Published in final edited form as:

Traffic. 2012 January ; 13(1): 30–42. doi:10.1111/j.1600-0854.2011.01293.x.

Mechanism of collapse of endoplasmic reticulum cisternae during African Swine Fever virus infection

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Summary

Infection of cells with African Swine Fever virus (ASFV) can lead to the formation of zipper-like stacks of structural proteins attached to collapsed endoplasmic reticulum (ER) cisternae. We show that the collapse of ER cisternae observed during ASFV infection is dependent on viral envelope protein, J13Lp. Expression of J13Lp alone in cells is sufficient to induce collapsed ER cisternae. Collapse was dependent on a cysteine residue in the N-terminal domain of J13Lp exposed to the ER lumen. Luminal collapse was also dependent on expression of J13Lp within stacks of ER where antiparallel interactions between the cytoplasmic domains of J13Lp orientated N-terminal domains across ER cisternae. Cisternal collapse was then driven by disulphide bonds between N-terminal domains arranged in antiparallel arrays across the ER lumen. This provides a novel mechanism for biogenesis of modified stacks of ER present in cells infected with ASFV, and may also be relevant to cellular processes.

Keywords

endoplasmic reticulum; cisternal collapse; viral envelope protein; African Swine Fever virus

Introduction

The nucleocytoplasmic large DNA viruses (NCLDV) such as poxviruses, African Swine Fever virus (ASFV) and iridoviruses (1) replicate and assemble in cellular inclusions called virus factories which form next to the centrosome (2-6). The factories contain viral genomes, major structural proteins and an ordered series of membrane-derived structures which are thought to form the inner envelope of the virus. For vaccinia virus these membranes first appear as open crescents of uniform radius which close during genome

packaging to form spherical immature viruses. For ASFV and the iridoviruses, small membrane crescents mature through a series of geometric angular structures representing specific stages in the assembly and envelopment of icosahedral viruses. Early work by Dales and colleagues (7) suggested that the viral envelopes for vaccinia virus were generated by *de novo* synthesis. More recent studies suggest that the lipids may in some way be derived from the endoplasmic reticulum (ER). For vaccinia virus, a pathway for transport of the viral membrane protein, A9, between the ER and the inner envelope has been demonstrated (8) and three dimensional deep etch EM suggests that the inner envelope may be formed from lipids extracted from the outer leaflet of the ER (9). Recent analysis by cryo EM and electron tomography has traced connections between precursors of the inner envelope of vaccinia virus and ER-derived vesicles (10).

In addition to the membrane-derived structures that are associated with intermediates in virus assembly, cells infected with NCLD viruses often contain long parallel stacks of membrane separated by electron dense zipper-like structures composed of major structural proteins. These are seen as concentric whorls for the iridovirus *Rana temporaria* (11), and as zipper-like stacks following infection by ASFV. The zipper-like stacks are found at low levels during infection but increase in number when gene deletions reduce the availability of specific structural proteins. Examples include vaccinia viruses lacking major core protein, A10L/P4a (12,13) and ASFV unable to synthesise the major capsid protein (14-16), or a viral chaperone essential for capsid assembly (17,18). The membranes are thought to be derived from the ER because many are attached to ribosomes and show positive immunogold labelling for protein disulphide isomerase (PDI) (14,15). In some cases the membranes attached to the zipper-like structures are separated by a clear luminal space suggesting assembly of linear arrays of structural proteins along ER membrane cisternae. In other images the cisternae show localised or complete collapse along the length of the arrays suggesting that assembly of viral proteins on the cytoplasmic face of ER membranes can result in collapse of ER cisternae.

In this study we have investigated how ASFV causes collapse of ER cisternae. We show that collapse is dependent on viral protein J13Lp/p54/ORF E183L (J13Lp), an inner envelope protein targeted to the ER. Expression of J13Lp alone in cells generates large arrays of organised smooth ER (OSER). Cisternal collapse is not generally observed in OSER generated by host ER proteins (19,20), nevertheless, we show that J13Lp induced a striking collapse of ER cisternae in the inner stacks of OSER. Collapse of ER cisternae was dependent on the orientation of J13Lp within OSER and required exposure of a cysteine residue in the N-terminal domain of J13Lp to the lumen of the ER. The results suggest that ER collapse occurs when disulphide bonds formed between N-terminal domains are orientated across the ER lumen. Virus driven collapse of ER membrane cisternae therefore represents a novel mechanism for biogenesis of modified stacks of ER that are present in cells infected with the NCLD viruses and may also be relevant to cellular processes.

