African swine fever virus assembles a single membrane derived from rupture of the endoplasmic reticulum

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Summary

Collective evidence argues that two members of the nucleocytoplasmic large DNA viruses (NCLDVs) acquire their membrane from open membrane intermediates, postulated to be derived from membrane rupture. We now study membrane acquisition of the NCLDV African swine fever virus. By electron tomography (ET), the virion assembles a single bilayer, derived from open membrane precursors that collect as ribbons in the cytoplasm. Biochemically, lumenal endoplasmic reticulum (ER) proteins are released into the cytosol, arguing that the open intermediates are ruptured ER membranes. ET shows that viral capsid assembles on the convex side of the open viral membrane to shape it into an icosahedron. The viral capsid is composed of tiny spikes with a diameter of ~5 nm, connected to the membrane by a 6 nm wide structure displaying thin striations, as observed by several complementary electron microscopy imaging methods. Immature particles display an opening that closes after uptake of the viral genome and core proteins, followed by the formation of the mature virion. Together with our previous data, this study shows a common principle of NCLDVs to build a single internal envelope from open membrane intermediates. Our data now provide biochemical evidence that these open intermediates result from rupture of a cellular membrane, the ER.

Introduction

Enveloped viruses acquire their membrane from the host by budding at cellular membranes or by wrapping, acquiring one or two membranes respectively. A prototypical example being the retrovirus HIV-1; this virus buds at the plasma membrane and fission requires the recruitment of endosomal sorting complexes required for transport (ESCRT) complex, a cellular machinery involved in scission of cellular membranes (reviewed in Welsch et al., 2007). Thus, viral membrane acquisition resembles cellular membrane dynamics and mechanisms of vesicle formation ensure the maintenance of closed membranes. Early transmission electron microscopy (TEM) images suggested that the large DNA virus vaccinia virus (VACV) maybe an exception. Dales and Mosbach proposed that the VACV envelope was made 'de novo', with open ends in the cellular cytoplasm (Dales and Mosbach, 1968). Because this model has no precedent in cellular biology, it was challenged by other TEM studies. Thus, Sodeik et al. proposed instead that VACV acquires its membrane by wrapping of a double-membraned cisterna derived from the endoplasmic reticulum (ER; Sodeik et al., 1993, Sodeik and Krijnse-Locker, 2002).

Preparation of specimens for conventional TEM requires the sample to be dry, thin and contrasted, which potentially leads to artefacts. Two membranes may appear as one due to dehydration-induced collapse and heavy-metal staining may disguise the underlying fine structure of the lipid bilayer. TEM imaging requires the samples to be cut in thin sections and the images represent two-dimensional projections of three-dimensional (3D) structures. To study the origin and 3D structure of cellular membranes at high resolution, the use of complementary electron microscopy (EM) methods that may overcome these limitations is thus a prerequisite (see e.g. Bleck et al., 2010).

We reinvestigated the membrane acquisition of VACV using cryo-EM of vitreous sections to image the VACV
membrane in a fully hydrated state without the use of heavy-metal contrasting. With electron tomography (ET), we analysed the VACV membrane in three dimensions, in particular putative connections to cellular membranes. These combined EM methods allowed us to conclude that VACV is composed of a single membrane coated by an 11 nm layer composed of the viral scaffold protein shaping it into a sphere with a diameter of about 350 nm. These cryo-EM observations confirmed former quick-freeze deep-etch analyses (Heuser, 2005). We used ET to show that the VACV membrane is open and is built from small open membranes to which it is connected (Chlanda et al., 2009). Although no continuities between the VACV membranes and cellular membranes could be detected, lipid analysis of purified virions suggested that the VACV membrane is derived from the ER (Sodeik et al., 1993; Krijnse Locker et al., 2013). Collectively, we proposed that vesicular carriers pinch off the ER rupture to form open intermediates that contribute to the formation of the single open membrane of VACV (Chlanda et al., 2009).

Membrane assembly using open intermediates has no cellular precedent and raises the following questions about the underlying molecular mechanism: which proteins mediate membrane rupture, how are the open membrane ends stabilized in the cytoplasm and how do the open intermediates contribute to membrane growth (Krijnse Locker et al., 2013). To address these questions, we asked whether other members of the nucleocytoplasmic large DNA virus (NCLDVs) family, to which VACV belongs, use the same unconventional membrane assembly pathway. NCLDVs are characterized by a large particle and genome size ranging from 150 nm to 1000 nm and from 100 kb to 1.2 Mb respectively (Colson et al., 2012; Yutin and Koonin, 2012). Comparison of their genomes strongly suggests that they are derived from a common ancestor; they comprise a conserved set of genes involved in DNA metabolism and transcription that enable for cytoplasmic metabolism and transcription that enable for cytoplasmic DNA replication in association with large viral factories (VF; Yutin et al., 2009; Colson et al., 2012; Yutin and Koonin, 2012; Filee, 2013). Recent data from our group as well as from Mutsafi et al., showed that the NCLDV mimivirus assembles its membrane similar to VACV (Mutsafi et al., 2013; Suarez et al., 2013). The collective data using ET (Suarez et al., 2013) and scanning mode ET (STEM-ET; Mutsafi et al., 2013) demonstrated that the mimivirus membrane is formed from open intermediates that is shaped into an icosahedron by the assembly of the viral capsid protein, the homologue of the VACV scaffold, on its convex side.

In this study, we ask whether the NCLDV African swine fever virus (ASFV) also uses this unconventional membrane assembly pathway in order to start looking for common denominators. We use TEM, ET, cryo-EM and STEM-ET to show that membrane acquisition of ASFV is very similar to VACV and mimivirus and involves open membrane intermediates to form an immature particle with a single open membrane. Importantly, we provide biochemical evidence for our membrane rupture model and show that luminal ER proteins are released in to the cytosol.

