Investigation of orf virus structure and morphogenesis using recombinants expressing FLAG-tagged envelope structural proteins: evidence for wrapped virus particles and egress from infected cells

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Orf virus (ORFV) is the type species of the genus Parapoxvirus, but little is known about the structure or morphogenesis of the virus. In contrast, the structure and morphogenesis of vaccinia virus (VACV) has been extensively studied. VACV has two main infectious forms, mature virion (MV) and extracellular virion (EV). The MV is wrapped by two additional membranes derived from the trans-Golgi to produce a wrapped virion (WV), the outermost of which is lost by cellular membrane fusion during viral egress to form the EV. Genome sequencing of ORFV has revealed that it has homologues of almost all of the VACV structural genes. Notable exceptions are A36R, K2L, A56R and B5R, which are associated with WV and EV envelopes. This study investigated the morphogenesis and structure of ORFV by fusing FLAG peptide to the structural proteins 10 kDa, F1L and ORF-110 to form recombinant viruses. 10 kDa and F1L are homologues of VACV A27L and H3L MV membrane proteins, whilst ORF-110 is homologous to VACV A34R, an EV membrane protein. Immunogold labelling of FLAG proteins on virus particles isolated from lysed cells showed that FLAG–F1L and FLAG–10 kDa were displayed on the surface of infectious particles, whereas ORF-110–FLAG could not be detected. Western blot analysis of solubilized recombinant ORF-110–FLAG particles revealed that ORF-110–FLAG was abundant and undergoes post-translational modification indicative of endoplasmic reticulum trafficking. Fluorescent microscopy confirmed the prediction that ORF-110–FLAG localized to the Golgi in virus-infected cells. Finally, immunogold labelling of EVs showed that ORF-110–FLAG became exposed on the surface of EV-like particles as a result of egress from the cell.

INTRODUCTION

Fifty years have passed since electron microscopy studies first described the orf virus (ORFV; genus Parapoxvirus) as having an ovoid structure with a spiral tubule protein wrapped around the outer surface. However, little is known today about its morphogenesis or the proteins that make up the virion. Early studies revealed that negatively stained preparations of ORFV appeared in two forms. In the capsular form where the stain penetrated the virion, a finely crenelate membrane appeared to surround an inner amorphous core, whereas virions that were impervious to the stain revealed a regular array of tubule-like structures arranged in a criss-cross manner (Nagington et al., 1962; Mitchiner, 1969). Where the virus has been propagated in cell culture, virions that appear in the medium are surrounded by a membranous structure. The complete sequence of the ORFV genome revealed that it has homologues of most vaccinia virus (VACV) structural proteins (Delhon et al., 2004; Mercer et al., 2006), which suggests that their morphogenesis and structure may be similar. VACV predominantly has one infectious form, the mature virion (MV), in which the outer membrane is derived from the endoplasmic reticulum (reviewed by Condit et al., 2006). In addition, wrapped virions (WVs) are also produced in which two additional membranes derived from the trans-Golgi network wrap the MV (Hiller & Weber, 1985; Schmelz et al., 1994). The outermost membrane of this form is lost during virus egress to produce the extracellular virion (EV) (Smith & Law, 2004). ORFV particles resembling MV and WV particles of VACV have been described in ultrathin sections of infected cells (Spehner et al., 2004). In VACV, specific structural proteins are associated with the envelope membranes of each form. ORFV has homologues of all of the VACV-encoded MV envelope-associated proteins except D8L, but only some of the VACV proteins
associated with WV and EV, which include A33R, A34R, F12L and F13L. ORFV does not have homologues of VACV A36R, A56R, B5R or K2, which are associated with either WV or EV.

The structural proteins of ORFV that have been most studied, the 10 kDa protein (ORF-104) (Naase et al., 1991; Spehner et al., 2004) and F1L (ORF-059) (Housawi et al., 1998; Scaglierini et al., 2002, 2004), are homologues of VACV A27L and H3L, respectively. Both A27L and H3L are expressed late during virus replication and are associated with the membrane envelope of the MV form. The ORFV 10 kDa protein is essential for the formation of an intact surface tubule that wraps the virus particle (Spehner et al., 2004), but it is not known whether it is incorporated into the surface tubule. ORFV F1L contains heparin-binding domains and has been implicated in adsorption of the virus to cells during infection (Scaglierini et al., 2002). In addition, F1L is a major immunodominant antigen (Czerny et al., 1997; Housawi et al., 1998). Little is known about the four other ORFV homologues of the VACV envelope-associated proteins A33R, A34R, F12L and F13L.

