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Review

African swine fever virus morphogenesis

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ABSTRACT

This review summarizes recent structural and molecular biology studies related to the morphogenesis of African swine fever virus (ASFV). ASFV possesses icosahedral morphology and is constituted by four concentric layers: the central nucleoid, the core shell, the inner envelope and the icosahedral capsid. The extracellular virus acquires an external envelope by budding through the plasma membrane. The genes coding for 19 of the 54 structural proteins of the ASFV particle are known and the localization within the virion of 18 of these components has been identified. ASFV morphogenesis occurs in specialized areas in the cytoplasm, named viral factories, which are proximal to the microtubule organization center near the nucleus. Investigations of the different steps of morphogenesis by immunocytochemical and electron microscopy techniques, as well as molecular biology and biochemical studies, have shed light on the formation of the different domains of the virus particle, including the recognition of endoplasmic reticulum membranes as the precursors of the virus inner envelope, the progressive formation of the capsid on the convex face of the inner envelope and the simultaneous assembly of the core shell on the concave side of the envelope, with the pivotal contribution of the virus polyproteins and their proteolytic processing by the virus protease for the development of this latter domain. The use of ASFV inducible recombinants as a tool for the study of the individual function of structural and nonstructural proteins has been determinant to understand their role in virus assembly and has provided new insights into the morphogenetic process.

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Abbreviations: ASFV, African swine fever virus; ER, endoplasmic reticulum; NCLDV, nucleo-cytoplasmic large DNA viruses; OSER, organized smooth endoplasmic reticulum.

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1. Introduction

African swine fever virus (ASFV) is a large, enveloped virus with icosahedral morphology and a double-stranded DNA genome of 170 to 190 kbp, containing ends with terminal inverted repeats and closed by hairpin loops (Salas, 1999). ASFV replication cycle is mainly cytoplasmic, but an early stage of replication in the nucleus has been described (García-Beato et al., 1992; Rojo et al., 1999). The role of the initial nuclear stage is not well understood at present, but the virus profoundly affects nuclear organization, including localized disassembly of the lamina network near the sites of nuclear DNA replication, and redistribution of several nuclear proteins, as well as dephosphorylation and subsequent degradation of RNA polymerase II (Ballester et al., 2012). The synthesis in the nucleus of small fragments of viral DNA that can be precursors of the larger cytoplasmic sequences and the existence of concatemer intermediates of full-length mature genomes have also been described (Rojo et al., 1999). Transcription of viral genes is strongly regulated and the synthesis of clearly recognizable classes of mRNAs, including immediate-early, early, intermediate and late transcripts, has been demonstrated (Almazan et al., 1992; Rodríguez et al., 1996b). Virus morphogenesis takes place exclusively in the cytoplasmic factories where the main late phase of DNA replication also occurs. Because of these replication characteristics and on the basis of comparative genome analysis, ASFV has been included within the proposed monophyletic group of nucleocytoplasmic large DNA viruses (NCLDV) consisting of the virus families *Poxviridae*, *Iridoviridae*, *Asfarviridae*, *Phycodnaviridae*, *Mimiviridae*, *Ascoviridae* and *Marseilleviridae*, infecting a broad variety of eukaryotes from unicellular marine protists to humans (Iyer et al., 2001). All the members of this group, except the poxviruses, possess icosahedral morphology, and all of them, including the poxviruses, conserve the major capsid protein, which in the case of poxviruses corresponds to the trimeric D13 protein that forms the transient scaffold covering the membrane crescent precursors (Szajner et al., 2005).

This review focuses on recent findings that demonstrate the complexity of ASFV morphogenesis, from the acquisition of viral precursor membranes to genome encapsidation and formation of intracellular and extracellular mature virions, and describes the development of new tools for the study of the role of individual structural and nonstructural proteins in the assembly process.

2. Structure and composition of African swine fever virus particle

The ASFV particle has a icosahedral morphology with an average diameter of 200 nm and is composed by several concentric domains: an internal core formed by the central genome-containing nucleoid coated by a thick protein layer designated core shell, an inner lipid envelope surrounding the core and the capsid, which is the outermost layer of the intracellular virions (Fig. 1A) (Carrascosa et al., 1984; Andrés et al., 1997). The extracellular virions possess an external envelope acquired by budding from the plasma membrane (Fig. 1B) (Breese and DeBoer, 1966).

Two-dimensional analysis of purified extracellular virus has identified 54 structural proteins with molecular weights ranging from 10,000 to 150,000 (Esteves et al., 1986). The virions also contain the transcriptional machinery for the synthesis, capping and polyadenylation of early RNA (Salas, 1999). The presence of other activities, like a protein kinase encoded by gene R298L (Baylis et al., 1993), two nucleoside triphosphate phosphohydrolases, an acid phosphatase, and a deoxyribonuclease, has also been described in the virus particles (Salas, 1999). Presently, 19 genes are known to code for structural proteins (Yanez et al., 1995; Dixon et al., 2012) and the localization of some of these proteins in the different domains of the virus particle has been identified, as described below and summarized in Fig. 1C.

2.1. The outer envelope

The morphology of the external envelope of extracellular virus is similar to the “unit membrane” characteristic of the plasma membrane, in accordance with the budding process for virus exit from the cell (Breese and DeBoer, 1966). The virus attachment protein p12 has been reported to localize into the outer envelope in immunoelectron microscopy studies of purified extracellular virus (Carrascosa et al., 1993). Another virus protein that putatively localizes at the outer envelope is the virus homologue of cellular CD2 (pE402R) (Dixon et al., 2012), which mediates the hemadsorption of infected cells (Rodríguez et al., 1993). Interestingly, a cellular protein designated p24 (Sanz et al., 1985) is present at the plasma membrane and is incorporated into the virus particles (see Section 2.3).

2.2. The capsid

The structure of the capsid has been studied in detail by different electron microscopic techniques, revealing that it is formed by about 2000 capsomers with the appearance of hexagonal prisms with a length of 13 nm, a wide of 5–6 nm and a central hole. The intercapsomer distance is of 7.4 nm (Carrascosa et al., 1984). Protein p72, encoded by gene B646L, is the major component of capsomers (García-Escudero et al., 1998) and accounts for about one third of the protein mass of the virus particle.

As indicated in Section 1, protein p72 presents similarity with the major capsid protein of iridoviruses, phycodnaviruses and mimiviruses, which also have icosahedral capsids surrounding an inner envelope (Iyer et al., 2001). The poxvirus protein D13 shares a domain conserved in all of these proteins, but lacks the C-terminal jelly roll domain characteristic of the capsid proteins of icosahedral NCLDVs and many other DNA and RNA viruses (Rossmann and Johnson, 1989).

Another component of the capsid is protein pE120R involved in the transport of the mature ASFV particles from the factory to the plasma membrane for exit from the cell (Andrés et al., 2001b). The structural membrane protein pB438L is also a likely component of the capsid, possibly forming part of the vertices (Epifano et al., 2006b).