Results

The assembly site of ASFV by thin-section EM and immunolabelling

The first evidence of ASFV morphogenesis is small curled membranes at the VF. Upon assembly of the major capsid protein p72, the homologue of the VACV D13L, on their convex side, these membranes give rise to icosahedral intermediates and then immature icosahedral particles (Andres et al., 1997; Garcia-Escudero et al., 1998). DNA and core protein uptake and core-protein processing results in the formation of the mature virion (MV), characterized by an electron-dense nucleoid enclosed by the core shell (Brookes et al., 1998; Andres et al., 2002a,b; Suarez et al., 2010b). Previous TEM studies suggested the ASFV membrane to be either one or two membranes, similar to VACV, which we addressed first using two complementary EM-embedding methods.

By thawed cryosections, the VF accumulated large amounts of curved structures composed of a single electron-lucent layer, surrounded by immature (IV) and mature (MV) icosahedral particles (Fig. 1A and Fig. S1A). The curved structures appeared as a railway track in high-pressure frozen/freeze substituted (HFP/FS) samples typical of a single bilayer where the stain is disposed on both sides of the membrane (Fig. 1B). In the same HFP/FS samples, the nuclear envelope displayed two railway-track-type structures (Fig. S2A), collectively suggesting that the viral structures were composed of a single bilayer.

The curved membranes were significantly labelled for the viral membrane protein p17 (Fig. 1C, Fig. S1B and E) and enclosed an electron density that labelled positive for anti-pp220, the precursor of several core proteins (Fig. S3). The convex side was sometimes studded with an electron-dense structure positive for anti-p72, the major viral capsid protein (Fig. S3C and D). Abundant anti-p72 labelling was also observed on IVs (Fig. 1D) and MVs as well as on membrane tails emanating from virus particles (Fig. 1D and Fig. S1C and D). As the VF is also the site of viral DNA replication, it is significantly labelled with anti-DNA. MVs with an electron-dense nucleoid were also strongly decorated with anti-DNA, IVs were sometimes positive while the viral membrane precursors were not significantly labelled (Fig. 1E and F).

Upon HFP/FS followed by embedding in epoxy resin, these features were not well resolved, the putative
Fig. 1. The ASFV-VF by thin-section EM and localization of the membrane protein p17 and the major capsid protein p72.
COS cells were infected with BA71V, fixed at 18 h post-infection and prepared for thawed cryosections (A, C to F) or HPF/FS and embedded in lowicryl (B) and immunolabelled with anti-p17 (C), p72 (D) or anti-DNA (E and F). In A, mature (MVs) and immature (IVs) virions surround membrane curts (arrows) sometimes coated with an additional layer on their convex side (arrowheads in A, B, E and F). B shows an area of a VF embedded in lowicryl, with MVs, IVs and curved membranes (arrows). The curved structures appear as a ‘railway-track’ typical of a single bilayer (arrows). In C, the curved structures label for anti-p17, an abundant and essential membrane protein of ASFV. In D, IVs label for the major capsid protein p72 as do some of the curved membranes shown in the Fig. S1C. The antibody also abundantly labels membrane extensions (black arrows) seemingly connected to immature virions. F and G. Anti-DNA labelling decorates MVs and some IVs, whereas the membrane precursors (arrows) are not significantly labelled. Scale bars A, B and F, −200 nm; C, −100 nm; D, −50 nm; E, −500 nm.

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membrane appeared somewhat fuzzy (Fig. S2B) and this method was not further used.

Thin-section EM and immunolabelling confirmed all the features observed previously (Andres et al., 1997; Garcia-Escudero et al., 1998; Suarez et al., 2010a) and strongly suggested that the ASFV envelope is a single bilayer, which we next analysed in three dimensions.

The viral precursor membranes arrange in strands of open membrane curls

Using ET, we studied the twisted membrane structures in 3D and their relation to assembling viruses. Figure 2A to D represents four 2.4 nm spaced slices of a dual-axis tomogram of HM20-embedded samples, showing viral membrane precursors surrounded by icosahedral particles (Fig. 2A to D and Video Clip S1). In 3D, the membranes precursors arranged as ribbons or serpentines (Fig. 2E and F and Video Clips S2 and S3) composed of collections of membrane curls (white arrows in Fig. 2E and F). They enclosed an electron density shown earlier to be positive for anti-pp220 (Fig. S3) and thus consisted of viral core proteins (see also Simon-Mateo et al., 1993; Andres et al., 2002b). Consistent with the anti-p72 labelling, some of the membrane curls were coated with capsid, shaping the membrane into icosahedrons. In 3D, capsid coats were detected as short segments on single membrane curls (black arrowheads in Fig. 2A to D), completely covering icosahedral particles or coated intermediates between these two structures (Fig. 2E and F). IVs typically contained an opening through which both ends of the internal envelope emanated into the cytoplasm (Fig. 2G, 2H 1 to 4).

Electron tomography of thawed cryosections resolved all of the features described in Fig. 2; membranes (black arrows in Fig. 3A to C and Video Clip S4) arranged as serpentines of open curls. Some of the curls were capsid-coated or found connected to almost complete icosahedral particles (Fig. 3D and Video Clip S5). Immature particles displayed an opening through which one or both ends of the internal membrane emanated (white double arrows, Fig. 3D to F). Compared with HM20-embedded samples, the membranes were generally more readily resolved and their continuities better revealed.

The membrane curls arranged as long ribbons were not the result of overexpression at late times post-infection as in small, early, factories they were also readily detected (Fig. S4A and B and Video Clip S6). In 3D, the twisted strands on small VFs looked indistinguishable from those of large VF described earlier (Fig. S4C and D and Video Clip S7), suggesting that in COS cells they may be a true early intermediate of viral membrane assembly (see Discussion).

We quantified our ET observations considering only those membrane structures whose ends were within the section volume. As shown in Table 1, a significant proportion of the membranes, both connected and not connected to the assembling particles, were open structures (60% and 44% respectively). Similar results were obtained using lowicryl sections (Table 1). The average diameter of the open membrane curls was 105 nm, exceeding that shown for VACV (around 50 nm) and mimivirus (around 90 nm) (Suarez et al., 2013; Table 1). We also analysed COS cells infected with a recombinant where the synthesis of the major membrane protein p17 is regulated by an inducible promoter and that fails to produce virus particles under restrictive conditions (Suarez et al., 2010a). Without the membrane protein p17 synthesis, open membrane curls were readily detected, suggesting that this viral protein is not involved in their formation (Video Clip S14).