To investigate whether ORFV produces extracellular EV-like particles, we constructed a recombinant ORFV expressing a FLAG-tagged version of ORF-110, a homologue of VACV A34R, which is associated with EV particles. In addition, recombinants were made that expressed FLAG-tagged versions of 10 kDa and F1L, homologues of the VACV genes A27L and H3L, which are associated exclusively with the envelopes of MV particles. FLAG-F1L and FLAG–10 kDa were detected on the surface of virus isolated from lysed cells by immuno-gold labelling, whereas ORF-110–FLAG could only be detected at the surface tubule. ORFV F1L contains heparin-binding domains and has been implicated in adsorption of the virus to cells during infection (Scaglierini et al., 2002). In addition, F1L is a major immunodominant antigen (Czerny et al., 1997; Housawi et al., 1998). Little is known about the four other ORFV homologues of the VACV envelope-associated proteins A33R, A34R, F12L and F13L.

A utility plasmid, pV47 SPL, derived from pSP70 (Promega), was used to make constructs harbouring the FLAG-tagged modified genes 10 kDa, F1L and ORF-110 of ORFV. This construct included left and right arms containing sequences flanking the vIL-10 gene, a synthetic late promoter (5'-TTTTTTTTTTTTTTTTTGGCATATAAA-3') (Hammond et al., 1997) and a reporter gene, β-glucuronidase (GUS), derived from Escherichia coli (Jefferson, 1989), under the control of the VACV H5 promoter (PH5) (Rosel et al., 1986). The right arm consisted of a 1473 bp fragment starting 34 bp downstream from the vIL-10 gene stop codon, whilst the left arm (1608 bp) comprised 1529 bp upstream of the vIL-10 gene plus the first 79 bp of vIL-10.

The genes for 10 kDa, F1L and ORF-110 were PCR amplified from ORFV strain NZ2 DNA (pVU85 and pVU87; Mercer et al., 1987) and cloned into restriction enzyme sites located between the synthetic late promoter and PH5/GUS of pV47 SPL. FLAG-tagged 10 kDa was amplified from pVU85 using primers 5'-CGCTCTAGAGCCACCCCAGTGGACTACAAGGACGACGATGACAAGATGGATGAAAATGAC-3' and 5'-CGCCGATCCCTAATATTCTCGTGTATCCTG-3' and cloned into the XbaI and BamHI sites of pV47 SPL (pV47 SPL--10 kDa). FLAG-tagged F1L was amplified from pVU87 with primers 5'-CCGCTCTAGAGCCACCCAGTGGACTACAAGGACGACGATGACAAGATGGATGAAAATGAC-3' and 5'-CGCTTCTAGAGCCACCCAGTGGACTACAAGGACGACGATGACAAGATGGATGAAAATGAC-3' and cloned into XbaI and NotI sites of pV47 SPL (pV47 SPL--F1L). The FLAG-tagged ORF-110 gene was amplified from pVU85 with the primers 5'-CGCTTCTAGAGCCACCCAGTGGACTACAAGGACGACGATGACAAGATGGATGAAAATGAC-3' and 5'-CGCTTCTAGAGCCACCCAGTGGACTACAAGGACGACGATGACAAGATGGATGAAAATGAC-3' and cloned into XbaI and NotI sites of pV47 SPL (pV47 SPL--ORF-110).

Recombinant ORFVs were generated in LT cells using procedures described previously (Savory et al., 2000). Recombinants were identified by their blue-plaque phenotype in the presence of 5-bromo-4-chloro-3-indolyl β-d-glucuronide (Glycosynth). Plaques were subjected to at least five rounds of plaque purification prior to preparation of virus stocks.