Results

J13Lp is required for collapse of ER cisternae during viral infection

The viral membrane protein, J13Lp, is required for the recruitment of membranes into virus factories during ASFV infection (21). In the absence of J13Lp, zipper-like stacks containing membranes and viral proteins accumulate at the cell periphery. Examination of these stacks by electron microscopy (Figure 1, panel a) shows zipper like stacks of electron dense material (arrow) formed from unprocessed ASFV matrix/core shell polyproteins attached to membrane cisternae. The membrane cisternae associated with these zipper-like structures (panel a) are dilated and have a clear luminal space (*). Reduced expression of the ASFV capsid protein also leads to the formation of zipper-like structures (14) and examples are

presented in panels b-f of figure 1. In many cases these zipper-like structures do not appear to be attached to membranes (figure 1 panel b) while in other images the stacks are attached to open membrane cisternae (figure 1 panel c and d). These zipper-like stacks formed in the absence of the major capsid protein have been examined in detail by Andres et al (14). The open membrane cisternae label with antibodies against ER marker proteins, and are in continuity with rough ER, and are therefore derived from the ER. Interestingly, the stacks that lack obvious membrane cisternae are nevertheless thought to be attached to membranes, but these are difficult to resolve unless viewed at high magnification at the edge of the stacks. Examples of high magnification images are presented in panels e and f of figure 1 and show two layers of electron density suggestive of membranes (panel f). It has been proposed that these membranes may represent collapsed ER membrane cisternae (14). Taken together, reduced expression of the major capsid protein produces a mixture of zipper-like stacks where the attached ER cisternae are either open and visible (panels c and d) or may be closely apposed (e and f). In the absence of J13Lp we failed to observe zipper like stacks with the appearance of closely apposed membranes, and all zipper like stacks were associated with open membrane cisternae. The results suggest that close apposition of membranes and consequent loss of cisternal space may require J13Lp, and that J13Lp may therefore be able to collapse ER cisternae.

J13Lp expressed alone in cells causes reorganisation of the ER

The relationship between J13Lp and the ER was examined by expressing J13Lp alone in cells (Figure 2). Approximately 8 to 16 hours after transfection 50 – 60% of cells expressing J13Lp (panel a) or J13Lp fused to monomeric YFP (panel b) exhibited a mesh-like network characteristic of the ER, showing that J13Lp is targeted to the ER. If the protein expression was allowed to continue to 24 hours, this mesh-like network redistributed to disks throughout the cytoplasm in all transfected cells (Figure 2, panels c and d). These disks resembled organised smooth ER (OSER) reported by Snapp et al (19) where whorls of ER membrane appear as disks of fluorescence in the cytosol. To see if the disks induced by J13Lp were derived from the ER, J13Lp-YFP was co-stained with ER marker PDI. Panels c and d show that J13Lp-YFP and PDI were co-localised, and that the remodelled disks of OSER induced by J13Lp were modified from *bona fide* ER. This result was also confirmed by co expressing J13LpYFP and DsRed-ER (supplemental fig 1)

J13Lp generates stacked membranes indicative of OSER with collapse lumen

The OSER generated by J13Lp was examined by transmission electron microscopy (Figure 2 panel e). J13Lp produced stacked membranes arranged in whorls, and less frequently in lamellar arrays (supplemental figure 2 and Fig 3). In many cases the whorls enclosed smaller whorls of stacked cisternae and/or cytoplasmic material including membrane bound vesicles. The ability of J13Lp to induce stacks of ER allowed us to determine if, as seen for membrane cisternae attached to zipper like stacks formed during virus infection, J13Lp would collapse ER cisternae. Careful examination of the images revealed two forms of cisternal stacking. The outermost peripheral stacks of OSER were less compact and a luminal space between membranes was visible (arrows in Fig 2 panel e, see also figure 7c). In addition, the peripheral stacks exhibited fenestrae or gaps (arrow heads). It has been proposed that OSER cisternae act as a template for the formation of successive cisternae (19). The presence of fenestrae in outer stacks suggest that each cisternal layer may begin as series of small cisternae that eventually fuse together, or that peripheral cisternae initially form a sheet with a series of holes that close during maturation.

When OSER was arranged in linear stacks, rather than closed concentric whorls, the ends of the ER stacks were exposed to the cytosol (figure 3, panel a and supplemental data 2). This allowed us to determine the orientation of membranes and inter-cisternal spaces at the

boundary between stacked membranes and the cytosol. A simple structure consisting of six stacks is shown in panels b-f of figure 3. The luminal space within the membrane cisternae at the edge of the stack was clearly visible (asterisk in panels d and cartoon in f), however in the centre of the stack (panel c) the membrane cisternae were collapsed with the upper and lower membranes of the stack touching in several places. The collapsed membrane cisternae were separated by a uniform electron dense layer 8-10nm across, and this inter-cisternal space was continuous with the cytosol (arrow panels d and f). Figure 4 shows membranes analysed following preparation of thin cryosections and immunogold labelling. Panels a and b show membrane stacks labelled with antibodies raised against PDI (small beads) or J13Lp (large beads). The region of interest (panel b) shows that immunolabelling for J13Lp was present throughout the stacks but labelling for PDI was restricted to peripheral stacks. Membranes appear white in thin cryosections. Single electron lucent lines, indicating membranes, were seen at the periphery of the stacks, these single membranes were separated by a cisternal space and labelled for PDI (arrows [i]). In the central stacks the electron lucent lines were closely apposed (arrows [ii]) suggesting loss of ER lumen through collapse of ER cisternae. The inner stacks were separated by a uniform 8nm intercisternal layer of electron dense material labelling for J13Lp which we believe represents antiparallel arrays of J13Lp. Progressive lowering of temperature provides a third means of observing membranes by electron microscopy. Membranes appear as grey/white lines because the counterstain adds electron density to proteins. Again a cisternal space is seen at the periphery of the stacks (long white arrows and white rectangle), and closely apposed grey/white lines representing membranes lacking cisternae, are seen in the centre of the stack (short white arrows). These are again separated by a uniform 8-10nm intercisternal layer of electron dense material.