Altogether, our quantitative ET data show that the ASFV precursor membranes are open intermediates that contribute to the formation of a single internal membrane, similar to what we previously showed for VACV and mimivirus.

Structure of IVs and MVs by room temperature (RT)-EM and cryo-EM of vitreous sections (CEMOVIS)

In 3D, we detected an abundant immature form shown in Fig. 4A that was also detected in early factories (Fig. S4). The capsid displayed a discontinuity through which both membrane ends protruded into the cytoplasm and that were associated with long ribbons of open membrane curls enclosing core proteins (Fig. 4A and Video Clip S8). Less frequently, the membrane ends of the IV arranged as a straight line or only one of the two ends protruded into the cytosol (Fig. 4B and C and Video Clips S9 and S10). Mature particles, characterized by an electron-dense nucleoid, contained an additional internal layer enclosing the nucleoid, corresponding to the viral core shell (Fig. 4D, Video Clip S11). In such virions, the capsid and membrane were closed. In thawed cryosections, the capsid and the inner envelope of immature and mature particles appeared as electron-lucent layers and in favourable slices the capsid exhibited the periodicity of the capsomers (arrowhead in Fig. 3E). The outer capsid layer was separated from the internal envelope by an electron-dense structure, which was not obviously resolved in lowicryl samples (Fig. 3E).

To address the discrepancy between Tokuyasu-type and HM20-embedded samples we used CEMOVIS. CEMOVIS (Al-Amoudi et al., 2004) enabled us to analyse virus assembly under native conditions, without dehydration and heavy-metal contrasting. We readily observed all of the features seen in RT-embedded samples,
Fig. 2. ET analysis of the ASFV-VF in semi-thin lowicryl sections. Infected cells fixed at 14 hpi were processed by HPF/FS and embedded in lowicryl as described in Experimental procedures. A to D are four 2.4 nm thick slices of a dual-axis tomogram from 200 nm thick sections. Curved membranes (arrows) are surrounded by mature- (MV) and immature viruses (IV). The membranes are bended by an additional layer to form an icosahedral intermediate (arrowheads). E and F show the rendering of a selected area of the VF; in E, the rendered image is merged with a slice of the tomogram. The curved membranes (green) are arranged in serpentine-like structures (white arrows). The curved membranes are coated with short segments of capsid protein (red; white arrowheads) or connected to the inner layer of IVs, emanating from both sides of the open capsid (rendered particle in top of E and F). The inset in F labelled with G shows the top middle particle from another orientation to more clearly display the opening in the capsid through which both membrane ends emanate. H shows higher magnification of four 2.4 nm spaced slices of the immature particle, boxed in A and B and rendered in G. The slices were taken from the Video Clips S1 to S3. Scale bars A to D, −200 nm; E and F, −100 nm; G and H, −50 nm.

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assembling capsids on membrane segments (that appear electron-dense by CEMOVIS) that were displayed as curls in thin sections (black arrows, Fig. 4E, I and J). Similar to the thawed cryosections, the IVs contained three distinct structures: an inner and outer electron-dense layer separated by an electron-lucent structure (Fig. 4F, I, J, K and L). The inner layer was connected to the uncoated membrane curls confirming of it being the viral membrane (Fig. 4F). MVs found within the VF (Fig. 4I) or budding at the plasma membrane (Fig. 4G and H) additionally contained a viral core. At first glance the three layers of the IVs displayed no distinct features besides being of different electron density. However, in thin sections (~50 nm in thickness), the structure connecting the inner to the outer most layer displayed thin striations (white arrowheads, Fig. 4F and L), whereas the capsid was composed of tiny spikes (black arrowheads in Fig. 4F and K).

Because CEMOVIS samples are without stain, we were able to measure the diameter of the different layers possibly without dehydration artefacts. We considered the curled precursor membranes, the layers of the immature particles, virions budding at the cell surface and the plasma membrane acquired during budding. The outer and inner mitochondrial membranes, used as control, measured around 6 nm. With around 5.5 nm, the plasma...
membrane was slightly thinner whereas the inner membrane of the IV and MV was around 5 nm. Finally, the capsid measured around 5 nm, whereas the electron-lucent structure connecting it to the membrane measured about 6 nm (Table 2).

Thus, the CEMOVIS data confirmed that the internal membrane is coated with two distinct structures, an outer layer connected to the internal envelope by a structure that appeared electron-lucent by cryo-EM and electron dense in thawed cryosections.

### ER rupture as origin of the ASFV membrane

We next sought to biochemically confirm membrane rupture as a mechanism to generate the open membrane precursors. Infected and control Vero cells were lysed by homogenization and membrane, cytosolic and nuclear fractions were analysed by Western blotting. This unexpectedly revealed significant amounts of protein disulfide isomerase (PDI) and binding of immunoglobulin protein (BIP), both lumenal ER proteins, into the cytosolic fraction in infected, but not mock, cells (Fig. 5). Cathepsin L, a luminal endosomal protein did not redistribute to the cytosol while the integral proteins, calnexin and the ASFV pE183L gene product, sedimented with the membrane fractions, indicative of the release of lumenal ER content rather than other effects on membranes. We repeated the experiment in the presence of cytosine β-arabinofuranoside (AraC), a well-described inhibitor of DNA replication. In ASFV-infected cells, AraC blocks DNA replication and consequently the synthesis of viral late proteins. The steps prior to replication, such as entry, transcription as well as the synthesis of about 58 early proteins occur normally. No substantial release of PDI into the cytosolic fraction was observed in the presence of AraC (Fig. S5), showing that its release required late viral protein synthesis as expected of a process related to virus assembly.

As this result supported the collective data suggesting that the ASFV membrane is derived from the ER (Andres et al., 1998; Rouiller et al., 1998), we performed ET to search for continuities between viral and ER membranes. However, in all TEM tomograms of 200–300 nm sections analysed, continuities were not observed (Video Clips S1, S4 and S6). We made use of STEM tomography that allows for the analysis of sections with a thickness of up to 1000 nm and thus for the analysis of larger volumes. Despite the larger volume analysed (450 nm and 750 nm sections), continuities with the ER were not observed although ER cisternae were observed in close proximity to the forming virions (arrows in Fig. 6A to D and Video Clips S12 and 13).