For preparation of purified virus stocks, virus was layered over a 40 % (w/v) sucrose cushion and ultracentrifuged at 46 000 g for 1 h at 4 °C. The pellet was resuspended in PBS.

**Preparation of viral DNA.** The techniques for viral DNA extraction, restriction endonuclease digestion, gel electrophoresis, cloning and transfer of DNA to nylon membranes were as described by Mercer et al. (1987).

**Digoxigenin (DIG)-labelled DNA probes.** The detection of the modified structural genes was performed using full-length 10 kDa, F1L and ORF-110 probes labelled with DIG–dUTP using a PCR DIG-Labeling Kit (Amersham). DIG-labelled DNA probes were added to a final concentration of 4 ng/μL to the reaction mixture.

**Solubilization of virus particles and Western blotting.** Purified virus particles were solubilized by a modification of the method described by Balassu & Robinson (1987). Briefly, 445 μL purified virus stock was solubilized with NP-40 and 2-mercaptoethanol (2-ME), added to a final concentration of 1 % each. The mixtures were sonicated and made up to 4 mL with ET buffer [0.025 M Tris/HCl (pH 8.0), 10 mM EDTA]. The solubilized membrane proteins were separated from the virus cores by ultracentrifugation over a 40 % (w/v) sucrose cushion at 30 000 g for 30 min at 4 °C. The insoluble pellet was dissolved in 50 μL 5 % (w/v) SDS and made up to 500 μL with ET buffer. Proteins were precipitated from the supernatants with 10 % trichloroacetic acid and pelleted by centrifugation. Precipitates were resuspended in 500 μL ET buffer. Proteins were resolved by SDS-PAGE (Laemmli, 1970).
Separated proteins were transferred onto nitrocellulose membranes and identified using Abs at the following dilutions: anti-FLAG mAb–HRP at 1 : 1000 or 8D7 mAb at 1 : 2000 for 1.5 h, and rabbit anti-mouse Ig–HRP at 1 : 5000. Incubations were performed at room temperature for 1 h unless otherwise stated. The immune complexes were detected using a SuperSignal Chemiluminescent Detection kit (Pierce Biotechnology).

**Preparation of EV particles.** EV particles were prepared using the following protocol, modified from Krauss et al. (2002). LT cells were infected with recombinant viruses at an m.o.i. of 0.1 and the supernatant was harvested at 48 h post-infection (p.i.). The cellular material was removed and the supernatant ultracentrifuged at 35 000 g for 1.5 h at 4 °C. The pellet was resuspended in 10 mM Tris/HCl (pH 9.0) and ultracentrifuged on a 36 % (w/v) sucrose cushion at 34 000 g for 80 min. Pelleted virus was resuspended in PBS.

**Immunogold labelling.** Purified viruses were adsorbed onto Formvar carbon-coated nickel grids treated with 0.01 % (w/v) poly-L-lysine and labelled using an immunogold labeller (Leica). Abs were used at the following dilutions: mouse anti-FLAG at 1 : 1000 and 8D7 at 1 : 10000 for 2 h at room temperature, and ultrasmall gold-conjugated goat anti-mouse secondary Ab at 1 : 100 for 1.5 h at room temperature. Gold particles were silver-enhanced (www.auron.nl/) and the viruses were negatively stained with 2 % (w/v) phosphotungstic acid before viewing in a Philips CM 100 transmission electron microscope.

**Immunofluorescence microscopy.** Immunofluorescence microscropy was used to determine whether ORF-110 localized to the Golgi during viral pathogenesis. The procedure was adapted from methods described by Lorenzo et al. (2000). LT cells grown on coated coverslips were infected with recombinant ORF-110 virus. The cells were permeabilized with methanol at various times p.i. FLAG-tagged protein was reacted with mouse anti-FLAG mAb (1 : 2000 dilution; Sigma) and detected with a secondary fluorescent Ab, Alexa Fluor 488-conjugated anti-mouse IgG (1 : 400 dilution; Invitrogen). The Golgi was stained with Alexa Fluor 594 conjugated to wheatgerm agglutinin (WGA; 1 μg ml⁻¹; Invitrogen). Nuclei were stained with 4'6-diamidino-2-phenylindole, dilactate (DAPI; 1 : 50 000 dilution; Invitrogen).