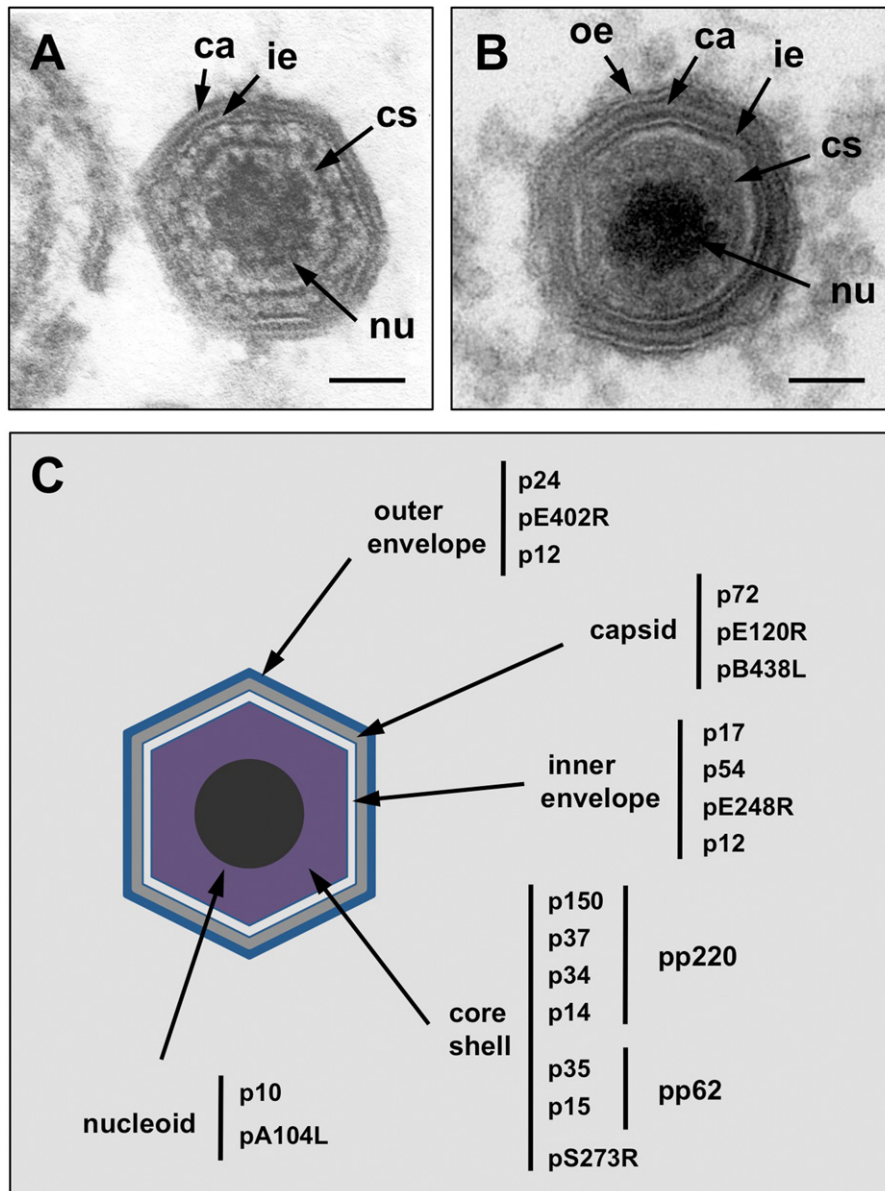


Fig. 1. Structure and protein composition of ASFV. Electron micrographs of an intracellular full ASFV particle (A) and an extracellular mature ASFV virion (B). The different structural domains are indicated. Outer envelope (oe), capsid (ca), inner envelope (ie), core shell (cs) and nucleoid (nu). Bars, 50 nm. Panel C illustrates the localization of different ASFV structural proteins inside ASFV as deduced from immunoelectron microscopy data and/or from EM studies with inducible ASFV recombinants.

2.3. The inner envelope

The inner envelope of the mature virus particle looks like a single lipid membrane when visualized by conventional EM methods (Carrascosa et al., 1984; Andrés et al., 1998) and modern cryotechniques (Hawes et al., 2008). Morphological and immunocytochemical evidence shows that the inner envelope is derived from the endoplasmic reticulum (Andrés et al., 1998; Cobbold et al., 1996; Rouiller et al., 1998) through a mechanism poorly understood at present (see below). The membrane proteins p54 (Rodríguez et al., 2004), p17 (Suarez et al., 2010a) and pE248R (Rodríguez et al., 2009) are constituents of this domain.

Interestingly, the virus attachment protein p12, which was initially located at the outer virus envelope (Carrascosa et al., 1993), is also a component of the inner envelope. Immunofluorescence microscopy of ASFV-infected cells at late times post-infection indicates that p12 localizes at the perinuclear virus factories as well as into virus particles spread throughout the cytoplasm and the cell

surface (Fig. 2A and D). Such subcellular distribution is nearly identical to that observed for the inner envelope protein p17 (Fig. 2B and C), but clearly different to that of the cellular protein p24 (Fig. 2E and F), which is localized at the plasma membrane. Furthermore, immunoelectron microscopy on thawed cryosections of infected cells indicates that p12 is associated to the viral membrane precursors (Fig. 2G), assembling particles (Fig. 2G) and intracellular mature viruses (Fig. 2H). At variance, protein p24 is detected at the plasma membrane and seems to be concentrated at the areas where ASFV particles bud (Fig. 2I), which suggests a selective recruitment of this cellular protein by the budding particle. Accordingly, p24 is detected at the outer envelope of the extracellular virions (Fig. 2J) after pre-embedding immunolabeling. Altogether, these results indicate that transmembrane protein p12 is located at the inner envelope of the ASFV structure where it could play a morphogenetic role. On the other hand, the specific recruitment of cellular protein p24 is consistent with a putative role in the budding event that deserves further studies.

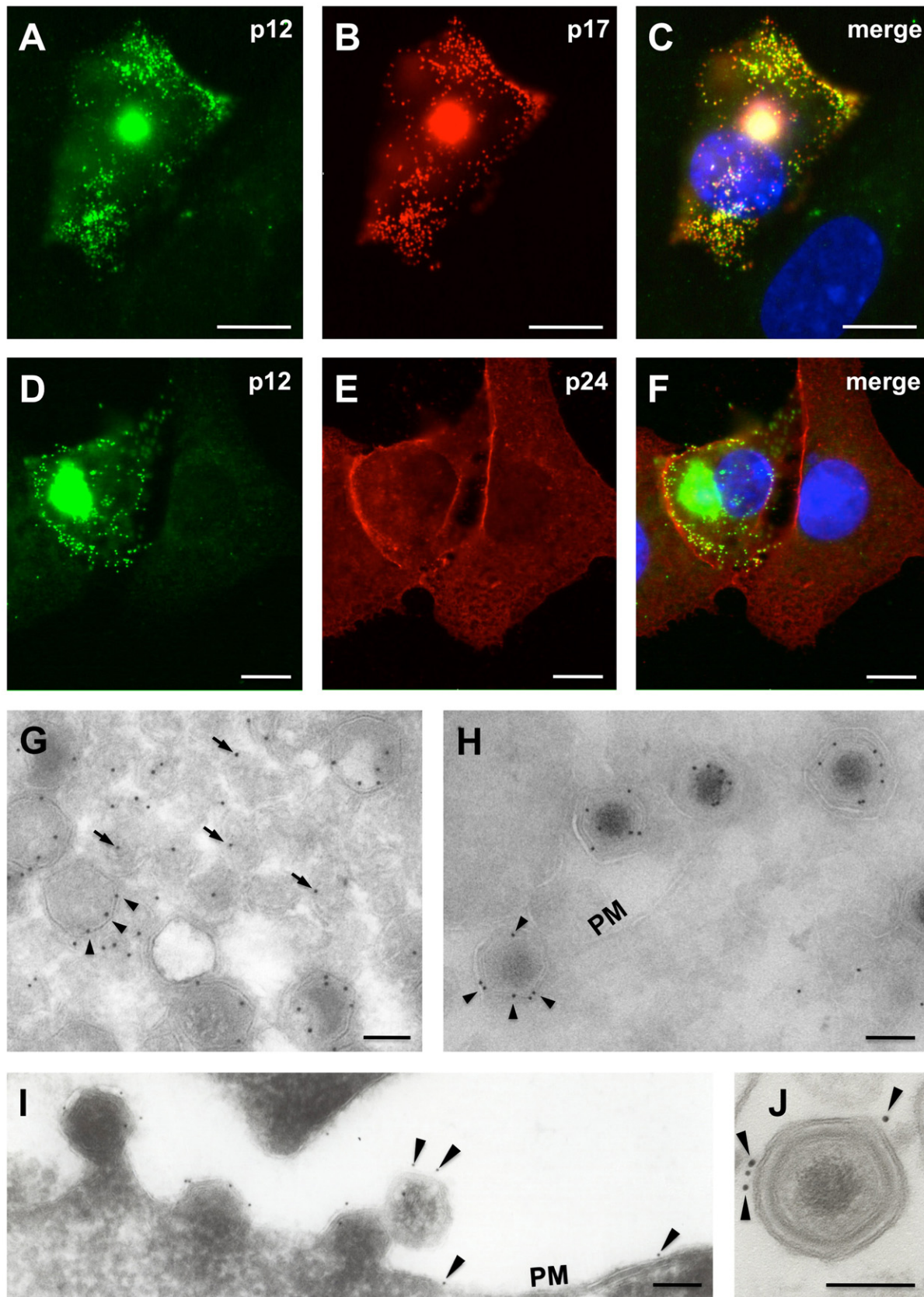


Fig. 2. Subcellular and subviral localization of ASFV structural membrane protein p12 and cellular membrane protein p24. Immunofluorescence microscopy of ASFV protein p12 and cellular protein p24 in ASFV-infected cells. ASFV-infected cells were immunolabeled at 14 h post-infection with antibodies against viral proteins p12 (panel A) and p17 (panel B) or with antibodies against viral protein p12 (panel D) and cellular protein p24 (panel E). As shown, antibodies against p12 label the perinuclear virus factories and virus particles spread throughout the cytoplasm and at the cell surface. This pattern is nearly identical to that of viral protein p17 (merge at panel C), which localizes at the inner viral envelope. On the contrary, cellular protein p24 localizes essentially at the cell surface where eventually colocalizes with p12 at budding virus particles (merge at panel F). Bars, 10 μ m. Immunoelectron microscopy of proteins p12 and p24 in ASFV-infected cells. Immunolabeling with protein A-gold conjugates was performed