The organisation of OSER with two different forms comprising open/dilated and collapsed cisternae suggested that these structures may be made by a multistep process involving a stacking event followed by collapse of cisternae in the centre of the stacks. J13Lp has a cytoplasmic domain, and this is consistent with previous reports that homotypic interactions between cytoplasmic domains play a crucial role in ER membrane stacking (19). However, J13Lp also has a predicted 17 amino acid N-terminal domain adjacent to the signal anchor transmembrane domain. We therefore decided to test whether the luminal and cytoplasmic domains played distinct roles in biogenesis of collapsed cisternae.

The cytosolic domain of J13Lp is required for membrane stacking while the luminal domain induces collapse of ER cisternae

To identify the domains of J13Lp responsible for producing OSER, we generated reporter proteins lacking the entire N-terminal luminal domain of J13Lp, or containing a substitution of the cytoplasmic domain with monomeric GFP which is unable to induce OSER (19). Reporters were expressed transiently in cells and panels a and b of figure 5 show that removal of the N-terminal luminal domain of J13Lp had no effect on targeting J13Lp to the ER or formation of OSER. However, when the entire cytoplasmic domain of the protein was replaced by monomeric GFP (J13Lp-□ C-mGFP, panel c) the protein was localized to the ER and nuclear envelope, but failed to induce OSER. These results demonstrated that interactions between cytoplasmic domains of J13Lp were required for OSER formation. Surprisingly, observation of the ultrastructure of OSER formed by the J13Lp lacking the N-terminal luminal domain by electron microscopy (figure 5panel d), revealed that the reorganised membrane stacks of ER consisted of dilated and fenestrated cisternae (asterisk) rather than the collapsed membrane cisternae observed for unmodified J13Lp. These results demonstrated that the luminal domain of J13Lp was responsible for producing collapsed ER membrane cisternae and may also play a role in cisternal continuity. To investigate whether the luminal domain was sufficient to induce cisternal collapse we fused the luminal and transmembrane domains of J13Lp to dimeric YFP (J13Lp-□C EYFP) which undergoes weak

anti-parallel dimerising interactions (22) that can generate OSER (19). Panel e of figure 5 shows that the protein induced OSER, but this time with collapsed internal cisternae. These results suggest that ER collapse was a property of the luminal domain. The luminal domain of J13Lp must either interact with resident ER proteins, or possibly with itself, to zipper together the membranes of the cisternae.

Collapse of ER cisternae requires disulphide bond formation between luminal domains

We hypothesized that cisternal collapse could result from homotypic antiparallel oligomerisation within the lumen of the ER, similar to the mechanism of cisternal stacking induced by cytoplasmic domains. Interestingly, the luminal domain of J13Lp contains the only cysteine residue in the entire protein. Since a luminal cysteine has the potential to form disulphides in the oxidising environment of the ER, and disulphide-bonded dimers of J13Lp have been detected in infected cells (21) we asked whether J13Lp expressed alone forms any disulphides and performed immunoblots of non-reducing SDS-PAGE gels (figure 5 panel f). The unmodified J13Lp migrated as a monomer at 25kDa and a dimer at 50kDa (lane 3). The full length protein fused to YFP also migrated as two bands now shifted to 50 and 100kDa (lane 7), strongly suggestive of monomers and dimers. Next we asked if the N-terminal domain was necessary for the formation of the two species. J13Lp lacking the N-terminal domain (lane 1), or when the luminal cysteine residue was converted to alanine (lane 2) migrated as a single band. Similarly, J13Lp with the cysteine-alanine substitution fused to monomeric YFP (lane 4) migrated as a single band at 50kDa but monomeric YFP could dimerise when fused to wild type J13Lp (*lane 7). Thus J13Lp existed as both monomer and a disulfide bonded dimer in cells. Dimerisation did not require the cytoplasmic domain of J13Lp, since dimers (*) were detected when the entire cytoplasmic domain of J13Lp was replaced by YFP (lane 5), and dimers were not inherently dependent on interactions between cytoplasmic domains since dimers formed when YFP was replaced with monomeric GFP (lane 6).