Scanning mode ET of thawed cryosections confirmed the details seen by ET and by CEMOVIS with an even better resolution. Using a high angle annular dark field (HAADF)-STEM filter (dark field detector), the internal membrane of the immature virions appeared as a discrete or continuous black line. Consistent with the TEM-ET shown earlier, the internal membrane was continuous with precursor membranes that protruded into the cytoplasm through an opening in the capsid layer (Fig. 6, slice 3, black double arrowheads). In favourable slices of the tomogram, the outer most layer revealed tiny spikes (Fig. 6, slice 3 and 4, black arrowheads), separated from the membrane by an electron-lucent layer that displayed thin striations (white arrowheads), confirming our observations made by CEMOVIS.

Similar to VACV, we propose that virally modified membranes are detached from their donor membranes prior to rupture, supported by our failure to detected continuities with the ER. Our biochemical data now for the first time provide strong evidence that the open membrane intermediates generated during ASFV infection are derived from rupture of a cellular organelle, more specifically of the ER.

### Discussion

#### Membrane assembly of NCLDVs from the ER

The origin and biogenesis of the ASFV membrane is a topic of intense debate (Sodeik and Krijnse-Locker, 2002; Salas and Andres, 2013). Early studies suggested that the inner ASFV envelope is a single lipid membrane (Stoltz, 1973; Carrascosa et al., 1984; Darcy-Tripier et al., 1984; Heppell and Berthiaume, 1992), consistent with more recent studies, using HPF/FS (Hawes et al., 2008). Other studies suggested that the ASFV membrane is
composed of two tightly apposed membranes derived from a collapsed ER cisterna (Andres et al., 1998; Rouiller et al., 1998), similar to what was proposed for VACV (Sodeik and Krijnse-Locker, 2002). Based on our present data, we propose that the ASFV particle assembles a single open membrane derived from open membrane intermediates. By ET, the ends of the membrane precursors were open, they were connected to the inner layer of...
Table 2. Average width of virus and cellular structures in CEMOVIS.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Immature particles (n = 16)</th>
<th>Mature virions (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner membrane</td>
<td>5.11 ± 1.29 nm</td>
<td>5.47 ± 1.12 nm</td>
</tr>
<tr>
<td>Capsid</td>
<td>5.07 ± 0.77 nm</td>
<td>5.37 ± 0.92 nm</td>
</tr>
<tr>
<td>Connecting layer</td>
<td>6.35 ± 1.63 nm</td>
<td>6.03 ± 1.36 nm</td>
</tr>
<tr>
<td>Outer envelope</td>
<td>–</td>
<td>5.32 ± 0.83 nm</td>
</tr>
</tbody>
</table>

Images acquired as in Fig. 4 were used to determine the average width of virus and cellular structures as explained in Experimental procedures. Immature particles and mature virions were identified based on the lack or presence of a nucleoid and core shell respectively.

a. The inner envelope of immature particles.
b. The outer layer surrounding the inner envelope that appears electron-dense by CEMOVIS.
c. The layer connecting the capsid and the inner envelope.
d. Outer envelope acquired upon budding of the mature virion at the plasma membrane as shown in Fig. 4G and H. As controls, mitochondrial membranes (n = 18) and the plasma membrane (n = 17) were used measuring 6.02 ± 1.19 nm and 5.48 ± 1.25 nm respectively.

growing particles that becomes progressively coated with the major capsid protein p72 to form an icosahedron. This membrane assembly is similar to what we, and others, showed for VACV and mimivirus (Chlanda et al., 2009; Mutsafi et al., 2013; Suarez et al., 2013). Previous data based on the presence of ER markers close to the viral membranes strongly suggest that the ASFV envelope is derived from the ER (Andres et al., 1998; Rouiller et al., 1998). Similarly, collective evidence for both VACV (Sodeik et al., 1993; Cluett and Machamer, 1996; Krijnse Locker et al., 2013) and mimivirus (Mutsafi et al., 2013) points to the ER as the most likely source of their membrane. Our biochemical data now provide strong support not only in favour of the ER as origin, but also for rupture of these membranes during ASFV infection. We could show that luminal ER proteins partially redistribute in the cytoplasm and that this release was specific for the ER. Similar experiments in VACV-infected cells failed to detect the release of ER proteins (data not shown) likely because its precursor membranes are much less abundant than in ASFV infection. For VACV (Chlanda et al., 2009), mimivirus (Mutsafi et al., 2013) and ASFV (present study) continuities between the viral membranes and the ER were, however, not detected. This suggests that the viral precursor membrane become separate entities, detached from the ER, before, or concomitant with, rupture. With the notion that rupture of the ER underlies the formation of the single-open NCLDV membrane, we are now able to search for common denominators that could mediate this assembly pathway. This process likely involves a complex of proteins and/or lipids encoded by the virus and the host that is regulated to mediate several consecutive events: rupture of ER membranes, the stabilization of open membrane ends in the cytoplasm, the targeting of the open membrane fragment to, as well as fusion with, the growing viral membrane. Our future goal is to find genes encoded by the three NCLDVs analysed, that have sequence or structure homology and study their potential role in membrane assembly.