2.4. The core shell

The core shell, a thick protein layer of about 30 nm, was defined as an independent domain of the virus core encompassing the central nucleoid (Andres et al., 1997, 2002a). This domain is mainly constituted by the processing products of polyproteins pp220 and pp62. This processing is catalyzed by a cysteine protease encoded by gene S273R that belongs to the SUMO-1-specific and related protease family, including the adenovirus and vaccinia virus I7 proteases (Andres et al., 2001a; Li and Hochstrasser, 1999). Protein pS273R, which is also a component of the core shell, has been shown to faithfully process the virus polyproteins in assays using transfected cells, extracts from transfected cells or the recombinant purified pS273R enzyme (Andres et al., 2001a; Rubio et al., 2003). Polyprotein processing occurs by an ordered sequence of proteolytic cleavages at Gly-Gly-X motifs, giving rise to six main components of the core shell: proteins p150, p37, p34 and p14, derived from polyprotein pp220 and proteins p35 and p15, products of pp62 (Andres et al., 1997, 2002a; Simon-Mateo et al., 1993, 1997). All these products are present in equimolecular amounts into the mature virus particle and collectively constitute 32% of the total mass of the virion. This mechanism of gene expression is unique among DNA viruses and might reflect the need to maintain a certain ordered arrangement of such structural proteins in the mature virions (see below).

2.5. The nucleoid

The nucleoid is an electron-dense structure of 80 nm containing the viral genome (Andres et al., 1997) and nucleoproteins such as the DNA-binding protein p10 (Andres et al., 2002b; Munoz et al., 1993) and protein pA104R (Andres et al., 2002b; Borca et al., 1996), which is similar to the histone-like proteins of bacteria. The nucleoid is also supposed to contain the transcriptional machinery for the synthesis and modification of early RNAs that includes the multi-subunit RNA polymerase, poly A polymerase, capping enzymes and early transcription factors (Salas, 1999).

3. ASFV morphogenesis

The assembly process of the ASFV particles takes place in specialized areas of the cytoplasm designated viral factories, located close to the cell nucleus and the microtubule organizing center (MTOC) (Heath et al., 2001). When the morphogenesis progresses, a gradual increase in the size of the factory is observed, occupying regions increasingly larger of the cytoplasm (Brookes et al., 1996). These areas are essentially devoid of cellular organelles, but are surrounded by ER membranes and enwrapped in a vimentin cage (Andres et al., 1998; Heath et al., 2001). Also an accumulation of mitochondria is observed in their periphery (Rojo et al., 1998). Viral factories resemble aggresomes, perinuclear inclusions of cellular protein aggregates that are close to the MTOC. As ASFV factories, aggresomes recruit chaperones and mitochondria and induce collapse of vimentin into distinct cage structures (Heath et al., 2001).

3.1. First steps in morphogenesis: formation of the inner envelope

The first morphological indication of viral assembly is the accumulation within the factory of viral membranes, which usually appear as small curved and open structures, and are the precursors of the inner envelope of the viral particle. The precursor

membranes can also be seen in parallel arrangements separated by electron-dense material and occasionally found between ER cisternae (Andres et al., 1998). In addition, some images were suggestive of the presence of two tightly apposed membranes within the membrane precursors (Andres et al., 1998) even though the resulting inner viral envelope looks as a unit membrane in the virus particle (Carrascosa et al., 1984; Andres et al., 1998; Hawes et al., 2008). Membranes with attached ribosomes in continuity with assembling virions are occasionally observed in the viral factory. Biochemical evidence showing the association of the capsid protein p72 with ER membranes has also been presented (Cobbold et al., 1996; Cobbold and Wileman, 1998).

Another type of structure can be observed both inside the assembly sites and outside but close to the viral factory. These aberrant viral forms that have been designated zipper-like structures (Fig. 3B, D and E), and will be further described below, consist of a elongated, 30-nm thick, protein domain similar to the core shell of the viral particle, which is flanked by lipid membranes at both sides. Such membranes are similar to the inner viral envelope in the zippers found inside the virus factories (Fig. 3B), whereas in the margins of the assembly sites they are ER cisternae with evident luminal spaces (Fig. 3D and E). This finding led to propose that the inner viral envelope derives from collapsed ER cisternae (Andres et al., 1998) and contains two tightly apposed membranes. Importantly, both kinds of zipper-like structures massively accumulate in infections with different inducible ASFV recombinants exhibiting defects in virus assembly (see later).

The origin of the viral membranes has been further analyzed by using markers of cell organelles in immunofluorescence and immunoelectron microscopy experiments. Studies reported by Andres et al. (1998) showed that luminal and membrane markers of the ER were excluded from the viral factory, but could be found strongly labeling the periphery of the assembly sites. Also, the luminal marker protein disulfide isomerase (PDI) was detected in the intracisternal spaces of marginal zipper-like structures. In addition, antibodies against membrane ER glycoproteins strongly labeled the membranes of these marginal viral structures and a low but significant labeling could be found associated with viral intermediates within the factory. The factory was also found to exclude markers of the Golgi complex and the endocytic-lysosomal pathway (Andres et al., 1998).

On the other hand, Rouiller et al. (1998) reported the presence of ER membrane proteins in membranes within the viral factory, as well as membranes in assembly intermediates, in mature virions and extracellular virus, using the antibody RxER that recognizes four membrane proteins of the ER. It is also of interest to mention that a member of multigene family 110, ORF XP124L, that encodes a protein predicted to translocate into the lumen of the ER, was found to be retained into the lumen after synthesis and was detected in viral assembly intermediates and mature virions. This was interpreted to mean that ASFV was wrapped by ER cisternae, supporting the idea that two membranes are present in the viral envelope (Rouiller et al., 1998).

In conclusion, the existing morphological and immunocytochemical evidence clearly indicates that the viral membrane precursors are derived from ER cisternae, which are recruited to the factory and modified by mechanisms still not well known. At this respect, since the appearance of the inner envelope in the mature virus particles is consistent with a single lipid bilayer, it can be hypothesized that the viral envelope derives from virus-modified ER membrane fragments, as it has been also proposed for poxvirus envelopment

on thawed cryosections of cells infected for 18 h. Labeling of p12 on viral membrane precursors (arrows in G), immature particle (arrowheads in G) and mature particles (arrowheads in H) is shown. At variance, cellular protein p24 localizes at the plasma membrane (arrowheads in I) and seems to be recruited at the areas of virus budding. Accordingly, p24 is detected at the outer envelope of extracellular particles (J) when pre-embedding immunogold labeling is performed. Bars, 100 nm.