In light of the results of the immunoblot in figure 5f we asked whether the mutation that prevented disulfide bond formation would also affect the morphology of ER cisternae. The top panels of figure 6 compare stacks generated by J13Lp lacking the luminal cysteine residue (a) with those formed by wild type J13Lp (b) and membrane cisternae failed to collapse when the cysteine was replaced with alanine, suggesting that collapse required disulfide bonding within the lumen of the ER. To rule out the possibility that the cysteine-alanine mutation had inadvertently caused misfolding of J13Lp, DTT was added to cells expressing wild type J13Lp to chemically reduce the lumen of the ER (panel c). DTT appeared to reverse the collapse of ER cisternae. High magnification images show the stacked membranes in more detail where the cysteine mutation (panel d) is compared with unmodified J13Lp (panel e) and cells incubated with DTT (panel f). The cartoons show the orientation of membrane cisternae and inter cisternal spaces. When the cysteine residue was replaced by alanine, or in the presence of DTT, the cisternal space was now visible in the central membrane stacks and had expanded to 10nm. Together the immunoblots, point mutations and results following addition of DTT strongly support the interpretation that homodimerization via the luminal cysteine of J13Lp is necessary for cisternal collapse.

Anti-parallel interactions between cytoplasmic domains on neighbouring ER stacks are required for collapse of ER cisternae

The experiments above have shown that collapse of stacked ER membrane cisternae requires disulphide bond formation between cysteine residues in the luminal domain of J13Lp. It is unlikely that disulfide bonding of adjacent J13Lp proteins on one side of a membrane cisternae (arrowhead in Figure 7 panel a) could achieve this. A zippering between opposing membranes leading to cisternal collapse could, however, occur if

disulfide bonds were orientated across the ER membrane cisternae (arrow in Figure 7, panel a). Orientation across ER cisternae could be achieved through anti-parallel interactions with cytosolic domains of J13Lp on neighbouring stacks above and below the stack in question (asterisk in Figure 7, panel a). This model predicts that cisternal collapse would require stacks of at least three membrane cisternae, and that collapse would not be observed in outer stacks, where interactions only occur on one side of the membrane cisternae. Inspection of several micrographs (figure 7 panels b, c and d) showed that this was the case. Panel b shows a relatively simple structure with 4 membrane stacks. The lumen of the cisternae are clearly visible, except at the bottom of the image (arrow) where the first signs of luminal collapse are evident, even so the lumen in the outer stacks remains dilated. In panel c the OSER is formed from 12 stacks and luminal space is only evident in the outer and inner most cisternae (arrows) and the lumen has collapsed in all the central stacks. Panels d and e show structures formed from two or three membrane cisternae. These images are particularly interesting because when the stacks contained two cisternae (* in panel e) the luminal space was clearly visible, however when there were three stacks the luminal space in the central stack showed evidence of collapse with the upper and lower membranes touching in several places (arrows). Taken together the images suggest that the collapse of membrane cisternae requires a minimum of three stacks, and that membrane cisternae do not collapse when they are on the outside of the OSER. One explanation is that collapse only occurs when the upper and lower surfaces of membrane cisternae are in contact with neighbouring stacks allowing J13Lp to be orientated across membrane cisternae. The ER lumen was observed to collapse in the central stacks of all OSER examined. Collapse was observed in simple structures of 3, 4 or 6 stacks and in complexes made from 12 or more stacks (Figures 3 and 7). If we assume that the number of stacks is proportional to the level of J13Lp expression, then ER collapse occurs over a wide range of J13Lp concentrations. Our observation that collapse occurs in simple structures argues against induction of ER collapse by protein over-expression.

Discussion

This study shows that viral envelope protein J13Lp can collapse ER cisternae. J13Lp is targeted to the ER by an N-terminal hydrophobic membrane anchor. The C-terminus contains the bulk of the protein and is exposed to the cytosol, while a short 17 amino acid N-terminal domain with a single cysteine residue enters the lumen of the ER. The cytoplasmic domain of J13Lp was able to maintain antiparallel interactions with cytoplasmic domains exposed on neighbouring ER stacks and, in common with host ER proteins, these induced stacks of OSER. When J13Lp was expressed alone in cells cisternal collapse was dependent on the cysteine residue in the N-terminal domain, and was reversed when cells were chemically reduced by DTT, showing that disulphide bond formation was necessary for ER collapse. While there are examples of cisternal ER collapse in the literature, such as the 'thin' subdomains of cortical ER that exclude luminal proteins during STIM1-activated calcium entry (23), the mechanism or effectors of the collapse remain unknown. Our study provides, for the first time, a mechanism of cisternal collapse where orientation of an ER membrane protein across ER cisternae allows disulphide bonds to collapse the ER lumen. We propose a model (Figure 8a) where J13Lp is recruited to the ER and antiparallel interactions between cytoplasmic domains lead to accumulation of J13Lp at the interface of ER cisternae (i). At this point disulphide bonds form between parallel N-terminal domains (panel b) but cisternae remain open. Continued delivery of J13Lp increases interactions between neighbouring ER cisternae and the generation of OSER. When stacks contain three or more cisternae, interactions between cytoplasmic domains can re-orientate N-terminal domains across the ER lumen (ii) and disulphide bonds between antiparallel N-terminal domains (panel b) lead to collapse of the cisternae (iii). Further delivery of J13Lp leads to increased stacking of ER cisternae and internal stacks again collapse when N-terminal

domains are re-orientated across the ER lumen (iv). This model is consistent with electron micrographs (figure 8c) where cisternal collapse is only observed when cisternae are sandwiched between upper and lower stacks. When cisternae are exposed to the cytosol either above or below central stacks or at the edges of lamella stacks (v) disulphide bonds are unable to collapse cisternae. The experiments do not provide an explanation for why J13Lp is unable to induce collapse of single cisternae. It is possible that disulphide bonds form preferentially between parallel N-terminal domains and that the force needed to convert these to an anti-parallel conformation can only be generated by anti-parallel interactions between cytoplasmic domains on neighbouring stacks.

