63, GalTrans, CD29 and β2-microglobulin were detected in cell extracts. Of these latter antigens, all except p63 were detected in EEV but not in IMV (Fig. 2). Again, the use of α-D8L and α-F13L mAbs served to confirm equal loading of virus samples and the purity of the IMV preparation.

Recombinant VVs that express marker proteins for cell compartments

To overcome the difficulty of low amounts of cellular proteins that are specific for some intracellular membrane compartments, we used recombinant VVs that express specific marker proteins. VVs expressing the IBV M protein and the MHV M protein have been described and these coronavirus M proteins were reported to locate to the IC and TGN, respectively (Klumperman et al., 1994). The glycoproteins G1 and G2 from Bunyamwera virus were also utilized. When expressed together these proteins co-localize to the Golgi, whereas if expressed separately they are located in the Golgi (G2) or the ER (G1) (Lappin et al., 1994). Recombinant VVs expressing these proteins were constructed as described in Methods and shown to express the Bunyamwera proteins of the predicted sizes that were detected with specific antiserum (Fig. 3). Lastly, a recombinant VV expressing the influenza virus HA (Smith et al., 1987) was used as a cell surface marker. All viruses were engineered to express the foreign gene from the same promoter (p7.5K) to ensure equivalent temporal and quantitative levels of gene expression.

Extracts from cells infected with each virus were shown by immunoblotting to express the relevant virus protein (Fig. 3). The control antibodies (α-D8L and α-F13L) showed again that the levels of IMV and EEV loaded were equivalent and that the IMV preparations were not contaminated with EEV or cell antigens. In each case, the foreign antigen was not associated with IMV preparations. The IC or ER-IC were proposed as the source of virus lipid used for the formation of the IMV envelope (Sodeik et al., 1993; Risco et al., 2002). If this is true, data presented here indicate that non-VV proteins are excluded from these membranes during VV morphogenesis. In contrast to IMV, EEV preparations contained several cell antigens: the MHV M protein and the influenza virus HA, which are located in the TGN and plasma membrane, respectively, were associated with EEV, whereas proteins located in the IC and cis/medial-Golgi were not present in EEV samples. These data are consistent with those shown in Fig. 2.

Incorporation of cellular proteins using a VV mutant K2X2-B5R in which the B5R protein is redirected to the ER

The VV B5R protein is a 42 kDa glycoprotein with a type I membrane topology that is present in the I EV, CEV and EEV envelope. It contains four domains related to the short consensus repeat (SCR) typical of complement control proteins and is required for the wrapping of IMV to form IEV. The
transmembrane domain and cytoplasmic tail of B5R are sufficient to direct the B5R protein fused to the HIV gp120 protein (Katz et al., 1997) or the enhanced green fluorescent protein (Ward & Moss, 2000; Hollinshead et al., 2001; Rodger & Smith, 2002) to the membranes used to wrap IMV particles to form IEV. Although the cytoplasmic tail is non-essential (Lorenzo et al., 1999), it affects the rate of B5R transport to the cell surface (Mathew et al., 2001). The B5R protein was re-directed to the ER by addition of the ER retrieval signal K\_X\_K to the C terminus (Mathew et al., 1999), but despite the relocation of B5R, IEV particles were formed that contained the usual virus proteins.

The K\_X\_K-B5R virus was used to assess whether there were differences in the incorporation of cellular proteins when the B5R protein was re-directed to the ER. IMV and EEV were purified and analysed as before (Fig. 4). Despite the relocation of the B5R protein to the ER, the incorporation of cellular proteins into EEV was indistinguishable from wild-type virus, and none of the proteins tested were present in IMV.