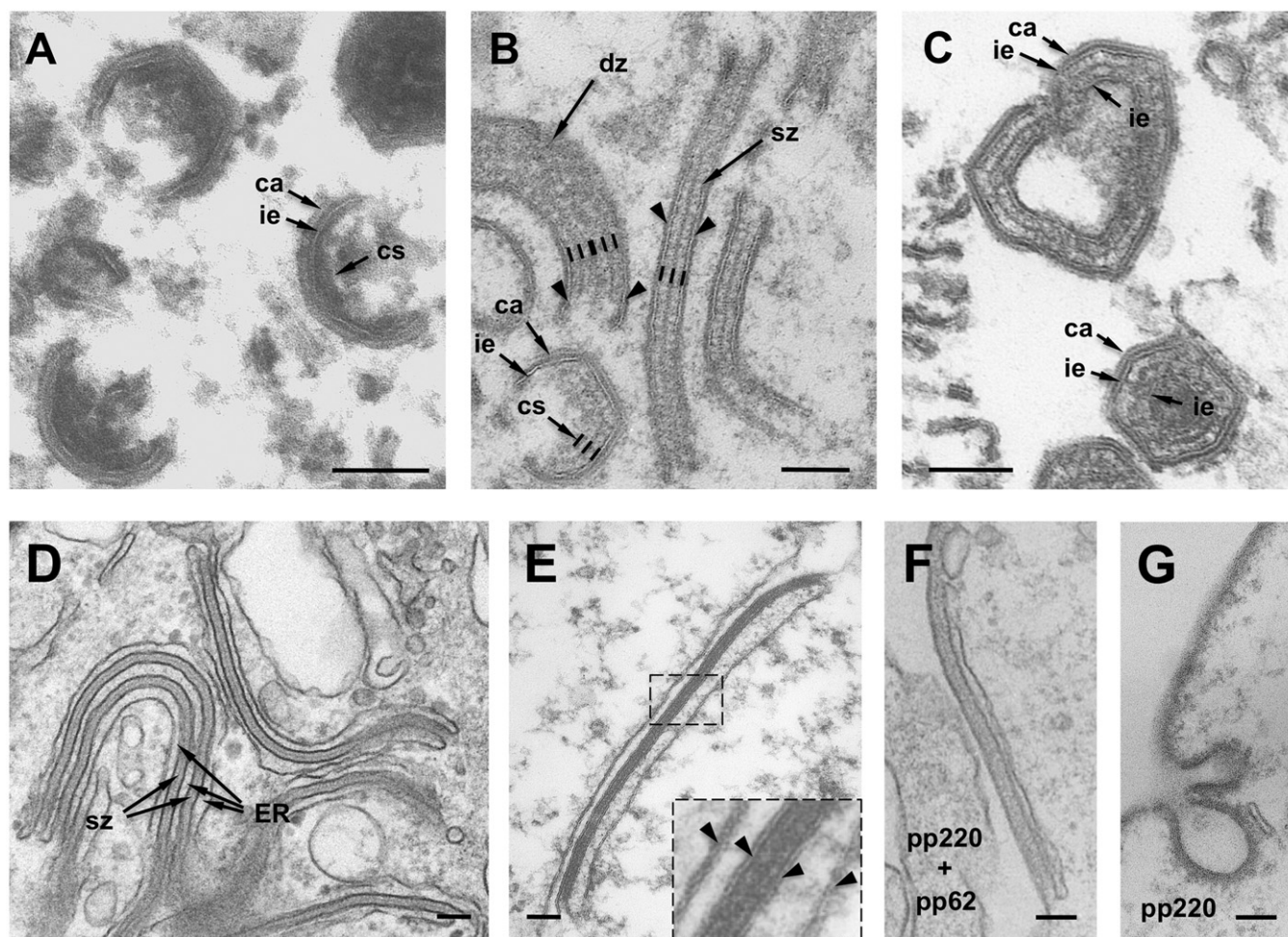


Fig. 3. Assembly of the capsid, the core shell and the zipper structures. (A) The capsid (ca) and the core shell (cs) are assembled concomitantly on and underneath, respectively, the inner envelope (ie). (B) Aberrant single (sz) and double zipper (dz) structures are constituted by one or two copies of an extended core-shell like structure (delimited by bars) flanked by membranes (arrowheads) resembling the inner envelope. These aberrant zippers accumulate inside the virus factory when expression of the capsid protein p72 is abrogated by means of a recombinant inducible virus. (C) Zippers inside the virus factory become polyhedral structures after capsid assembly following induction of the expression of protein p72. As a consequence, double-enveloped virus particles are detected. (D) Another kind of single zippers appear associated with ER cisternae at the cytoplasm surrounding the viral factories. These zippers accumulate when expression of protein p54 is abrogated by using a recombinant inducible virus. (E) Structure of a single zipper bound to two adjacent ER cisternae. The inset shows a higher magnification of the limiting membranes (arrowheads). (F) Zipper structure formed following transient co-expression of polyproteins pp220 and pp62 in COS-7 cells. (G) Membrane-bound dense coat formed by transient expression of polyprotein pp220 alone. Bars, 100 nm.

(Chlanda et al., 2009), or that one of the two membranes resulting from a putative collapsed cisternae is lost by a mechanism unknown at present.

3.2. Formation of the capsid

Electron microscopic analysis of the viral factories shows that the capsid, a layer of regularly arranged subunits displaying a hexagonal pattern (Carrascosa et al., 1984), is progressively assembled on the convex face of the viral membranes, which thus become polyhedral forms (Fig. 3A) (Andrés et al., 1997; García-Escudero et al., 1998), this being an ATP and calcium-dependent process (Cobbold et al., 2000). Besides the major capsid protein p72, capsid assembly depends on protein pB602L, a non-structural protein that acts as a chaperone for the folding of the major protein p72 (Cobbold et al., 2001; Epifano et al., 2006a) and on protein pB438L, a minor capsid component probably involved in the formation of the capsid vertices (Epifano et al., 2006b).

3.3. Formation of the core shell

Simultaneously to the capsid assembly, the core shell is formed underneath the concave face of the viral envelope (Fig. 3A). This domain seems to be constituted by two regular arrays of globular subunits of 10 nm subdivided by a thin electron-dense layer (Fig. 1A). Such organization can be clearly seen in images of assembling particles and aberrant zippers (Fig. 3B), although it is much less, if any, evident in images of intracellular and extracellular mature virus, which suggests further core maturation (Andrés et al., 1997). As indicated above, the main constituents of the core shell domain are the proteolytic products of the two virus polyproteins, pp220 and pp62. The presence also of the pS273R protease in this domain and the subcellular distribution of the polyproteins and their products in pulse-chase experiments indicates that polyprotein processing is coupled to the assembly of the core (Andrés et al., 2002a).

The available data also indicate that polyproteins pp220 and pp62 interact with each other to form the core shell underneath the inner lipid envelope. Transient co-expression of both polyproteins leads to the assembly of zippers (Fig. 3F), which are

symmetrical core shell-like structures (30-nm thick) delimited at both sides by lipid membranes (Andres et al., 2002a). At variance, when expressed alone, polyprotein pp220 binds to lipid membranes through its N-terminal myristic moiety giving rise to dense coats (24-nm thick) limited by one membrane (Fig. 3G) (Andres et al., 2002a). Thus, it is tempting to speculate that zipper structures consist of two membrane-bound pp220-containing opposite layers interconnected by the precursor pp62. Interestingly, while zippers appear preferentially associated with ER cisternae in ASFV infections, they are bound predominantly to the plasma membrane and endolysosomal membranes in the transient expression experiments. This finding suggests that proper membrane targeting of polyprotein pp220 involves other factors besides its acylation.

A similar organization to that described for zippers might also occur at the developing core shell of normal ASFV particles. However, unlike the zippers, the proper core shell is an asymmetrical structure delimited at the outer side by a lipid envelope and at the inner side by the DNA-containing nucleoid. This major difference could be explained by additional structural changes occurring during the assembly of the virus particle, where core and capsid formation are concomitant events. At this respect, aberrant zippers constituted by unprocessed pp220 and pp62 polyproteins accumulate in infections with a number of conditional lethal mutant viruses arrested directly or indirectly in the formation of the capsid (see below). Therefore, it seems probable that the progressive assembly of the capsid layer on the inner envelope determines in some way the proper assembly of an asymmetrical core shell beneath the envelope.

3.4. Formation of the nucleoid

The formation of the nucleoid is likely to be the last step in morphogenesis. In the viral factories icosahedral particles with and without an electron-dense central nucleoid are observed. Two models can be proposed regarding this step of virus assembly. One of them suggests that electron-dense nucleoprotein complexes, designated pronucleoids, are preformed in the factory bound to membranes near the “empty” particles, and are then encapsidated at a single vertex into the “empty” particles (Brookes et al., 1996, 1998). This mechanism, however, has not been confirmed in other studies, and the alternative model favors the idea that the viral DNA is first encapsidated, possibly together with nucleoproteins, and then condensed inside the assembling virus particles to produce the “full” mature virions observed in the factory. In support of this view, experiments with recombinant viruses arrested in the expression of polyproteins pp220 (Andres et al., 2002b) and pp62 (Suarez et al., 2010b) have shown that the incorporation of the viral genome and the nucleoproteins p10 and pA104R to the virus particle depends on the previous assembly of the core shell (see also below).

Whatever the mechanism, this process does not seem to be very efficient, since “empty” virus particles can be frequently seen at the plasma membrane to be released from the cell by budding.