**Bioinformatic analyses.** Sequence alignments and comparisons were performed using CLUSTAL W (in the MEGALIGN program of DNASTAR Inc.). Hydrophilicity plots were generated using the Protein program from the same package. The PredictProtein program (http://www.predictprotein.org/) was used for the prediction of secondary structures. Transmembrane domains were predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM). Golgi type II membrane predictions were obtained from the Golgi Predictor IMB UQ website (http://ccb.imb.uq.edu.au/golgi/). N- and O-linked glycosylation sites were predicted using http://www.cbs.dtu.dk/services/.

**RESULTS**

**Prediction of secondary structure and topology of ORFV structural proteins**

In order to identify ORFV proteins to which foreign peptides could be fused and thereby incorporated and detected on the surface of infectious virus particles, we selected three ORFV structural proteins for examination. 10 kDa and F1L are major proteins of the virion (Czerny et al., 1997; Housawi et al., 1998; Scagiarini, et al., 2002; Spehner et al., 2004) and are homologous with the MV envelope-associated proteins of VACV A27L and H3L, respectively. ORF-110 is a homologue of VACV A34R and is one of few ORFV proteins predicted to be associated with the outer envelope of EV particles.

The VACV A27L gene encodes a 14 kDa fusion protein that forms a triple coiled-coil structure and interacts with VACV 21 kDa (A17L), a virus membrane protein, through a C-terminal α-helix (Vazquez et al., 1998). We compared the ORFV 10 kDa protein with the VACV A27L protein using CLUSTAL W alignment and secondary structure prediction analysis (Fig. 1a). The 10 kDa protein was 21 aa shorter than A27L with the apparent deleted sequences being located within the N terminus. The 10 kDa protein was 26.4 % identical to VACV A27L, with the majority of the sequence similarities seen within the C-terminal end of the molecules.

Our analyses revealed two predicted α-helical domains in both VACV A27L and ORFV 10 kDa, located in the middle and at the C termini of the proteins. As with VACV A27L, we found no evidence of a transmembrane domain in ORFV 10 kDa. The indirect association of 10 kDa with the viral membrane could occur via interaction with ORF-094, a homologue of VACV A17L, at the leucine zipper motif spanning residues 62–76. This interaction has been described previously for VACV (Vazquez et al., 1998).

The VACV H3L protein is a member of the C-terminal anchor family and inserts into the membrane of virus particles during their maturation (da Fonseca et al., 2000). We compared the sequence of VACV H3L with ORFV F1L. A CLUSTAL W alignment showed that F1L was 27.5 % identical to H3L overall (Fig. 1b) and that the proteins were most similar within the C-terminal regions (Housawi et al., 1998). F1L is 10 aa longer than H3L, with the additional amino acids located within the near N terminus. H3L possesses a structure-less N-terminal region that precedes two GAG-binding domains spanning residues 103–110 and 170–175 (Lin et al., 2000). A hydrophilicity plot of F1L showed that it contains short N-terminal and long C-terminal hydrophobic domains similar to H3L (Housawi et al., 1998; da Fonseca et al., 2000). These observations suggested that F1L, like H3L, has type I topology and that the C-terminal region anchors the protein in the membrane, with the N terminus exposed on the outside of the virus particle.

The VACV A34R gene encodes a C-type lectin-like glycoprotein of 22–24 kDa with type II membrane topology (McIntosh & Smith, 1996; Wolfe et al., 1997). ORFV ORF-110 was composed of 165 aa, three amino acids fewer than A34R, and was 21.4 % identical (Fig. 1c). Sequence analysis predicted a transmembrane helix spanning aa 20–39. The hydrophilicity profile was similar to that of A34R, which has a transmembrane domain that spans aa 15–37. A34R is reported to localize to the Golgi...
Fig. 1. Alignment of the polypeptide sequences of the ORFV structural proteins 10 kDa (a), F1L (b) and ORF-110 (c) with their VACV homologues. Alignments were created using the default settings of CLUSTAL W. Shaded areas indicate amino acids identical to the ORFV protein and boxed regions show predicted GAG-binding domains (Hsiao et al., 1998; Lin et al., 2000). Transmembrane (TM) domains are indicated by a bold line above the ORFV sequence or below the VACV sequence. The TM domains for F1L and VACV H3L have been reported previously (Lin et al., 2000; Scaglioni et al., 2002). Secondary structure predictions are denoted by ‘$\alpha$’ (α-helices) or ‘$\beta$’ (β-sheets). Hydrophilicity plots of the ORFV proteins are displayed under each sequence alignment.
during morphogenesis (Lorenzo et al., 2000). Analysis of ORF-110 using the Golgi predictor program also demonstrated that ORF-110 is likely to be directed to the Golgi, with an index value of 21.58. Proteins with index values above the threshold of 20.005 are predicted to localize to the Golgi.