**Immunoelectron microscopy**

The presence of proteins in EEV preparations that had been purified by sucrose density gradient centrifugation does not prove that the protein is part of the EEV envelope. This was illustrated with the VV A36R and F12L proteins. Originally, A36R was found associated with EEV (Parkinson & Smith, 1994), but subsequent immunoelectron microscopy showed this protein was on IEV but not CEV and EEV (van Eijl et al., 2000). Similarly, the F12L protein is present in EEV preparations but immunoelectron microscopy showed that it is on IEV but not CEV/EEV particles (Zhang et al., 2000; van Eijl et al., 2002). Therefore, the presence of virus and cellular proteins in EEV and CEV was investigated by immunoelectron microscopy.

**The VV HA is absent from one-third of IEV and EEV particles**

Two different VVs that yielded greater amounts of EEV than strain WR were used. These were the IHD-J strain and a mutant of VV strain WR called vSCR1-3, which has SCR domain 4 of B5R deleted (Mathew et al., 1998). Cryo-sections of these preparations were stained with antibodies against A33R, A34R, A36R, A56R, B5R and F13L. Staining of each virus preparation gave indistinguishable data. Fig. 5 shows that the proteins encoded by VV genes A33R, A34R, A56R, F13L and B5R are present in the EEV envelope, whereas A36R is not. For the A56R protein, it was noted that whereas some EEV particles stained well with the \( \alpha \)-A56R mAb and showed many gold particles per virion, others did not (Fig. 5d). Therefore, this was investigated quantitatively. By cryo-immunoelectron microscopy 33-2% of EEV particles (n = 196) were negative for gold particles. Similarly, analysis of pre-embedded sections of VV strain WR-infected HeLa cells showed that 34-6% (n = 107) of CEV particles were negative for HA. In contrast, F13L and B5R proteins were present on all EEV particles. This result raises the question of whether the A56R protein is a true EEV protein, and in this regard it is notable that this protein is present predominantly on the surface of cells infected with VV or Semliki Forest virus expressing A56R (Lorenzo et al., 2000), rather than in the intracellular wrapping membranes.

**Incorporation of CD29, CD46, CD59 and CD71**

The incorporation of cell proteins into EEV particles was investigated in a similar way. IHD-J and vSCR1-3 EEV cryo-preparations were stained with \( \alpha \)-CD29, \( \alpha \)-CD71, \( \alpha \)-CD46 and \( \alpha \)-CD59 (Fig. 6). For CD46 and CD59 staining of some EEV particles was detected and this often had a capped appearance (Fig. 6c, e) as reported previously (Vanderplasschen et al., 1998). However, for CD29 and CD71 fewer gold particles were seen on EEV and many virions were negative (Fig. 6a, g). This was investigated further by cutting thin sections of pre-embedded, labelled HeLa cells that had been infected with VV.
Fig. 5. Immunoelectron microscopy. EEV was purified from the supernatant of VV strain IHD-J- or vSCR1-3-infected cells and processed for cryo-immunoelectron microscopy as described in Methods. EEV samples were stained with antibodies for the A33R, A34R, A36R, A56R, B5R and F13L proteins followed by appropriate secondary antibodies and protein A conjugated to 9 nm gold particles. Scale bars, 200 nm.
Fig. 6. For legend see facing page.
strain WR for 12 h (Fig. 6b, d, f, h). A virion not stained by gold particles indicates only that the technique did not detect the antigen in that thin section, rather than throughout the virion. This analysis gave similar data with only low levels of staining for α-CD29 and α-CD71; however, in this case the level of staining of EEV could be compared with the plasma membrane. This comparison showed that for CD29 the number of gold particles per µm of membrane was 2.8 for EEV membrane and 9.4 for plasma membrane. Table 1 summarizes an extensive electron microscopy study for CD29, CD46, CD59 and CD71 and the proportion of CEV particles associated with gold particles compared to the proportion following incubation with immunogold conjugate alone without primary antibody. A statistical analysis indicated that the staining of CD29, CD46 and CD59 was significantly above control (P < 0.05, Student’s t-test) for CEV, and staining for CD46 and CD71 was significantly greater than control for IEV. Overall, the low level of staining indicates that these host proteins were present at only low levels in IEV and CEV/EEV particles.