3.5. Virus egress

ASFV particles exit the host cell by budding at the plasma membrane, which yields extracellular virions enveloped by an additional lipid membrane (Breese and DeBoer, 1966). This mechanism of egress involves the transport of intracellular particles from the virus factories to the cell surface through a microtubule-mediated mechanism (de Matos and Carvalho, 1993) depending on the motor protein conventional kinesin (Jouvenet et al., 2004) and on the capsid protein pE120R (Andres et al., 2001b). Once at the cell surface, ASFV particles can also induce actin nucleation and can be detected in the tip of filopodia-like projections (Jouvenet et al., 2006).

Interestingly, ASFV infection evolves toward cell lysis at very late times of infection (Breese and DeBoer, 1966), which might represent an alternative mechanism of viral egress. The release of intracellular mature virions, which are infectious (Andres et al., 2001b) and structurally and antigenically different to the extracellular enveloped virions, may have important implications in the host immune response against ASFV.

4. Study of the role of viral proteins in morphogenesis making use of ASFV inducible recombinants

The use of ASFV inducible recombinants has allowed investigating the function of viral proteins in morphogenesis. These recombinants have been obtained by introducing the *Escherichia coli lac* repressor/operator system inducible by IPTG in the ASFV genome, as described by Garcia-Escudero et al. (1998). The inducible system consists of the *lac I* gene, encoding the repressor protein, introduced in the thymidine kinase locus of ASFV BA71V strain, under the control of the ASFV early/late promoter *pU104L* (Almazan et al., 1992), and an IPTG-inducible promoter, formed usually by the 41 bp region of the *p72* promoter, designated *p72.4*, (Garcia-Escudero and Vinuela, 2000), followed by the operator sequence at a distance of 2 or 8 nucleotides, replacing the promoter of the gene of interest. In this way, the expression of the analyzed protein is IPTG-dependent. The spacing between the operator and the transcription initiation site at the promoter is important for the level of repression and of IPTG-induced expression. Greater repression but reduced induction is obtained with the shorter distance and vice versa (Garcia-Escudero et al., 1998). It should be mentioned that the *p72.4* promoter could be too strong to achieve inducible expression of genes that are expressed in low amounts during infection. This will be commented further in the section describing the virus recombinant inducibly expressing the protease gene.

4.1. The inner envelope proteins p54 and p17 are involved in the acquisition and formation of viral membrane precursors

The early stages of morphogenesis that include the recruitment of ER membrane cisternae and their modification to produce the viral membrane precursors are still poorly understood processes. The functional characterization of membrane structural proteins residing in the inner envelope of the virus particle is providing insights into the mechanisms underlying these initial steps of virus assembly. One of these proteins is protein p54 encoded by gene E183L, which is expressed late in infection and is essential for virus viability (Rodriguez et al., 1994, 1996a). In vitro and in vivo studies have indicated that protein p54, with a size of 25 kDa, is a type I transmembrane protein, with the small N terminus inside the luminal space and the large C terminus facing the cytoplasm (Rodriguez et al., 2004). The protein is targeted to the ER membranes in transfected cells, while, in infected cells, it is found in the ER-derived membrane precursors inside the factories as well as in intracellular and extracellular mature particles. Protein p54 forms disulfide-linked dimers through its unique cysteine residue present in the N terminus, the dimers being more abundant in extracellular virions. To investigate its function, a conditional lethal mutant, vE183Li, with an inducible copy of gene E183L, was constructed and analyzed (Rodriguez et al., 2004). Under conditions that repress the synthesis of p54, the viral factories appear essentially devoid of membrane precursors and other viral structures (Fig. 4B) found in normal infections (Fig. 4A), while at the periphery of the factories zipper-like structures associated to open ER cisternae (Fig. 3D) and large inclusion bodies, also frequently enclosed by cisternal membrane profiles, accumulate. Both aberrant forms contain unprocessed core polyproteins pp220 and pp62. Protein

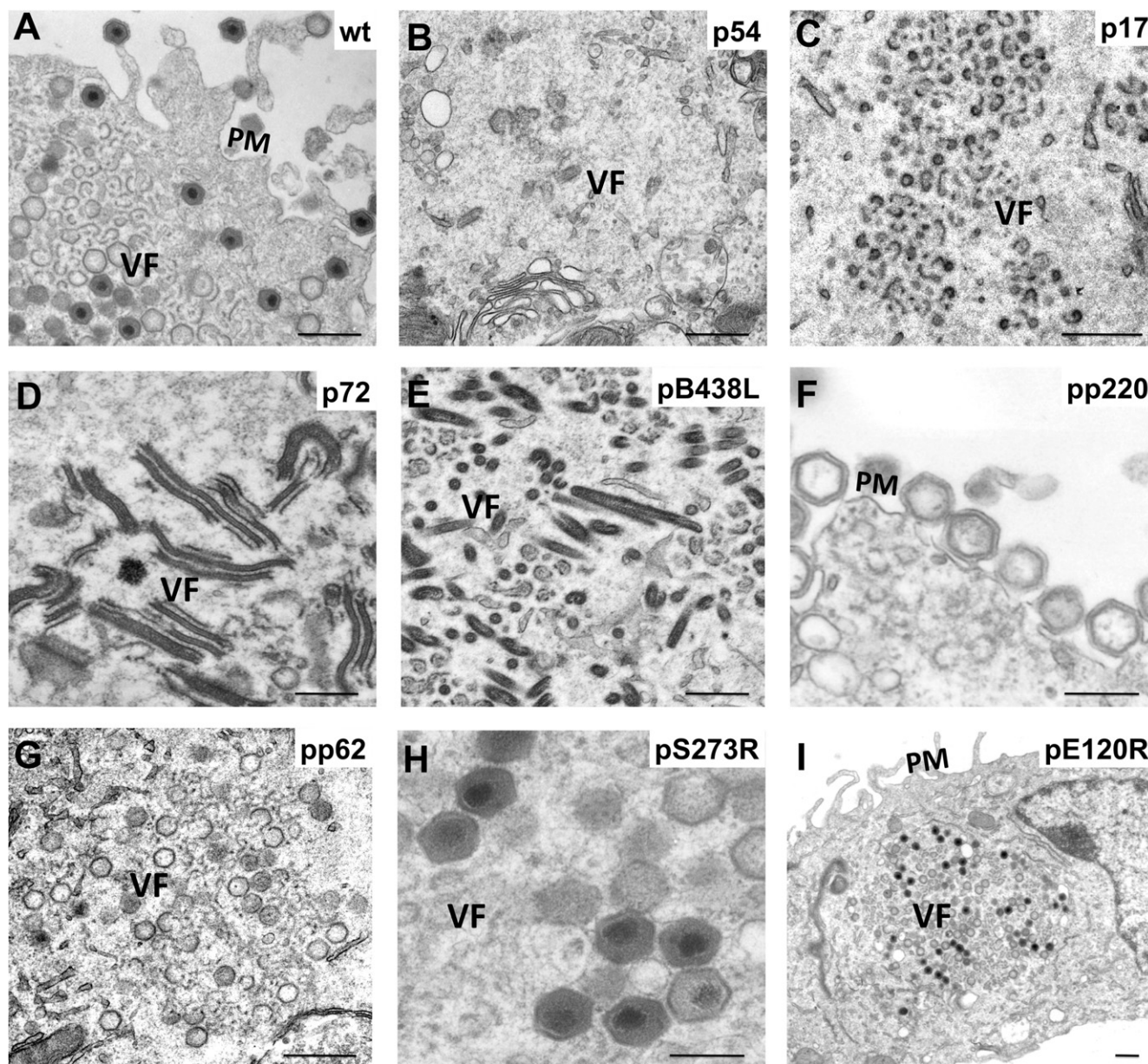


Fig. 4. Ultrastructural phenotype of inducible ASFV recombinants. Electron microscopy of cells infected under restrictive conditions with various inducible ASFV recombinants. Micrographs illustrate the ultrastructural phenotype observed when silencing the expression of the inner envelope transmembrane proteins p54 (B) and p17 (C), the major capsid protein p72 (D), the minor capsid protein pB438L (E), the core polyproteins pp220 (F) and pp62 (G), the polyprotein proteinase pS273R (H) and the capsid protein pE120R (I). Mutant phenotypes include defects in the formation of precursor membranes (B, p54), the capsid assembly (C, p17; D, p72; E, B438L), the assembly of the core (F, pp220; G, pp62; H, pS273R) and the microtubule-mediated transport of intracellular particles to the cell surface (I, pE120R). As a reference, a detail of a cell infected with parental ASFV strain BA71 V is shown (A, wt). Bars, 500 nm (A, B, C, D, E, I), 200 nm (F, G).

p72 is also found delocalized and, in post-induction experiments, assembles into icosahedral capsids at the border of the inclusion bodies.