Based on the above analyses, we predicted that we could fuse a FLAG peptide to the N termini of 10 kDa and F1L, and to the C terminus of ORF-110 for surface display.

**Construction of ORFV recombinants expressing FLAG fusion proteins**

Recombinant ORFVs were made in which genes encoding FLAG–F1L, FLAG–10 kDa and ORF-110–FLAG were inserted by homologous recombination within the vIL-10 locus of the ORFV strain NZ2 genome (Fleming et al., 1997). Schematic representations of each construct are shown in Fig. 2(a). The construction strategy we chose should result in recombinant viruses expressing two versions of the selected envelope protein: one unmodified and one with the N- or C-terminal FLAG extension.

Digestion of FLAG–10 kDa recombinant virus genomic DNA with KpnI revealed loss of the 10.3 kb KpnI-E fragment accompanied by the appearance of two new fragments of 1.65 and 10.6 kb as predicted (Fig. 2b). The latter band, along with a 14.4 kb band harbouring the native 10 kDa gene, hybridized with the 10 kDa probe (Fig. 2c).

The FLAG–F1L recombinant virus was predicted to contain one new KpnI site, resulting in loss of the 10.3 kbp fragment and the appearance of fragments of 11.3 and 1.65 kb (Fig. 2b). KpnI digestion of FLAG–F1L recombinant virus genomic DNA revealed the predicted two new fragments, the larger of which hybridized with the F1L probe, as did an 8.6 kb fragment harbouring the native F1L gene (Fig. 2c).

KpnI digestion of ORF-110–FLAG recombinant virus genomic DNA revealed two new fragments of 10.8 and
1.65 kb as predicted (Fig. 2b). The larger of these hybridized with the ORF-110 probe, as did a 14.4 kb fragment harbouring the native ORF-110 gene (Fig. 2c).

PCR analyses confirmed the integrity of the ORFV recombinants and their purity (Fig. 2a, d). PCR with the primer pair 149 and 92B_r generated a product of 840 bp spanning the vIL-10 gene of the parental virus that was not observed in the recombinant viruses (results not shown). The presence of the 10 kDa gene within the vIL-10 locus was confirmed with the primer pairs 149 and 10-kDa_4r together with 10-kDa_1f and 78B_r. The presence of the FLAG–F1L gene within the vIL-10 locus was confirmed with the primer pairs 149 and F1L_3r and also F1L_1f and 78B_r. The incorporation of ORF-110–FLAG within the same locus was determined using the primer pairs 149 and ORF-110_3r as well as ORF-110_1f and 78B_r.