Discussion

The incorporation of foreign proteins into the IMV and EEV forms of VV has been investigated. Antigens analysed were from specific membrane compartments. These were either cellular or were of viral origin and were expressed by recombinant VVs. No foreign proteins were found incorporated into IMV particles, even though some of these proteins were from membrane compartments allegedly utilized during formation of the IMV envelope, and even if the proteins were overexpressed from VV. This indicates that during the morphogenesis of IMV, there is likely to be a mechanism to exclude foreign proteins from membrane compartments used to form the IMV envelope.

For EEV, the situation was more complex. Immunoblotting showed that proteins were associated with EEV if they derived from membrane compartments that were from the TGN or further downstream in the exocytic pathway. In contrast, proteins were not associated with EEV if they were derived from ER, IC or cis/medial-Golgi. Although proteins associated with EEV were detected easily by immunoblotting, immunoelectron microscopy found only low levels of proteins associated specifically with the EEV membrane, and in some cases the density of these proteins per unit length of membrane was lower than that present on the plasma membrane. Possible interpretations are (i) that the cellular proteins are predominantly associated with membrane fragments that co-purify with the EEV particles on sucrose density gradients, (ii) that the levels in IEV are low because by the time the majority of virus particles is being released from the infected cell, the synthesis of host protein has been inhibited for several hours and the levels of these proteins have been reduced, (iii) the combination of low levels of protein and the antibodies (Abs) used was insufficient to detect these proteins efficiently by immunogold electron microscopy, or (iv) cell antigens are excluded from EEV because of the density of virus proteins in these membranes.

It is hard to distinguish between these possibilities, but it is known that the presence of proteins not considered strict EEV antigens varies with the strain of virus used. For example, using

Table 1. Labelling of IEV and CEV particles with specific antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Labelled CEV (%)</th>
<th>Labelled IEV (%)</th>
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<tbody>
<tr>
<td>α-CD29</td>
<td>20 (n = 65) $P = 0.016$</td>
<td>15·9 (n = 63) $P = 0.421$</td>
</tr>
<tr>
<td>α-CD46</td>
<td>20·6 (n = 68) $P = 0.005$</td>
<td>19·4 (n = 72) $P = 0.016$</td>
</tr>
<tr>
<td>α-CD59</td>
<td>20·7 (n = 58) $P = 0.015$</td>
<td>17·5 (n = 57) $P = 0.398$</td>
</tr>
<tr>
<td>α-CD71</td>
<td>15·3 (n = 59) $P = 0.069$</td>
<td>24·1 (n = 79) $P = 0.048$</td>
</tr>
<tr>
<td>Rabbit α-mouse</td>
<td>α-gold conjugate</td>
<td>α-gold conjugate</td>
</tr>
<tr>
<td>protein A–gold conjugate</td>
<td>61 (n = 66)</td>
<td>13·1 (n = 61)</td>
</tr>
</tbody>
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Fig. 6. Immunoelectron microscopy. (a, c, e and g) Cryo-immunoelectron microscopy. EEV was purified from the supernatant of VV strain IHD-J- or vSCR1-3-infected cells and processed for cryo-immunoelectron microscopy as described in Methods. EEV samples were stained with antibodies for CD29, CD46, CD59 and CD71 followed by appropriate secondary antibodies and protein A conjugated to 9 nm gold particles. (b, d, f and h) Pre-embedding labelling. HeLa cells were infected with VV strain WR at 10 pfu per cell and were stained with the antibodies followed by secondary antibodies and protein A conjugated to 9 nm gold particles as in (a, c, e, g). Subsequently, the samples were processed for electron microscopy as described in Methods. Scale bars: (a, c, e, g), 200 nm; (b, d, f, h), 100 nm.
the IHD-J strain of VV, little of the IEV-specific A36R protein or cell actin was found associated with the EEV preparations, whereas with WR strain WR greater amounts of these proteins were detected in EEV (van Eijl et al., 2000). This observation might be explained by the differing amounts of EEV and CEV made by these VV strains. With the WR strain, more CEV remains bound to the cell surface and as these CEV particles are driven away from the cell by polymerization of actin tails, the CEV particle might eventually be torn away from the tip of an extended microvillus while still associated with fragments of cell membrane. In contrast, the IHD-J strain releases more EEV due to a mutation in the C-type lectin domain of A34R (Blasco et al., 1993). It is possible that the enhanced release of EEV by IHD-J is associated with lower levels of contaminating fragments of cellular membranes. In accord with this idea, electron microscopy of EEV preparations from WR and IHD-J showed greater levels of membrane contamination with WR (data not shown).