The above results indicate that protein p54 is required for membrane recruitment to the viral factories. However, protein p54 has not been found to colocalize with the ER that surrounds the viral factory, which raises the question of how can the protein accomplish its proposed function. One plausible explanation of the role of p54 in membrane recruitment is that the nascent p54 polypeptides are cotranslationally inserted into the ER proximal to the factories and that these virus modified ER membranes would be rapidly recruited and converted into the membrane precursors inside the factories. On the other hand, the finding that viral proteins are excluded from the assembly sites when p54 is repressed and the factories are devoid of precursor membranes has been interpreted

to mean that the recruited ER membranes play a pivotal role for the anchorage of the virus proteins in the factory (Rodríguez et al., 2004).

Another question arisen by the observation that in the absence of p54 the zipper-like structures found outside the factories are bound to ER cisternae with evident luminal spaces, while those present in a normal infection inside the factories are delimited by apparently collapsed cisternae, is whether protein p54 plays a role in cisternal collapse. Direct evidence for this has been provided in a recent study (Windsor et al., 2012) showing that the collapse of ER cisternae is induced by expression of p54 alone in transfected cells, and that the collapse is dependent on the N-terminal cysteine residue. The authors showed under these conditions the generation of large arrays of organized smooth ER (OSER) and that in the inner stacks of OSER the ER cisternae were collapsed by a

mechanism requiring exposure of the cysteine residue in the N-terminal domain of p54 to the lumen of the ER and formation of disulphide bonds between these domains arranged in anti-parallel arrays across the ER lumen. Whether the p54-induced collapse of ER cisternae described in this study plays any role in the development of the ASFV inner envelope during viral infection is unknown at present.

Protein p17, encoded by the late gene D117L (Simon-Mateo et al., 1995), is also an integral membrane protein (Cobbold et al., 1996) with the C terminus predicted to be inside the luminal space and the N terminus facing the cytoplasm. The protein is structural (Simon-Mateo et al., 1995) and has been found to localize at the inner envelope of the virus particle and in membrane precursors at the factory (Rouiller et al., 1998). The presence of p17 in the membranes delimiting zipper-like structures and in the inner envelope of aberrant filamentous virus has also been reported (Epifano et al., 2006a,b). As in the case of other ASFV proteins that localize at the inner envelope or in viral membrane precursors, protein p17 has an intrinsic affinity for the ER, being targeted to this compartment when expressed in transfected cells out of the context of the viral infection (Suarez et al., 2010a). As has been discussed, this is probably related to the process of recruitment and modification of ER-derived membranes for their conversion into viral precursor membranes.

In cells infected with the virus recombinant v17i that inducibly expresses protein p17, it was found, under restrictive conditions, an accumulation in the factory of viral precursor membranes, which are indistinguishable, at the electron microscopy level, from those present at very early times of infection with the parental BA71 V virus or the recombinant v17i virus under permissive conditions (Suarez et al., 2010a). However, in the absence of p17, the virus morphogenesis is blocked at this stage and the precursor membranes do not evolve towards icosahedral particles (Fig. 4C), whereas under permissive conditions, or in the infection with the parental virus, icosahedral intermediates and immature and mature particles are produced beyond this morphogenetic stage. Moreover, repression of protein p17 leads to the delocalization of the capsid proteins p72 and pE120R and of the core shell polyproteins, which remain unprocessed, indicating a direct or indirect defect in the correct interaction of the capsid and core shell components with the precursor membranes, which impedes the formation of icosahedral intermediates and thus the progression of morphogenesis.

The structure of the viral membrane precursors has been investigated by analyzing ultrathin serial sections of cells infected with the recombinant virus under restrictive conditions (Suarez et al., 2010a). However, this study could not detect any continuity between the viral membrane precursors, which was interpreted to mean that these precursors are so convoluted that it is not possible to identify any continuity between them. A similar analysis performed with cells infected with the parental ASFV or the recombinant v17i under permissive conditions, allowed the surprising identification of large helicoidal membrane structures. These forms, which can also be found in natural infections of porcine macrophages or soft ticks (Kleiboeker et al., 1998, 1999), have been described as two-strand helicoidal structures formed by a long ribbon of inner membrane precursor disposed around a rod of core-like material following a helicoidal trajectory (Suarez et al., 2010a). Moreover, immature icosahedral particles can be frequently seen at one of the ends of the helicoidal structures and several images have suggested a pathway for the formation of immature particles during ASFV assembly. The discovery of these novel helicoidal structures raises the question of their relevance as intermediates in morphogenesis and of the need of other viral proteins, in addition to p17, for their formation.

4.2. Role of proteins p72, pB602L and pB438L in the formation of the icosahedral capsid

The first inducible recombinant generated was vA72 that expresses the major capsid protein p72 in an IPTG-dependent way (García-Escudero et al., 1998). The report on this recombinant showed the usefulness of the inducible gene expression system to investigate the role of ASFV genes in virus assembly. Repression of p72 leads to the accumulation in the viral factories of two types of zipper-like structures (Fig. 4D). The most abundant of them is constituted by a copy of an extended core shell-like domain, and is referred to as “single”, while the “double” zipper is formed of two copies. Induction of protein p72 expression, after a period of repression, allowed to observe the progressive building of the capsid on these structures, which became polyhedral forms and lead to the eventual assembly of ASFV particles containing one or two inner envelopes and occasionally a central electron-dense nucleoid-like domain (Fig. 3C).

The protein encoded by the late gene B602L is also required for the formation of the icosahedral capsid of the virus particle (Cobbold et al., 2001; Epifano et al., 2006a). Protein pB602L is non-structural and has been described as a chaperone for the folding of the capsid protein p72 (Cobbold et al., 2001). Accordingly, repression of protein pB602L in cells infected with the inducible virus vB602Li produces an effect on morphogenesis similar to that observed when the expression of p72 is inhibited. Thus, the factories contain zipper-like structures indistinguishable from those seen when the capsid protein p72 is repressed. These structures contain the inner envelope protein p17 and unprocessed core shell polyproteins pp220 and pp62, but lack the capsid proteins p72 and pE120R. Surprisingly, protein pB602L is localized in the cytoplasm of the infected cell. This poses a dilemma regarding its role as a chaperone of p72, since the capsid protein is found in the viral factory. A possible explanation could be that the two proteins transiently interact shortly after their synthesis either at the viral factory or the cytoplasm, but alternative possibilities should be considered in future studies to clarify this quandary.

Construction of the icosahedral capsid is also dependent on the late structural protein encoded by gene B438L (Epifano et al., 2006b; Galindo et al., 2000). The precise localization of protein pB438L (also designated p49) in the virus particle has not been identified because of the lack of adequate antibodies, but it is known that the protein associates with membranes in the infected cells and behaves as an integral membrane protein, even though the protein does not contain a hydrophobic region and no post-translational modifications that might account for its membrane association seem to occur. It has been hypothesized that protein pB438L might associate with membranes by interacting with another viral protein and, in connection with this, it has been found that in the viral particle the protein forms disulphide bonded oligomers with a size of 150 kDa, which might correspond to a complex formed with the capsid protein p72, since this complex is recognized by antibodies directed against both proteins (Epifano, 2005). The *in vivo* association of protein pB438L with membranes raises the possibility that in the viral particle the protein might be localized in the inner envelope through its binding to an integral protein of the envelope, thus allowing its interaction with protein p72.

Using a recombinant ASFV that inducibly expresses protein pB438L, it was found that, in the absence of the protein, large tubular structures and other aberrant forms with a bilobulate structure were generated (Fig. 4E), instead of the normal icosahedral particles (Epifano et al., 2006b). The tubular structures had a length of 1 μ m or more and a diameter of 80 nm. The morphological and immunocytochemical studies showed that the filamentous structures are covered by a capsid-like layer, which does not present an icosahedral morphology, but contains the major capsid protein p72

and protein pE120R, this latter allowing the movement of the tubular forms from the viral factory to the budding sites at the plasma membrane. The tubules also possess an inner envelope that contains the viral membrane protein p17 and an internal domain that resembles the core shell of the icosahedral particle, but with the two viral polyproteins in an unprocessed state. In addition, a central electron-translucent channel of 25 nm lacking the viral DNA is found inside these structures.