The necessity to compare different embedding and EM imaging methods

In this study, we compared Tokuyasu-type cryosections, HPF/FS lowicryl-embedded samples and CEMOVIS. We observed that the former result in a better resolution of structures and membrane continuities compared with HPF/FS. Thawed cryosections may have two advantages over substituted samples. During sample preparation and sectioning the sample is not dehydrated, whereas FS samples are dehydrated in the first steps of embedding. Only at the very last step of sample preparation are the sections air-dried and embedded in methyl cellulose to prevent the collapse of the structure during drying. Consequently, the Tokuyasu method of embedding may suffer less from dehydration artefacts (e.g. collapse of structures/membranes). Second, FS samples are incubated ‘en block’ with heavy metals, such as osmium tetroxide and uranyl acetate. These associate with cellular structures and potentially disguise the underlying structure. In thawed cryosections, contrasting occurs at the very final step for a short time creating a uniform uranyl

![Mock and ASFV](image)

![Calnexin, BIP, PDI, Cathepsin L, Lamin B1, AKT, pE183L](image)
acetate stain where membranes are negatively contrasted. This results in the appearance of membranes as a single white line of which continuities may be easier to track. The latter is exemplified in Table 1, showing that the percentage of membrane ends that were not unequivocally resolved is higher in lowicryl-embedded samples after HPF/FS. We are aware of the fact that thawed cryosections may be particularly sensitive to shrinkage during tilt series acquisition (Bos et al., 2014). As similar data were obtained with different embedding and imaging methods, we feel confident that this limitation does not significantly affect our conclusions drawn. The resolution in thawed cryosections was particularly striking when using STEM tomography, displaying the fine structure of the capsid. The observation that small details in thick sections are better resolved in STEM than in TEM tomography is in line with earlier observations (Hohmann-Marriott et al., 2009; Hohn et al., 2011). The blurring of small structures in thick sections in conventional TEM is due to chromatic aberration caused by electron energy loss caused by multiple inelastic scattering of electrons. This problem increases with increasing section thickness. In STEM, there are no image forming lenses after the sample, and therefore chromatic aberration cannot occur and even electrons that have lost energy can contribute to the image. In addition, when a small convergence angle of the primary beam is used (Biskupek et al., 2010), depth of focus is higher than with regular TEM that is especially useful at high tilt angles.

Whereas our data show a preference for thawed cryosections to image membranes by ET, CEMOVIS is the method of choice to study cellular and viral structures under native conditions (Al-Amoudi et al., 2004). However, because such samples are beam-sensitive and CEMOVIS sections are difficult to produce, we propose that ET on thawed cryosections are the method of choice to generate many tomograms, allowing for quantification of the observations. An important advantage is that they can be immunolabelled to identify and image structures by ET with specific antibodies. Altogether, our collective

Fig. 6. HAADF-STEM tomography of 450 to 750 nm sections reveals no continuities of the ER with the viral membrane precursors. Infected COS cells were fixed and prepared for cryosectioning at 14 h post-infection. A and B are two 3.9 nm spaced slices of a single-axis STEM tomogram of a 750 nm thick section; C and D correspond to two 3.4 nm slices of a tomogram of a 450 nm thick section. No continuities between ER cisternae (black arrows), the viral precursor membranes (white arrows and circled in B) or the forming viral particles (black arrowheads) are detected. The images 1 to 4 show a higher magnification of the boxed areas in C and D to demonstrate the fine structure of the IV. By HAADF-STEM the inner membrane appears as a continuous black line (white arrow) connected to the precursor membrane (double-headed arrow in 3). The outer layer (white arrowheads in 3 and 4) displays tiny spikes and is connected to the membrane by a structure with thin striations (black arrowheads). Spikes and striations are best visible in 3. Slices were taken from Video Clips S12 and 13 respectively. Scale bars A and B, 180 nm; C and D, 90 nm; 1 to 4, 100 nm.
data demonstrate the necessity to combine and compare several EM-embedding and imaging methods to study the fine structure and 3D organization of cellular and viral membranes.

The structure and role of the membrane serpentines during NCLDV assembly

A common feature of the three NCLDVs was the 3D arrangement of the precursor membranes into serpentine-like structures composed of open membrane curls. The reason for this particular arrangement remains elusive; it could stabilize the open membrane ends in the cytoplasm, perhaps shielding them from host defence mechanisms. As already noticed for mimivirus, the membrane curls arrange as if they previously formed a cisterna that was cut at distinct intervals, hence forming curls with a defined diameter (50 nm for VACV, 90 nm for mimivirus and 105 nm for ASFV). This would require a machinery that not only ruptures ER membranes but does so at specific sites. Whatever the reason and mechanisms, in this study we could show that the ASFV membrane precursors adopted a clear topology; the concave side associated with core proteins and the convex side with the capsid. We believe that this topology reflects differences between the (previously) lumenal and the cytosolic sides of the membrane curls. Binding to either side of the membrane may be mediated by the membrane protruding ends of the protein p17, an abundant and essential membrane protein that localizes to the membrane curls (Rouiller et al., 1998; Suarez et al., 2010a). Its sequence predicts a single transmembrane domain possibly exposing its N-terminus lumenal and the C-terminus cytosolic (Simon-Mateo et al., 1995; Yanez et al., 1995 and C. Suarez, pers. comm.). The polyprotein pp220, the core precursors, may also bind to the cytosolic side of the membrane by virtue of its myristoylation (Simon-Mateo et al., 1993). Membrane rupture exposes the lumenal side of the protein p17 to which we propose capsid may bind. This scenario is consistent with the observation that in the absence of the protein p17 synthesis viral membrane precursors collect that fail to form icosahedrals and that lack the major capsid protein p72 (Suarez et al., 2010a). A striking observation was that the membrane serpentines were often associated with immature particles and the abundance of this structure suggested an important role during virus assembly. As the serpentines enclosed core proteins, we propose that they mediate the packaging of core proteins after which the particle can proceed to mature and form a core shell.

A model on ASFV assembly and particle structure

A model on the sequence of events during ASFV assembly is depicted in Fig. 7 (Andres et al., 1997; Garcia-Escudero et al., 1998). Collections of open membranes are formed by rupture of ER membranes. They are targeted to the VF and become shaped on their convex side by the scaffold/capsid protein. More open membranes are added to (fuse with) the growing virus that is progressively coated by the viral capsid to form an icosahedral particle. How membrane rupture and fusion, two processes that seemingly exclude each other, is regulated is currently unknown. As mentioned earlier core protein uptake may be facilitated by the serpentine membrane, upon which the particle closes and matures resulting in an identifiable core shell that surrounds the nucleoid. Our data did not unequivocally answer as to how and when the viral genome is taken up. One model postulates that the prepackaged genome is inserted once the particle is completed (Brookes et al., 1998). Another model proposed that the viral DNA is inserted and packaged together and bound to core proteins (Andres et al., 2002b). We would favour the latter model as our tomograms failed to detect an identifiable structure located at the particle’s opening, unlike what we showed before for VACV (Chianda et al., 2009). The fact that the concave side of the membranes enclosing core proteins was not significantly labelled with anti-DNA can be explained by the affinity of the antibody, accessibility of the antigen or too little antigen to be detected by EM