**Incorporation of the FLAG fusion proteins into virus particles**

ORFV particles can be partially solubilized with mild detergents such as NP-40 and 2-ME (Easterbrook, 1966). After such treatment, proteins associated with the core of the virus particle (insoluble fraction) are pelleted during ultracentrifugation through a sucrose cushion, whereas proteins associated with viral envelope membranes (soluble fraction) remain in the supernatant. Recombinant viruses grown in LT cells were released by freeze–thaw cycles and sonication and purified using sucrose gradients. After solubilization of virus particles and separation of the insoluble (C) and soluble envelope (E) fractions, we examined by Western blotting with anti-FLAG mAb whether the recombinant proteins were incorporated into the virus particles (Fig. 3). This analysis revealed that
FLAG–F1L and FLAG–10 kDa were incorporated into the virus particles at high levels and that both were associated mainly with the soluble fraction. In contrast, ORF-110–FLAG was found entirely in the insoluble fraction. This result was unexpected, suggesting that this protein was highly insoluble under the conditions used to solubilize the viral membranes and therefore was partitioned into the insoluble core fraction. Analysis of the ORF-110 sequence revealed at least one N-glycosylation site, which could explain the multiple bands seen for ORF-110–FLAG where there were additional bands larger than the 22 kDa band. The Western blots were also examined with anti-F1L mAb (8D7) in order to confirm separation of the insoluble and soluble fractions. This showed that the 39 kDa F1L protein was detected in the unfractionated virus (UF) and soluble fractions of each virus preparation, although there was some carry-over of envelope proteins into the core fraction of 10 kDa, consistent with the result of the anti-FLAG Ab (Fig. 3).

In addition, a smaller band of 33 kDa reacted with 8D7 in the wild-type (wt) virus and in the recombinants. A 28 kDa protein was also seen in the recombinants expressing the FLAG-tagged version of F1L. These proteins are thought to be proteolytic cleavage products of F1L. The anti-F1L Western blot revealed double bands for the F1L recombinant but not the other viruses. We believe this represents detection of both the native F1L protein and the 40 kDa FLAG–F1L fusion. This allowed us to determine that FLAG–F1L was present at approximately 30 % of the level of native F1L by densitometric analysis of the bands in lane E (Fig. 3).

Detection of FLAG peptide on the surface of virus particles by immunogold labelling

Western blot analyses revealed that the FLAG–F1L and FLAG–10 kDa proteins expressed by the recombinant viruses were mostly associated with the viral envelope, whereas ORF-110–FLAG appeared to be associated with the insoluble core fraction. We next tried to detect the presence of the FLAG peptide on the surface of the recombinant virus particles by immunogold labelling and electron microscopy. The wt and recombinant particles were reacted with 8D7 (anti-F1L) or anti-FLAG primary mAb together with a secondary anti-mouse gold-conjugated mAb. The results showed that the FLAG peptide could readily be detected on the surface of the recombinant FLAG–F1L and FLAG–10 kDa viruses (Fig. 4a). However, the number of gold grains on F1L recombinant virus particles labelled with anti-FLAG mAb was greater than that detected on 8D7-labelled particles from the same recombinant virus. This discrepancy may be due to the FLAG peptide being more accessible to the anti-FLAG mAb and also the higher affinity of this mAb to the peptide. In addition, approximately 30 % of particles did not label with anti-FLAG mAb or 8D7.

We could not detect ORF-110–FLAG by immunogold labelling of virus particles from lysed cells. We speculated that ORF-110–FLAG, like VACV A34R, could be associated with additional membranes derived from the trans-Golgi network and that ORF-110–FLAG was not accessible to the anti-FLAG mAb. Virus particles were therefore isolated and gently purified from the supernatants of virus-infected cells by a method used to purify VACV Western Reserve (WR) strain EV particles. Immunogold labelling of this preparation revealed ORF-110–FLAG on the viral membrane. In many cases, the membrane appeared to have partially separated from the virus particle, and here labelling could clearly be seen to associate only with the membrane and not with the intact virus particle. In addition, labelling with anti-F1L showed that this structural protein was only associated with the virion particle and not the EV membrane (Fig. 4b). Immunogold labelling of an EV preparation of F1L recombinant virus using anti-FLAG revealed that FLAG–F1L was not incorporated into the EV membrane (Fig. 4c).

Localization of ORF-110–FLAG to the Golgi

In VACV, A34R localizes to the Golgi (Lorenzo et al., 1994). The MV forms of ORF-110 recombinant virus appeared to be
Fig. 4. Electron micrographs of immunogold-labelled recombinant ORFVs. (a) Purified virus preparations of MV particles were reacted with anti-FLAG or anti-F1L mAb, followed by labelling with a gold-conjugated secondary Ab. EV preparations of ORF-110–FLAG recombinant virus (b) and F1L recombinant virus (c) were labelled with anti-FLAG or anti-F1L mAb. Viruses displaying partial or complete detachment of the EV membranes are shown. Gold particles were silver enhanced and viewed under a transmission electron microscope. Bars, 200 nm.
wrapped by an additional membrane that had ORF-110–FLAG protein incorporated into it. To determine whether ORF-110 localized to the Golgi, immunofluorescence assays were carried out.