A mechanism for the collapse of ER cisternae attached to zipper-like structures formed during viral infection is provided in Figure 8d. In the absence of the major capsid protein, structural proteins, such as the matrix core shell are recruited to the cytoplasmic face of the ER (14). They are unable to assemble correctly and produce zipper like arrays of electron density associated with ER cisternae (i). J13Lp is targeted to these ER cisternae by an N-terminal hydrophobic membrane anchor (ii). The short N-terminal domain containing the cysteine residue enters the ER allowing disulphide bonds between J13Lp N-terminal domains orientated across the ER to cisternal collapse (iii-v). While we provide a mechanism for cisternal collapse, the mechanisms that orientate J13Lp across ER cisternae during viral infection are not known. A clue may come from the work by Andres et al (14) who show that the major capsid protein and the inner matrix/core shell of ASFV form protein coats on opposite sides of ER membrane cisternae. The mechanisms that drive asymmetric assembly of capsid and matrix proteins across the ER (indicated by the open rectangle in (iv-vii) in figure 8d) could allow structural proteins to bind the cytoplasmic tail of J13Lp and orientate J13Lp across ER cisternae.

Cells infected with modified vaccinia virus unable to express the major core protein, A10L/P4a, also generate zippered ER stacks (12,13). The stacks are composed of linear arrays of viral protein with closely apposed ER cisternae. The interior stacks exclude host ER proteins suggesting collapse of ER cisternae. The similarities between these stacks and those produced by ASFV, suggests that a similar mechanism for generation of zipper-like stacks of ER operates for vaccinia virus lacking A10L. Recombinant vaccinia viruses engineered to express J13Lp show defects in generation of spherical immature viruses (24) from crescents, raising the possibility that J13Lp interferes with a vaccinia counterpart.

The formation of stacks of OSER by J13Lp allowed us to observe collapse of ER cisternae. It is important to note that the induction of OSER by J13Lp does not imply that formation of OSER plays a role in ASFV assembly or envelopment. While zipper-like stacks of ER associated with structural proteins are seen in cells infected with NCLD viruses, their role in virus life cycle remains to be resolved. Studies on vaccinia virus lacking A10L gene expression demonstrate partial rescue of assembly when expression of A10L/P4a is restored (12) suggesting that zippers may represent frozen assembly intermediates (12,13). For ASFV linear zippers containing matrix/core shell proteins associate with ER cisternae in the absence of the major capsid protein (14). The major structural proteins of ASFV are recruited onto the cytoplasmic face of the ER at early stages in assembly (25-28). It is possible that in the absence of the major capsid protein, the matrix/core shell proteins assemble into non-productive complexes on ER cisternae and that the zipper-like stacks represent a storage site for proteins unable to assemble into viruses. These linear structures are not, however, dead-end complexes because they transform into angular structures indicative of assembly intermediates when expression of the major capsid protein is restored (11). Zipper-like structures associated with ER membranes are also observed in cells infected with (+) strand RNA viruses (29). When, for example, the 1a and 2a^{pol} replicase proteins of brome mosaic virus are present at the correct ratio, they assemble into a complex

on the cytosolic face of the ER that generates spherical invaginations into the ER lumen. If the ratio is perturbed the proteins induce stacks of OSER (30). This makes it possible that the zipper-like stacks associated with ER membrane cisternae seen during viral infection could also reflect a generalised cellular response to a build up of excess viral protein on the cytoplasmic face of the ER. This leads to stacking of ER cisternae similar to that seen for formation of OSER by increased levels of host ER proteins, but in the case of ASFV, the viral envelope protein J13Lp can cause collapse of ER cisternae.

Materials and Methods

Antibodies and reagents

The rabbit antibody recognising J13Lp and mouse monoclonal antibody recognizing p73 have been described previously (25). The monoclonal antibody against PDI (1D3) was prepared from cell supernatants obtained from the hybridoma cell line available from ATCC. Rabbit anti-GFP was purchased from Abcam. Alexa fluor conjugated secondary antibodies were purchased from Molecular Probes, The Netherlands. Other reagents were purchased from Sigma UK unless otherwise stated.