![Fig. 7. A model for the assembly of ASFV. We propose that membranes modified by viral membrane proteins pinch off from the ER and are ruptured to form open membrane curls that arrange in ribbons (1 and 2). These are targeted to the VF and become coated with capsid (red), shaping the membrane in an icosahedral form, and that enclose viral core proteins (brown; 2). Growth of the internal membrane is mediated by the recruitment of additional open membrane intermediates that may fuse with the forming viral envelope to form an IV (3). In the IV, both ends of the internal membrane emanate into the cytosol through an opening in the capsid. They are associated with the curled open membranes in the cytosol, arranged in serpentines, and that enclose viral core proteins (3). Through the opening in the immature, virion viral core proteins (with or without the viral DNA) may be taken up. Once uptake is completed, the membrane and the capsid are closed (5), likely using an intermediate where the two membrane ends become tightly apposed (4).](image-url)
immunolabelling. Finally, we also contribute to the fine structure of the viral capsid. By thawed cryosections as well as cryo-EM, we identify two layers with distinct densities. In thawed cryosections using ET, in CEMOVIS and in particular by STEM-ET, the outer layer displayed tiny spikes and was seemingly connected to the internal membrane by a structure displaying small striations. Compared with mimivirus, which is also icosahedral, ASFV is exceedingly less complex; it misses the additional layers that associate with maturing mini-virions, including the glycosylated fibres (Klose et al., 2010).

In summary, our data argue that ASFV, being a member of the NCLDV family, acquires its membrane in a way similar to Mimi and VACV. This unconventional way involves open membrane intermediates that are derived from cellular membranes and our data for the first time provides evidence for membrane rupture to generate the ASFV precursors. The challenge will be to find common denominators that mediate rupture and the biogenesis of a single open membrane.

Experimental procedures

Cells and viruses

COS-1 or Vero cells (American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS). Cells were infected at a multiplicity of infection of 5 pfu per cell with the BA71V strain of ASFV (Enjuanes et al., 1976) or with the recombinant virus v18i in absence of isopropyl-D-1-thiogalactopyranoside (IPTG) (Suarez et al., 2010a) for 1 h at 37°C and further incubated in DMEM supplemented with 2% FCS.

Sample preparation for EM

BA71V (ASFV strain used in this work) is a biosafety level 2 pathogen in Spain but biosafety level 4 in Germany. Biosafety level 4 pathogens cannot be imaged without prior chemical fixation, and therefore infected cells were chemically fixed prior to embedding. At the indicated times of infection, BA71V-infected cells were fixed by adding an equal volume of 2% glutaraldehyde (GA) (electron microscopy sciences, cat# 16220) in 2 mM thylene glycol tetraacetic acid (EGTA) (pH 6.9) directly to the medium. After 1 h at RT, the cell pellet was washed three times with 1× PHEM buffer and stored at 4°C until further processing. For Tokuyasu cyrosectioning, BA71V- or v17i-infected cells were fixed with 4% paraformaldehyde (PF) (electron microscopy sciences, cat# 15710) and 0.1% GA in PHEM buffer for 1 h at RT and kept in 4% PF in PHEM until processing. For high-pressure freezing, BA71V-infected cells, fixed with GA in PHEM buffer, were washed five times with 50 mM glycine in PHEM buffer. The pellet was mixed with an equal volume of 40% (w/v) dextran (40 kDa; Fluka, Heidelberg, Germany) for embedding in lowicryl and for CEMOVIS or 20% (w/v) bovine serum albumin (BSA) (Sigma, cat# A2153) for embedding in epoxy resin (Epon) in PHEM buffer and the samples were frozen using high pressure by a HPM010 (Baltec) as reported previously (Suarez et al., 2013). For Epon-embedding, freeze substitution was in 2% (w/v) osmium tetroxide, 0.1% (w/v) uranyl acetate, 5% (v/v) water in glass-distilled acetone (EM sciences) by slowly warming the samples from −90°C to 0°C over 20 h (Buser and Walther, 2008). Samples were kept a 0°C for 1 h, washed four times with cold glass-distilled acetone at RT and stepwise embedded with increasing concentrations of epoxy resin mixed with acetic. Samples were polymerized overnight at 65°C. Freeze substitution followed by lowicryl HM20 (Polysciences, Inc) embedding was carried out as described in Suarez et al. (2013).

Sectioning and immunolabelling

HM20- or Epon-embedded samples were sectioned with a Leica Ultracut S for TEM (60 nm thick sections) or ET analyses (250 to 750 nm sections) with or without post-contrasting with lead citrate. For Tokuyasu-type cryosections, BA71V- or v17i-infected cells were processed as described in Ghosh et al. (2003). Tokuyasu-type cryosections were immunolabelled as described previously (Suarez et al., 2013) with anti-DNA (Roche), mouse monoclonal antibody 198B2 against the major capsid protein p72 (Sanz et al., 1985), polyclonal antibody raised to the core protein precursor pp220-derived products p150 and p34 (Simon-Mateo et al., 1993) and mono-specific rabbit polyclonal sera against the membrane protein p17 described in Suarez et al. (2010a). For CEMOVIS, the vitrified blocks were sectioned with a Leica EM UC7 ultramicrotome at −150°C. The sections were cut with a 25° diamond knife (Diatome) at a clearance angle of 6°, a nominal speed of 1 mm s−1 and a thickness of 40 to 120 nm. The ribbons were manipulated with an eyelash and placed on 100 mesh grids (C-flat holey carbon film for high-resolution TEM, cat#71150, Electron Microscopy Sciences). The grids were stored in liquid nitrogen until further analysis.