Recombinant ORF-110 virus-infected LT cells grown on coverslips were fixed and permeabilized with methanol at various times p.i. Previous studies have shown that new infectious particles are detected at 14–16 h p.i. (Balassu & Robinson, 1987). The cells were then stained for the presence of FLAG peptide, and nuclei were stained with DAPI and the Golgi with WGA (Fig. 5). In mock-infected and recombinant ORF-110 virus-infected cells harvested at 1 h p.i., a perinuclear distribution of the Golgi was observed. In recombinant ORF-110 virus-infected cells at 3 h p.i., the Golgi appeared more condensed (results not shown), whilst at 12 and 18 h p.i., red staining was observed throughout the cytoplasm, indicative of fragmentation of this organelle. However, a major portion of the Golgi was still observed to be closely associated with the nuclei. Merged images showed the accumulation of ORF-110–FLAG protein in the Golgi at 12 h p.i. and this build-up was evident until 24 h p.i. Punctate staining with anti-FLAG was observed in the cytoplasm from 18 h p.i. and may correspond to virus particles being transported to the cell periphery for virus egress. The results suggest that ORF-110 localizes to the Golgi during the late stage of ORFV infection.

DISCUSSION

Sequence analysis of the ORFV genome has revealed homologues of almost all of the VACV structural genes, suggesting that the structures of the two viruses may be similar. However, there are some notable exceptions, including genes encoding proteins associated with the WV and EV envelopes, that suggest possible differences in their morphogenesis, intracellular movement and cell–cell transmission.

Immunogold labelling of purified recombinant virus particles isolated from lysed infected cells and extracellular
virus suggested that, like VACV, ORFV has at least two types of infectious particles, MV and EV. FLAG–F1L and FLAG–10 kDa were detected by immunogold labelling on virus particles isolated from lysed cells. However, it was noted that a proportion of purified MV particles did not label with anti-FLAG or anti-F1L mAb. A possible explanation was that the epitopes were masked by the double membranes of the WV form and these were not ruptured during the purification process. ORF-110–FLAG could not be detected on the surface of such particles despite the abundance of this protein in virus from lysed cells, whereas ORF-110–FLAG could readily be detected on extracellular virus or membranes that were shed from the virus. The VACV homologue A34R is associated with the two outermost membranes of WV, that is, before it egresses from the cell. In the outermost membrane, the C terminus of A34R faces inwards, whereas on the inner membrane of WV, the C terminus of A34R faces outwards. During virus egress, the outermost membrane of WV fuses with the cell membrane (reviewed by Smith et al., 2002), thus exposing A34R on the surface. Like VACV, it would appear that ORFV also loses one of the two outermost membranes when it egresses from the cell, exposing the C terminus of ORF-110 on the outer surface of the virion envelope. ORFV has never been observed to bud (Hiramatsu et al., 1999), supporting our view that the mechanism of egress from the infected cell is through fusion with the plasma membrane. Many of the extracellular particles labelled with gold also showed the membrane detaching from the virus particle. The EV membranes are fragile, and it is possible that the purification technique caused the membranes to detach completely, producing gold-decorated circlets. These appeared to be labelled on both sides of the membrane; however, this could be an artefact of the preparation in which the membranes had twisted prior to recircularizing and resealing. In addition, on particles where the membrane had detached, F1L could be detected on the virion by immunogold labelling with anti-F1L mAb.

The finding that we could only detect ORF-110–FLAG once the particle egresses from the infected cell also suggests that, like VACV A34R, ORF-110 is concealed between the two membranes derived from the Golgi that form WVs. Although there was a high abundance of ORF-110–FLAG detected by Western blotting in virus particles from lysed cells, there were few EV-like particles detected in the cell-culture medium. In VACV, EV particles are generally produced at very low levels compared with MV, and this appears also to be the case with ORFV, although we could be underestimating their levels due to their fragile nature. In VACV, a large proportion of WVs become membrane associated to form cell-associated enveloped virus. It is possible that this form is also produced in ORFV.