Plasmids—The open reading frame for E183L (BA71 strain of ASFV) was inserted into the pcDNAtm3.1/V5-His TOPO TA vector (Invitrogen) to give a C-terminal V5 tag (J13LV5). The pEYFPC1 vector (CLONTECH laboratories, Inc) provided a C-terminal YFP. This was modified to give the monomeric form of YFP (J13LmYFP) by site-directed mutation of leucine 221 to lysine using the Quick-change kit from Stratagene (23). The N terminal deletion to J13Lp (J13LΔN) excised the first 26 amino acids and the C terminal deletions (J13LΔC) removed the last 129 amino acids. Either EYFP or mGFP were added to J13LΔC to generate J13LΔC-EYFP or J13LΔC-mGFP. The cysteine residue at position 17 was mutated to an alanine using the Quick-change kit (Stratagene). DsRed-ER was purchased from Clontech.

Viruses and cells—Vero (ECACC 84113001) cells were cultured and infected with the BA71 strain of ASFV as described previously (12,13). Infections with the ASFV IPTG-inducible viruses vA72 and vE183Li were carried out as described previously (14,21).

Protein expression and immunofluorescence—Cells were grown to 70% confluence and transfected using Transfast (Promega, UK). Immunofluorescence images were obtained by using either a Leica SP2 confocal laser scanning head attached to a DMRXA2 microscope or by digital deconvolution of 0.2 μm optical sections using a Nikon E800 microscope and Improvision Openlab 2.1.3 or 3.1 software. For Western blot. Vero cells were transfected with J13Lp constructs under the control of a T7 promoter for 16 hours. Protein expression was induced by infection with vaccinia virus strain MVA-T7. Cells were incubated in PBS containing 20mM N-ethyl maleimide (NEM) for 10 minutes on ice prior to lysis in 50mM Hepes pH 6.8, containing 200mM NaCl, 20mM NEM, 2% CHAPS and 1μg/ml leupeptin, pepstatin, antipain and chymostatin. Debris was removed by centrifuging for 15 minutes at 16000g and diluted in non-reducing sample preparation buffer (250mM Tris pH 6.8, SDS 10%, Glycerol 50%, 50mg/ml bromophenol blue). Proteins were resolved by SDS-PAGE followed by Western blot analysis, using rabbit anti GFP (Abcam) or mouse anti-V5 (Invitrogen) antibodies.

Electron microscopy

Conventional Epon section: Vero cells were infected with 10 PFU per cell of ASFV recombinants vA72 or vE183Li and fixed at 24 hpi with 2 % glutaraldehyde in 200 mM HEPES (pH 7.4) for 1 h at room temperature. Post-fixation was carried out with 1% OsO₄

and 1.5% $K_3Fe(CN)_6$ in H_2O at $4^\circ C$ for 30 min. Samples were dehydrated with acetone and embedded in Epon according to standard procedures. Specimens were examined with a Jeol 1010 microscope. For examination of OSER cells expressing J13Lp were grown on 13 mm diameter Nunc Thermanox coverslips (VWR International) to 80% - 90% confluency. Cells were fixed in 2% glutaraldehyde solution followed by aqueous 2% osmium tetroxide, at room temperature. After dehydration in an ethanol series the cells were washed with propylene oxide before being infiltrated with epoxy resin (Agar Scientific). After 18 hours polymerization at $60^\circ C$ the Thermanox coverslips were peeled away leaving the cell monolayer inside the resin block. Sixty micrometer sections were cut and stained using an EMStain (Leica Microsystems) before being imaged at 100kV in an FEI Tecnai 12 TEM with TVIPS F214 digital camera.

Immunogold labeling of thin cryosections: Cell monolayers were fixed in 2% paraformaldehyde/0.05% glutaraldehyde at room temperature for 2 hours. The fixative was replaced with warm 2% gelatin solution. Cells were scraped into suspension, suspended in warm 12% gelatin and placed on ice to harden. $1mm^2$ blocks were infiltrated overnight in 2.3M sucrose, affixed to cryopins and frozen in liquid nitrogen. 70nm sections were cut at $-100^\circ C$, collected on Formvar coated grids, blocked for 30 minutes in 0.5% bovine serum albumin in PBS, and labeled overnight with the rabbit antibody recognising J13Lp or the ID3 monoclonal antibody against PDI. Antibodies were visualised using appropriate secondary antibodies conjugated to 10nm gold or 5nm gold beads (Amersham Biosciences). The samples were washed in PBS and briefly fixed in phosphate buffered 2% glutaraldehyde, then grids were held in a matrix of 2% methyl cellulose containing 3% uranyl acetate and allowed to dry. Samples were imaged at 100kV in an FEI Tecnai 12 TEM with TVIPS F214 camera.