Microscopy

Thin sections (60 nm) were examined with a Zeiss EM10 transmission electron microscope at 80 kV equipped with a Megaview charge coupled device (CCD) camera (Olympus). For dual-axis ET, Tokuyasu-type cryosections or lowicryl sections of 250 to 300 nm in thickness were collected on 50 mesh or slot grids using protein A coupled to 15 nm gold as fiducials. Where indicated, cryosections were immunolabelled and the gold particles of the immunolabelling were used as fiducials. Dual-axis tilt series were acquired as described previously (Suarez et al., 2013) using a Fischione dual-axis holder and a Tecnai TEM (FEI) with field emission gun (FEG) operating at 200 kV equipped with an Eagle bottom mounted 4K camera (200 kV, HS CCD 4 port read-out; FEI) at a magnification of 25 000 or 29 000×, binning two corresponding to a pixel size of 0.89 and 0.7642 nm respectively. For STEM, the tilt series (−72° to +72°, single axis, increment 1°) of 450 nm or 750 nm thick sections were recorded with a 300 kV STEM, FEG, (Titan 80–300 TEM, FEI) by use of a HAADF detector (Fischione, Export, PA, USA). Images were recorded with 1024×1024 pixels at an illumination time of 12 s as described in Villinger et al. (2014) with a camera length of 301 mm. Three-dimensional reconstructions of dual-axis ET or single-axis STEM tomography data were computed from the tilt series using IMOD software (Kremer et al., 1996). Rendering of

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the 3D surface of the tomograms was performed manually assigning colours to certain structures (capsid, membrane, core, nucleoid) based on densities. Videos of tomograms were made using IMOD and Image J. For cryo-EM, the grids with vitreous sections were transferred into a cryo-holder (model 626; Gatan) and were analysed with the Tecnai-FEG operated at 200 kV and a temperature below −170°C. Micrographs were taken under low-dose conditions provided by serial EM (http://bio3d.colorado.edu/SerialEM/) at a defocus range from −1 to −10 μm.

Quantification and processing of digital images
Quantification of open versus closed membranes, the diameter of the open membrane curds and of the virus layers in CEMOVIS was performed as described in Suarez et al. (2013). Briefly, only those membrane structures whose ends were contained in the full volume of the tomogram were considered for quantification. The measurements of the thickness and diameter of viral structures were carried out in Image J using micrographs with a known pixel size. Digital 2D and 3D images were processed with the Adobe Photoshop, Image J and Illustrator software.

Subcellular fractionation
Subconfluent Vero cells grown on 100 mm dishes were mock-infected or infected with BA71V. The ASFV-infected cells were incubate in presence or absence of 40 μg ml−1 AraC. After 13 or 18 h post-infection, cells were harvested by scraping, centrifuged at 400 × g for 10 min and washed twice with homogenization buffer (20 mM HEPES, pH 7.2, 1 mM ,2′,2″,2‴-ethane-1,2-diylidinitro)tetraacetic acid (EDTA), 0.25 M sucrose) supplemented with protease inhibitor cocktail (Complete, Roche). All subsequent fractionation steps were performed at 4°C. After 10 min on ice, the cells were mechanically lysed by passing them through a 23 G needle. Nuclei and cellular debris were sedimented at 500 000 × g for 5 min. The post-nuclear supernatant was then centrifuged at 100 000 × g for 30 min to separate the cytosolic fraction from the post-infection, cells were harvested by scraping, centrifuged at 400 × g for 10 min and washed twice with homogenization buffer (20 mM HEPES, pH 7.2, 1 mM ,2′,2″,2‴-ethane-1,2-diylidinitro)tetraacetic acid (EDTA), 0.25 M sucrose) supplemented with protease inhibitor cocktail (Complete, Roche). All subsequent fractionation steps were performed at 4°C. After 10 min on ice, the cells were mechanically lysed by passing them through a 23 G needle. Nuclei and cellular debris were sedimented at 500 000 × g for 5 min. The post-nuclear supernatant was then centrifuged at 100 000 × g for 30 min to separate the cytosolic fraction from the membrane/particulate fraction. All fractions were dissolved in reducing Laemmli buffer. Equal amounts of each fraction were analysed by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western immunoblotting on 0.2 μm polyvinylidene fluoride (PVDF) membranes (BioRad). The following antibodies were used: rabbit anti-calnexin (StressGene, 1:1000), rabbit anti-cathepsin L (BioAss, 1:1000), goat anti-lamin B1 (Santa-Cruz, 1:200), mouse anti- AKT (Abcam, 1:1000), rat anti-pE18SL (Rodriguez et al., 2004; 1:1000) and rabbit anti-p32 (Prados et al., 1993, 1:500). Signal was developed using enhanced chemiluminescence (ECL) Prime western kit (Life Technologies).

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References


Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Immunolabelling identifies the curved structures as ASFV membranes. COS cells were infected with ASFV as in Fig. 1 and prepared for cryosectioning. In A, an overview of the VF showing how the electron-dense mature particles surround the collections of open membranes. In B and E, the membranes and the virus particles are abundantly labelled with an antibody to the essential viral membrane protein p17. In C, immature virions (IVs) label for the major capsid protein p72 (black arrowheads) as do some of the curved membranes. The antibody also abundantly labels membrane extensions (arrow in D) seemingly connected to immature virions. MV, mature virion. Scale bars A, –1 μm; B, –500 nm; C, D and E, –100 nm.

Fig. S2. Appearance of the nuclear envelope in HM20-embedded cells and ASFV after epoxy resin embedding. A. In ASFV-infected cells embedded in lowicryl resin, the nuclear envelope adopts two ‘railway-tracks’ typical of two apposed bilayers (arrows). In B, viral membranes (arrows) of ASFV-infected cells embedded in epon resin are poorly resolved and the icosaedral particles show few details. N, nucleus. Scale bars, 100 nm.

Fig. S3. Localization of the polyprotein pp220. Tokuyasu-type cryosections of ASFV-infected cells were immunolabelled with anti-pp220. Gold particles decorate the core of mature particles (MV in A and D) and the forming core of immature particles (IV in A, B and C). The curved membranes enclose an electron density that also labels for anti-pp220 (C, E and F), indicating that the electron density is composed of core proteins. B and D are a higher magnification of the squares in A respectively. Scale bars, 100 nm.