In conclusion, the results indicate an essential role for protein pB438L in the construction of the icosahedral form of the ASFV capsid. The lack of knowledge on the precise localization of the protein in the virus particle has impeded to provide an explanation for its mechanism of action. Nevertheless, taking into account that the inner domains of ASFV are not required for the correct assembly of icosahedral particles (see below), it has been proposed that a defect in the external domains, the inner envelope or the capsid, is the reason why an icosahedral capsid can not be correctly assembled in the absence of protein pB438L, suggesting a localization of the protein in these sites. This idea is supported by the above mentioned finding that protein pB438L associates with membranes as well as with the capsid protein p72. Taking all this into account, it has been hypothesized that protein pB438L may form part of the vertices of the icosahedral capsid. Nonetheless, a better knowledge of the organization and composition of the external domains of ASFV will be required to clarify the mechanism by which protein pB438L carries out the construction of an icosahedral capsid instead of a tubular structure.

4.3. Role of polyproteins pp220 and pp62 and polyprotein processing in the formation of the core shell

As indicated before, the virus core is constituted by the core shell and the nucleoid, and the core shell is composed of the processing products of the two ASFV polyproteins, pp220 and pp62. The protease that catalyzes the proteolytic processing is also localized in this domain. It was therefore expected that the expression and processing of the polyproteins would play a relevant role in the construction of the core.

4.3.1. In the absence of polyprotein pp220 “empty” icosahedral particles are assembled

Repression of polyprotein pp220 in cells infected with the inducible recombinant v220i leads to the assembly of “empty” icosahedral particles that lack the virus core shell and nucleoid, but conserve the external layers formed by the inner envelope and the icosahedral capsid (Andrés et al., 2002b). Consistent with this, the coreless particles can be transported to the plasma membrane for budding and exit from the cell (Fig. 4F), and contain the peripheral proteins p72, pE120R, p12 and p17, but lack the core shell products of pp220 and pp62 polyproteins, the pS273R protease and the nucleoid proteins p10 and pA104R as well as the viral DNA. The results mean that the construction of the icosahedral capsid on the inner envelope is a process independent of core maturation, and that polyprotein pp220 plays a pivotal role in core assembly, including the steps leading to genome encapsidation and condensation and the uptake of the viral nucleoproteins. It is also to be noticed that, under these conditions, polyprotein pp62 is not incorporated into the virus particle and its processing is blocked, although both the polyprotein and the processing protease are normally expressed (Andrés et al., 2002a). This supports the idea that only after the proper interaction of both polyproteins inside the assembling virus polyprotein processing occurs.

4.3.2. Polyprotein pp62 is required for core development

The role of polyprotein pp62 in ASFV morphogenesis has been also analyzed using a virus recombinant that inducibly expresses

the polyprotein (Suarez et al., 2010b). Repression of polyprotein pp62 does not affect the assembly of the inner envelope and the outer capsid, but the viral factory shows an accumulation of abnormal particles presenting different levels of core development (Fig. 4G), indicating that polyprotein pp62 is essential for the correct assembly and maturation of the viral core. On the other hand, polyprotein pp220 is proteolytically processed but delocalized, being mainly found dispersed throughout the cytoplasm, possibly because expression of polyprotein pp62 is needed for the recruitment or the retention of polyprotein pp220 in the assembling virion. Taking into account that the processing of polyproteins is tightly associated with virus assembly, the present model is that polyprotein pp220 is first encapsidated and proteolytically processed, but, in the absence of pp62, the products of pp220 are not stabilized in the developing particle and will move out and accumulate in the cytoplasm of the infected cell.

4.3.3. Inhibition of polyprotein processing leads to the assembly of icosahedral particles with aberrant cores

As mentioned above, pulse-chase experiments indicate that proteolytic processing of polyproteins is concomitant with particle assembly. Moreover, it has been shown that when the expression of several structural or nonstructural proteins is repressed, the two viral polyproteins, present in zipper-like structures or inclusion bodies, are not processed. Also, in the absence of pp220, polyprotein pp62 does not assemble into the virus particle and remains unprocessed. These findings establish a tight correlation between virion assembly and polyprotein processing.

The effect of inhibiting polyprotein processing on the formation of the viral core has been examined using the virus recombinant vS273Ri that inducibly expresses the protease. For the construction of this recombinant it was necessary to replace the p72.4 promoter sequence by the endogenous protease promoter, consisting of a 42-bp sequence that contains the transcription initiation site of gene S273R and the promoter elements described by Garcia-Escudero and Vinuela (2000). This was followed by the *E. coli lac* operator sequence as described (Alejo et al., 2003). It was first shown that proteolytic processing of both polyproteins was severely impaired by repression of pS273R expression (Alejo et al., 2003). Moreover, under these conditions non-infectious particles with an aberrant core were generated. The aberrant core consists of a disorganized core shell of unequal thickness and a misplaced nucleoid of irregular shape and highly condensed (Fig. 4H). Furthermore, the purified extracellular mutant particles contained the pp220 and pp62 unprocessed polyproteins, as well as the two known nucleoid proteins, p10 and pA104L, indicating that the incorporation of the polyprotein precursors and of at least some of the major components of the nucleoid does not depend on polyprotein processing. This is in keeping with the finding that the proteolytic cleavage of pp220 and pp62 precursors occurs after their incorporation into the assembling particles (Andrés et al., 2002a). It can be concluded from these studies that the ASFV protease is essential for the production of infectious virus particles in correlation with the severe inhibition of the proteolytic processing of the two virus polyproteins.

Taken together, the results indicate that polyprotein processing is involved in a late maturational step required for the correct organization of the core shell and nucleoid. In addition, it has been proposed that this process may depend on the interaction of components of the internal nucleoid with motifs unmasked in the proteolytic products of the polyproteins. The reason for the lack of infectivity of the defective vS273Ri particles is unknown at present, although several explanations have been proposed. One interesting possibility is that some early step in infection, such as the uncoating process is affected. This could be due to the fact that, in the absence of processing, the entire core remains bound to the inner envelope through the N-terminal myristic moiety of the unprocessed pp220