Progressive lowering of temperature: Cell monolayers on 3mm sapphire discs were fixed for 60 minutes in 2% paraformaldehyde and 0.05% glutaraldehyde and dehydrated at progressively lower temperatures ($+4^\circ C$ to $-50^\circ C$). Samples were infiltrated with Lowicryl HM20 resin at $-50^\circ C$ overnight. Following UV polymerisation over 48 hours at $-50^\circ C$ the temperature was raised to $20^\circ C$ and continued for a further 24 hours. Seventy micron sections cut from polymerised blocks were stained with uranyl acetate and lead citrate before being imaged in the TEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the strategic Biotechnology and Biology Research Council core grant to IAH Pirbright laboratories and grants from the Spanish Ministerio de Ciencia e Innovación (AGL2010-22229-C03-02) and by an institutional grant from Fundación Ramón Areces to the CBMSO. Erik Snapp was an Ellison Medical Foundation New Scholar in Aging and is supported by NIA 1R21AG032544-01, NIGMS 1R01GM086530-01 and NIDDK 2P01DK041918-16. We thank G. Andrés for advice on electron micrographs, E.M. Forero for technical assistance and J. Bashiruddin for help with figures.

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Figure 1. Expression of J13Lp is necessary for collapse of endoplasmic reticulum membrane cisternae during viral infection

Panel a. Cells were infected with recombinant ASFV (vE183Li) where expression of the J13Lp reading frame (E183L) is under control of the *E. coli lac* operator/repressor. The images were obtained from cells incubated in the absence of IPTG to repress expression of J13Lp. Zipper like structures are separated by an electron dense layer (arrows) and flanked by cisternal membrane profiles. Open endoplasmic reticulum cisternae are indicated by asterisks. Panels b-f. Vero cells were infected with recombinant ASFV (vA72) where the gene for the major capsid protein is under control of the *E. coli lac* operator/repressor. The images were obtained from cells incubated in the absence of IPTG to repress expression of the capsid protein. Panels b- d. Zipper-like structures of electron dense material formed from matrix and core shell proteins are flanked by membrane profiles that show closely apposed membranes (b) or open cisternae (c-d). The high magnification image in panels e and f show double membrane profiles indicated by arrows. The diagrams to the right of the electron micrographs show the orientation of endoplasmic reticulum cisternae. Open cisternae are indicated by white blocks, closed cisternae as three parallel lines. Grey blocks indicate the protein coat between cisternae. Images show Ultrathin Epon sections of Vero cells 24 hours after infection with virus.

Figure 2. J13Lp causes reorganization of the endoplasmic reticulum

Panels a and b. Vero cells expressing J13Lp alone (a) or J13Lp fused to monomeric YFP (b) were fixed and permeabilized 16 hours following transfection. The distribution of J13Lp was determined by immunofluorescence using secondary antibodies conjugated to Alexa fluor 594 (a) or from the natural fluorescence of YFP (pseudo coloured green) (b). Panels c and d. Vero cells expressing J13LpYFP were fixed and permeabilized 24 hours following transfection and labeled with antibodies specific for protein disulphide isomerase (PDI). Protein distribution was determined from the natural fluorescence of YFP pseudo coloured green (c) and secondary antibodies conjugated to Alexa fluor 594 (PDI red) (d). Panel e, Vero cells expressing J13Lp were examined by transmission electron microscopy of Ultra

thin Epon sections. A low power view shows concentric whorls of organized ER. The outermost stacks show fenestrae (arrow heads) and have open cisternae (arrows), while the inner stacks show increased electron density.

Figure 3. J13Lp generates organized smooth endoplasmic reticulum with collapsed cisternae
 Transmission electron micrographs were taken at the boundary between stacked cisternae and the cytosol. Panel a. Boundary between complex lamella array and cytosol showing outermost stacks with fenestrae and open cisternae (see also supplemental figure 2). As seen for concentric whorls the inner-most stacks show increased electron density. Panel b. Boundary between the cytosol and simple lamella array composed of six stacks. Regions of interest are indicated and shown at higher magnification with associated annotation in panels c-f. Panels d and f. Black asterisks show open membrane cisternae at the edge of the stack and the arrow indicates electron dense inter-cisternal space connected to the cytoplasm. Panels c and e show collapse of ER cisternae in internal stacks. White asterisks in panel c

show collapsed membrane cisternae. The diagram to the left in e shows orientation of cytoplasm, ER membranes and ER lumen and electron dense intercisternal space.

Figure 4. Analysis of reorganized endoplasmic reticulum by immunogold electron microscopy and progressive lowering of temperature

Panels a and b. Thin cryosections prepared from Vero cells expressing J13Lp were incubated with antibodies specific for J13Lp and protein disulphide isomerase (PDI). J13Lp was visualized by secondary antibodies coupled to 10nm gold beads. PDI was visualized by secondary antibodies coupled to 5nm gold beads. PDI labeling (black PDI arrows) was present in fenestrae with open cisternae located at the periphery of the stack and absent from central stacks. J13Lp was present throughout the stack. Arrows (i) indicate membranes at the edge of the stack separated by a cisternal space. Arrows (ii) indicate closely apposed membranes that lack cisternal space in the interior of the stack. Panels c and d. Vero cells expressing J13Lp were processed by progressive lowering of temperature followed by negative staining. The region of interest indicated in the low power image in panel c is presented at high magnification in panel d. Long arrows indicate membranes at the edge of the stack separated by a cisternal space (white rectangle). Short arrows indicate closely apposed membranes that lack cisternal space in the interior of the stack.