Fig. S4. ET of an early factory. A and B shows two 1.35 nm spaced slices of a dual axis tomogram made from 200 nm thick Tokuyasu-type cryosections of ASFV-infected cells immunolabelled with anti-DNA as in Fig. 3. Gold particles were used as fiducials for tomogram reconstruction. The small factory displays a few curved membranes (black arrows) located close to immature particles (black arrowheads). The factory also displays open membranes (white arrows) in green arranged as serpentine cofaces (double arrows) to an immature particle (white arrowheads) as shown in the rendering in C and D. The concave side of the membrane encloses the putative core in brown. The slices were taken from Video Clips S6 and S7. Scale bars A and B, 100 nm; C and D, 50 nm. c, immature core; ca, capsid; ie, internal envelope.

Fig. S5. Analysis of the distribution of PDI in ASFV-infected cells in presence or absence of AraC by subcellular fractionation. Mock- or ASFV-infected cells in presence or absence of AraC were harvested at 13 hpi and fractionated by centrifugation into nuclear (N), cytosolic (C) and membrane (M) fraction. Equal amounts of each fraction were analysed by Western blotting for the ER luminal protein PDI, the ER membrane protein calnexin, the late viral protein pE183L and the early viral protein p32. In the presence of AraC, the synthesis of the late viral membrane protein pE183L is blocked as expected for AraC treatment, whereas the expression of early viral cytosolic protein p32 is not affected. Under these conditions, PDI is not significantly mislocalized into the cytosolic fractions.

Video Clip S1. 3D view of a viral factory region. Dual-axis tilt series was generated from 200 nm thick sections embedded in lowicryl tilted from –60° to +60°. The tilt series was reconstructed in iMOD. The video corresponds to the slices shown in Fig. 2A–D. Scale bar, 200 nm.

Video Clip S2. The viral precursor membranes arrange in long strands. Tomogram and partial rendering of the same area are shown in Video Clip S1. Membranes are green, the capsid layer in red and the core in brown. The video shows how the ends of the precursor membranes are coated with capsid, whereas the uncoated membranes consist of open membrane curls arranged in long ribbons or serpentine. The video corresponds to the slice shown in Fig. 2E. Scale bar 100 nm.

Video Clip S3. 3D view of the precursor membranes. Partial rendering of the VF area is shown in Video Clip S1. The video corresponds to the slices shown in Fig. 2F. Scale bars, 100 nm.

Video Clip S4. 3D analyses using Tokuyasu-type cryosections labelled with anti-DNA. The movie is a tomogram through an ASFV factory region. Dual-axis tilt series was generated from 200 nm thawed cryosections tilted from –70° to +70° and immunolabelled with anti-DNA, followed by rabbit anti-mouse and protein A coupled to 15 nm gold particles. The latter were used as fiducials for stack alignment in iMOD. The video corresponds to the slices shown in Fig. 3A–C and E. Scale bar, 200 nm.

Video Clip S5. 3D analyses of the precursor membranes in Tokuyasu-type cryosections. Partial rendering of the area is shown in Video Clip S4. It shows long ribbons (green) composed of open membrane curls. The curls are continuous with the ends of the internal membrane (also in green) of the growing particle (in red). The putative viral core (in brown) is associated with the concave surface of the forming particles. The video corresponds to the slices shown in Fig. 3D and F. Scale bar, 200 nm.

Video Clip S6. The long membrane ribbons are also made in small factories. The movie is a tomogram of a small ASFV factory. Dual-axis tilt series was generated from 200 nm Tokuyasu-type cryosections from –70° to +70° and labelled anti-DNA as for Video Clip S4. The video corresponds to the slices shown in Fig. S4A and B. Scale bar, 100 nm.

Video Clip S7. Long membrane ribbons shown in 3D after rendering of the Video Clip S6. In 3D, the curled membranes (in green) appear to consist of open membranes arranged in 3D as ribbons. The ends of the ribbons are connected to the growing particle. The video corresponds to the slices shown in Fig. S4C and D. Scale bar, 50 nm.

Video Clip S8. Rendering of the immature virus is shown in Fig. 4A where both open ends of the internal envelope (in green) emanates into the cytosol and are connected to curled membranes (green). The curled open membranes arrange into long ribbons and enclose the core proteins (brown). Red, capsid. Scale bar, 200 nm.

Video Clip S9. Rendering of the immature particle shown in Fig. 4B where only one of the two open membrane ends protrudes into the cytosol. ca, capsid in red; ie, internal envelope. Scale bars, 50 nm.

Video Clip S10. Rendering of the immature particle shown in Fig. 4C. The ends of the internal envelope (green) of the growing particle protrude into the cytosol. Rather than being curled, they align in two closely apposed straight membranes, appearing as a tail (arrow). Red, capsid. Scale bar, 100 nm.

Video Clip S11. Rendering of a mature particle shown in Fig. 4D, showing the nucleoid surrounded by concentric closed

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layers. c, core (brown); ca, capsid (red); ie, internal envelope (green); N, nucleoid (white). Scale bars, 50 nm.

**Video Clip S12.** The ER is not continuous with the ASFV precursor membranes. HAADF-STEM tomogram of the peripheral region of a BA71V factory. Tilt series was generated from 750 nm Tokuyasu-type cryosections tilted from $+72^\circ$ to $-72^\circ$. Several ER cisternae that are not continuous with forming virions are shown. The video corresponds to the slices shown in Fig. 6A and B. Scale bar, 90 nm.

**Video Clip S13.** HAADF-STEM tomogram on 450 nm thick section of BA71V-infected cells. Several ER cisternae are shown that are not continuous with the viral membrane precursors. The tomogram reveals viral membranes and icosahedral particles with very good resolution. The video corresponds to the slices shown in Fig. 6C and D 1 to 4. Scale bar, 90 nm.

**Video Clip S14.** Rendering of the precursor membranes of the recombinant virus v17i in absence of IPTG. The tomogram used for rendering was generated from dual-axis tilt series and was generated from 200 nm thawed cryosections tilted from $+70^\circ$ to $-70^\circ$ and immunolabelled with anti-DNA, followed by rabbit anti-mouse and protein A coupled to 15 nm gold particles. The latter were used as fiducials for stack alignment in iMOD. The expression of the protein p17 is repressed during the infection. Scale bar, 100 nm.