As well as being influenced by the strain of virus, the pattern of cell proteins that are associated with an EEV preparation is influenced by the cell type (Payne, 1978). This observation might also be explained by the ease of release of EEV because cell lines differ in the amount of released EEV (Payne, 1978).

The failure to detect the proteins from the ER, IC and cis/medial-Golgi with EEV preparations indicates that either these membranes are not used to form either IMV or wrap IMV to form IEV, or that there are mechanisms to exclude non-viral proteins from the lipid envelope during morphogenesis.

The possibility that VV proteins present in the IEV or EEV envelope affected the incorporation of cellular proteins into VV particles was addressed using a collection of VV mutants lacking individual proteins. Specific interactions between different IEV proteins have been noted (Röttger et al., 1999), but data addressing interactions of VV and cellular proteins were not available. It was found that loss of the A33R, A34R, A35R, A56R and B5R proteins did not affect the association of cellular proteins with EEV. Therefore, if these VV proteins have interactions with host proteins, the recruitment of cellular proteins is not dependent upon any single virus protein. Finally, a virus mutant with the B5R protein redirected to the ER by the attachment of the ER retrieval sequence to its C terminus was studied. In this case too the incorporation of cellular proteins with EEV was not altered. Notably, IMV particles were still wrapped by membranes to form IEV and the membrane used seemed not to be altered because protein markers of the ER were not included.

The functional significance of cell antigens in EEV remains to be determined. The only property investigated so far is the resistance to complement (Vanderplaschen et al., 1998), whereas with other viruses, such as HIV, cellular proteins have been shown to affect virus neutralization by Ab and the virus infectivity (Arthur et al., 1992; Fortin et al., 1997).

The incorporation of both GalTrans and CD71 (transferrin receptor) into EEV might seem surprising because these proteins label either the TGN or early endosomes, respectively. However, this observation is consistent with reports that show, on the one hand, that TGN membranes are used to wrap IMV particles (Schmelz et al., 1994) and, on the other hand, that the wrapping membranes contain fluid phase markers (Tooze et al., 1993; van Eijl et al., 2002). It is well established that a pathway from either the early or late endosomes to the TGN exists (Duncan & Kornfeld, 1988; Goda & Pfeffer, 1988; Néeffes et al., 1988) and it was shown that this pathway is upregulated in VV-infected cells (Tooze et al., 1993; Schmelz et al., 1994). This would explain the incorporation of both cell surface markers into the EEV membrane. It is possible that either the TGN or early endosomes may be used as the wrapping membrane for IEV.

Finally, the presence of VV proteins in EEV was investigated. We confirm that the A36R protein is not a component of EEV; previously, this was indicated by confocal microscopy of EEV and immunoelectron microscopy of CEV (van Eijl et al., 2000, 2002). In addition, it is shown that the A56R protein was absent in one-third of EEV particles, raising the possibility that A56R is not a true EEV protein. A possible explanation for the presence of A56R in some but not all EEV particles is that the protein does not accumulate in the intracellular wrapping membranes (Schmelz et al., 1994; Lorenzo et al., 2000) and so its incorporation into IEV and thereby CEV/EEV is dependent upon sufficient A56R protein trafficking through these intracellular membranes at the time wrapping takes place.

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