A number of VACV proteins associated with EV and WV membranes are glycosylated, whereas glycosylation of MV-associated proteins has not been reported. Our findings showed that, unlike F1L and 10 kDa, ORF-110 was modified by a post-translational event that most likely involves glycosylation. An N-linked glycosylation site was predicted for ORF-110. A notable difference between ORF-110 and A34R was that ORF-110 has a predicted Golgi retention signal sequence that is not found in VACV A34R. In VACV, B5R, whose homologue is absent in ORFV, interacts with A34R and this complex is localized to the Golgi by F13L (Röttinger et al., 1999; Husain and Moss, 2001). There is also evidence that B5R alone translocates A34R to the Golgi (Lorenzo et al., 2000). The prediction that ORF-110 localizes to the Golgi in ORFV-infected cells was confirmed by fluorescence microscopy. Whether it actually localizes there by itself has yet to be determined. Nevertheless, our findings suggest that the mechanism of translocation of ORFV proteins to the Golgi is different from that of VACV.

Our results showed that ORFV has wrapped particles of MV, despite the lack of a homologue of VACV B5R, which has multiple functions including a role in membrane wrapping of MV. ORFV has homologues of the other VACV genes known to be involved in wrapping that include F13L and A27L (Blasco & Moss, 1991; Smith et al., 2002), suggesting that the mechanism of wrapping could be different from that of VACV. In addition, ORFV lacks A36R, A56R and K2L, which are associated with either the WV or EV envelope. B5R and F13L are involved in the movement of WV on microtubules to the cell periphery (Ward & Moss, 2001a, b), whilst A36R is critical for the movement of VACV from cell to cell by actin tail formation, but is not required for EV production (Frischknecht et al., 1999), which may explain why actin tail formation has not been reported for ORFV. A34R and B5R disrupt the EV envelope prior to fusion during entry (Law et al., 2006), whilst A56R and K2L interact with A16L and G9R to prevent fusion of infected cells (Wagenaar et al., 2008). The lack of homologues of some of the VACV genes described above suggests that ORFV has evolved other mechanisms that allow intracellular movement and entry into neighbouring cells.

Western blot analysis of solubilized virus particles revealed that native 39 kDa F1L and 40 kDa FLAG–F1L fusion proteins were partially cleaved, producing a 33 kDa protein that was detected by anti-F1L mAb (8D7) in all virus preparations and a 28 kDa protein that was detected by anti-FLAG in the FLAG–F1L recombinant. A 33 kDa cleavage product of F1L was also detected with 8D7 by Housawi et al. (1998). The above cleavage products suggest that there are at least two cleavage sites and that the epitope to which 8D7 binds is located towards the C terminus of the molecule. The smaller product of 12 kDa had been detected (not shown). We have expressed FLAG–F1L using a eukaryotic expression vector in HEK293 EBNA cells where a 28 kDa product was also detected with anti-FLAG, suggesting that cleavage was due to a cellular protease. Multiple bands of different apparent molecular masses have been reported for VACV H3L in which it was suggested that the forms differed in folding (Takahashi
et al., 1994) and for the FWPV homologue where proteolytic cleavage might be responsible (Boulanger et al., 2002).

Our data also provide an indication that it might be possible to genetically fuse antigens to ORFV structural proteins for vaccine development. Studies have shown that fusing immunogenic epitopes of human immunodeficiency virus type 1 with major antigenic envelope proteins of VACV B5R, A27L and the core protein A4L elicits Abs with higher specificity and strong neutralizing activity (Katz & Moss, 1997; Collado et al., 2000; Loewinger & Katz, 2002). We are exploring the potential of ORFV for vaccine development using the above approach.

We have shown that ORFV has multiple enveloped forms and that specific virus-encoded proteins are associated with these envelopes. Like VACV, our results suggest that ORFV MV is wrapped by two additional membranes derived from the Golgi, and that the WV particles egress from the cell by fusion to the cell membrane to release MV particles wrapped by a single membrane. Our findings suggest that ORFV is able to spread from cell to cell within the infected host by producing EV-like particles.

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