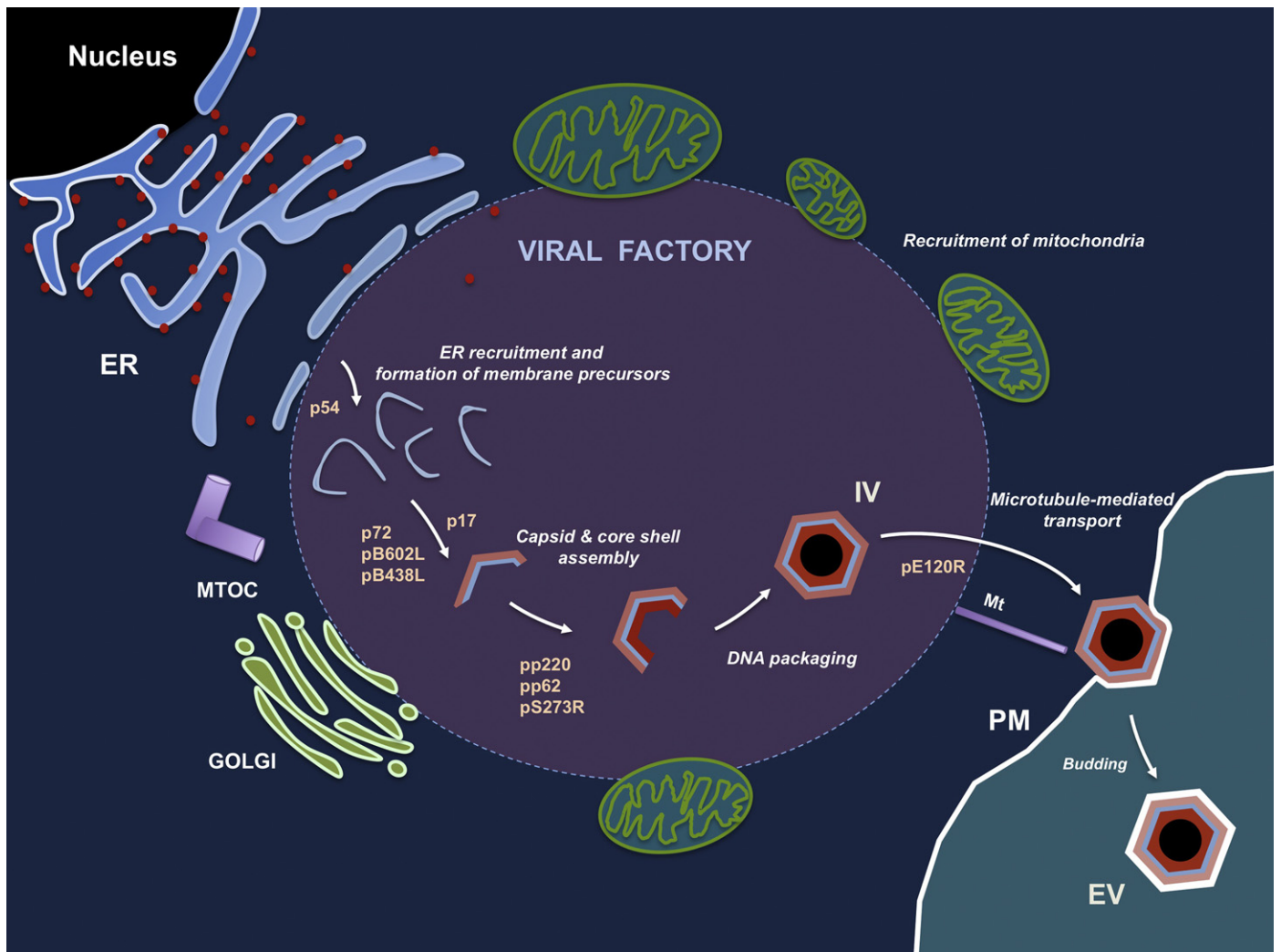


Fig. 5. Model of ASFV assembly. ASFV morphogenesis occurs at perinuclear viral factories close to the Golgi complex and the microtubule organizing center and surrounded by ER cisternae and recruited mitochondria. The first indication of ASFV assembly is the formation of viral membrane precursors from ER cisternae through a poorly understood mechanism depending on the transmembrane viral protein p54. Viral membrane precursors become polyhedral intermediates after progressive assembly of the capsid. This event critically depends on the expression of inner envelope protein p17, the major capsid protein p72, the p72 chaperone pB602L and the minor capsid protein pB438L. Concomitantly to the capsid formation, the viral core is assembled under the viral inner envelope. Polyproteins pp220 and pp62 and the viral polyprotein processing proteinase pS273R play a key role in this event. Morphogenesis of intracellular particles is completed when packaging of viral DNA and associated nucleoid proteins take place. Intracellular mature virus moves from the virus factory to the cell surface through a microtubule-dependent process dependent on viral capsid protein pE120R. Finally, ASFV particles exit the host cell by budding from the plasma membrane (PM) thus acquiring an extra lipid envelope. Abbreviations: ER, endoplasmic reticulum; MTOC, microtubule organizing center; PM, plasma membrane; IV, intracellular virus; EV, extracellular virus.

polyprotein, as suggested by the observation of a tight association between the viral core and the inner envelope in the mutant particles, while a retraction of the core can be noticed in the normal virions. This abnormality in the mutant virus might impede the normal uncoating of the virus particle. The protease activity itself might play a role in the disassembly of the virus particle, as suggested by the presence of this enzyme in the mature virions and by the demonstration of a similar role for the adenovirus protease during virus entry in the cells (Greber et al., 1996). A defect in early transcription could also explain the lack of infectivity of the mutant virus.

4.4. The capsid protein pE120R is required for virus transport from the factory to the plasma membrane

Protein pE120R, also designated p14.5, is a late protein localized in the capsid layer of the virions where it interacts with the major capsid protein p72 (Martinez-Pomares et al., 1997; Andres et al.,

2001b). Protein pE120R exists in several forms, ranging from 14.5 to 22 kDa when present in the cytosol, and from 12 to 25 kDa after its incorporation in the virus particle (Andres et al., 2001b). This size heterogeneity seems to be due to post-translational modification by acetylation at the N-terminal Ala residue (Alfonso et al., 2007) and phosphorylation (Martinez-Pomares, 1990). The role of protein pE120R was studied using a virus recombinant that inducibly expresses the protein. In its absence, intracellular infective virus is produced, but the virus particles remain confined into the factory indicating the requirement of this protein for virus transport from the factory to the plasma membrane (Fig. 4I), where they are released by budding thus acquiring the additional envelope of extracellular virus. Since the transport of ASFV to the cell periphery is dependent on microtubules (de Matos and Carvalho, 1993; Jouvenet et al., 2004), it has been speculated that protein pE120R could be involved in the virus-microtubule interaction. However, further studies are needed to establish this mechanism for virus exit from the factory and release from the cell for virus dissemination.

5. Concluding remarks and future directions

Recent studies on ASFV morphogenesis have provided important advances in our understanding of the mechanisms involved in the formation of mature virions, as summarized in the model presented in Fig. 5. We know that the viral precursor membranes are derived from ER cisternae and that the inner envelope protein p54 is required for membrane recruitment to the factory. However, the process that leads to the conversion of a membrane cisterna into a single lipid bilayer remains to be explored. In the case of vaccinia virus, it has been proposed that single membrane sheets are produced by rupture of ER membranes, but the mechanisms underlying this process and the proteins involved have yet to be investigated (Chlanda et al., 2009). At very early stages of infection, or when the synthesis of the envelope protein p17 is repressed, factories containing exclusively precursor membranes can be seen. In the following step, the precursor membranes develop into icosahedral intermediates by the simultaneous assembly of the icosahedral capsid and the core shell domain on opposite sides of the envelope, this progression being dependent on the envelope protein p17, which seems to be needed to recruit or retain the capsid proteins and the core shell polyproteins. Repression of the major capsid protein p72, or of its chaperone pB602L, also interferes, as expected, with this event, while repression of the polyproteins does not impede the formation of icosahedral particles, which lack the core shell and nucleoid, but possess the external layers of the virion. The key role of polyprotein processing in the development of infective virus with a proper core has also been described, as well as the coupling of processing to virus assembly, suggesting a tight regulation of the protease catalyzing polyprotein processing. As has been discussed here, the novel helicoidal membrane structures recently identified can also be morphogenetic intermediates by enwrapping the core domain and interacting with the capsid proteins at one of the ends to produce the icosahedral particle, but whether this is the main morphogenetic route or only an alternative pathway remains to be clarified. The last step of DNA encapsidation and condensation is still a poorly understood feature and should be further investigated by studying the role of nucleoproteins composing the nucleoid. In this connection, it should be mentioned that the ASFV gene B354L codes for a protein with similarity to the vaccinia virus A32 ATPase required for DNA packaging into virions (Iyer et al., 2001). Finally, the virus exits the factory and is transported to the cell surface by a mechanism dependent on the capsid protein pE120R and microtubule motor kinesin (Andrés et al., 2001b; de Matos and Carvalho, 1993; Jouvenet et al., 2004). At the plasma membrane the virus is released from the cell by a budding process, acquiring thus the outer envelope of the extracellular virus. Such exit event is poorly understood as no viral or cellular proteins at the plasma membrane have been shown to be involved at present. A structural and antigenically distinct form of the virus can be liberated by cell lysis and the relevance of this form in pig infection merits to be investigated. This study will be facilitated by the use of the virus recombinant that produces only the intracellular virus form by repression of protein pE120R required for virus exit from the factory.

Thus far, the genes of 19 of the 54 structural components of the virion have been identified, but we only know the function of 9 of them. Additionally, as in the case of the chaperone protein pB602L, other non-structural proteins may play a role in morphogenesis. One possible example is the ASFV prenyltransferase, which is localized in the precursor membranes where the enzyme catalyzes the synthesis of geranylgeranyl diphosphate, a product that could modify the viral membranes (Alejo et al., 1999). Further studies on the ASFV sulfhydryl oxidase, another non-structural protein encoded by gene B119L, (Rodríguez et al., 2006) regarding its possible role in the formation of disulfide bonds in structural

proteins will also be of great interest to understand the importance of these bonds in virus assembly. Also, many details of the morphogenetic process remain enigmatic, as for example the biogenesis of the viral membranes. Future studies should be directed to mapping the genes coding for other proteins of the viral particle, determining their localization within the virion and examining the role of the proteins by using inducible virus recombinants. Further evaluation of the interactions between structural proteins will be an area of interest for subsequent investigations. The application of new techniques of electron microscopy, such as electron tomography, should also be determinant for our understanding of virion structure and assembly.

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