Figure 5. Role played by cytosolic and luminal domains of J13Lp in membrane stacking and cisternal collapse

Vero cells expressing unmodified J13Lp (panel a), or J13Lp lacking the N-terminal luminal domain (J13Lp-ΔN, panel b) generate OSER. Panel c. Replacement of the cytosolic domain of J13Lp with monomeric GFP (J13Lp-ΔC-mGFP) prevents formation of OSER. Panel d. Electron micrographs of ultra thin Epon sections show that J13Lp lacking the N-terminal luminal domain (J13Lp-ΔN) generate OSER with open cisternae (asterisk) separated by an electron dense intercisternal layer (arrows). Panel e. Electron micrographs of ultrathin Epon sections show that replacement of the cytosolic domain of J13Lp with dimerising EYFP (J13Lp-ΔC-EYFP) generates OSER where cisternae collapse in central stacks. Panel f. Analysis of J13Lp disulphide bond formation. The lane numbers cross reference to numbered stick diagrams showing orientation of cytosolic (C), membrane anchor (M) and luminal (N) domains of J13Lp fused to dimerising (EYFP) or monomeric (mYFP)

fluorescent protein. N* indicates removal of cysteine residue. Proteins expressed in Vero cells were separated by non reducing PAGE and analysed by Western blot. (*) indicates proteins able to generate disulphide bonds.

Figure 6. Collapse of ER cisternae by J13Lp requires disulphide bond formation

Panels a-c compare micrographs of ultrathin Epon sections of Vero cells expressing J13Lp carrying the cysteine to alanine substitution (panel a) with wild type J13Lp (panel b) and wild type J13Lp expressed in cells reduced by addition of 5mM DTT for 2 hours, prior to fixation and processing (panel c). Panels d-f, compare high power images. The ER lumen (-*) is visible following replacement of the cysteine residue (d), or reduction of cells with DTT (f), but not visible for unmodified J13Lp (e). The cartoons below the images show the orientation of electron dense intercisternal space (grey box), open ER cisternae (white box) and collapsed ER cisternae (three parallel lines). ER cisternae do not collapse when the cysteine in the N-terminal domain of J13L is converted to alanine or when cells are reduced with DTT.

Figure 7. Collapse of ER cisternae by J13Lp requires anti-parallel interactions between cytoplasmic domains on neighbouring stacks
 Panel a. Diagram showing possible orientation of J13Lp and disulphide bonds in OSER. Asterisks indicate antiparallel interactions between cytoplasmic domains on neighbouring stacks. Open arrowheads show disulphide bonds between adjacent proteins. These do not extend across the ER lumen. The block arrow shows that disulphide bonds maintained across ER cisternae could lead to ER collapse and loss of luminal volume. Panel b. Simple stacked ER where cisternal collapse is evident in internal stacks (arrow). Panel c. Complex stacked ER where all internal stacks show collapsed cisternae while fenestrae at periphery of stacks show open cisternae (arrows). Panel d and e. Comparison of stacks of two or three

cisternae. High power images (e) show that the luminal space is present throughout stacks of two cisternae (*). Collapse of the inner cisternae is evident (arrows) when there are three stacks.

Figure 8. Models for the collapse of ER cisternae induced by J13Lp

Panel a. Collapse of ER cisternae during the formation of OSER. J13Lp (zig zag) is targeted to ER cisternae by an N-terminal hydrophobic membrane anchor (i) and the short N-terminal domain enters the ER. Disulphide bond formation occurs between N-terminal cysteine residues on adjacent proteins, but does not result in ER collapse. Increased delivery of J13Lp to the ER results in antiparallel interactions between cytoplasmic domains of J13Lp on neighbouring ER cisternae that induce formation of OSER (ii). The antiparallel interactions orientate J13Lp across ER cisternae in central stacks and disulphide bond formation results in collapse of ER (iii-iv and insert b). ER stacks that border the cytosol are unable to reorientate disulphide bonds across the ER lumen and have ER have open cisternae (v). **Panel b.** Parallel and antiparallel orientation of N-terminal domains in lumen

of the ER. N-terminal domains have parallel orientation in open cisternae and antiparallel orientation in collapsed cisternae. **Panel c.** Electron micrograph of a simple stack of ER exposed to the cytosol providing reference for the diagram in panel a. **Panel d.** Collapse of ER attached to zipper-like structures. Structural proteins, such as the matrix/core shell (grey box) are recruited to the cytoplasmic face of the ER (white box). They are unable to assemble correctly and produce zipper like arrays of electron density associated with ER cisternae (i). J13Lp is targeted to ER cisternae by an N-terminal hydrophobic membrane anchor (ii) and is orientated across ER cisternae (iii-iv). Formation of disulphide bonds between J13Lp N-terminal domains leads to cisternal collapse (open arrow iv-v). The mechanisms that drive asymmetric assembly of capsid and matrix proteins across the ER (indicated by the open rectangle in (iv-vii) may orientate J13Lp across the ER.