Novel insights into cryo-EM structure of African Swine Fever Virus

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Abstract African swine fever virus (ASFV) is a giant and complex DNA virus that causes a highly contagious and lethal swine disease for which there is no vaccine available. Here we describe the cryo—electron microscopy (cryo-EM) structure of ASFV, the first virus (to our knowledge) that has been found to use two lipid membrane layers and two protein shells to encapsidate and protect its genome. Using an optimized image reconstruction strategy, we solved the AFSV capsid structure up to 4.1-angstroms, which is built from 17,280 proteins, including one major (MCP) and four minor capsid proteins (M1249L, p17, p49 and H240R), and organized into pentasymmetrons and trisymmetrons. The atomic structure of the MCP informs putative conformational epitopes, which determine the specific differences between the virus types, being valuable for epitope-focused immunogen design against ASFV infection. The minor capsid proteins form a complicated network below the outer capsid shell, stabilizing the capsid by holding adjacent capsomers together. Acting as core organizers, 100-nm-length M1249L proteins, running along each edge of the trisymmetrons and bridging two neighboring pentasymmtrons, form extensive intermolecular networks with other capsid proteins to guide the formation of capsid framework. These structural details unveil the basis of capsid stability and assembly, opening up new avenues for ASF vaccine development.

Keywords African swine fever virus; Major capsid proteins; Minor capsid proteins; Capsid assembly
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1 Introduction

African swine fever (ASF), first described in Kenya in 1921 [1], is a highly contagious viral disease of swine with mortality rates approaching 100%. Over the past decade, ASF has spread through many countries of the Caucasus, the Russian Federation, and Eastern Europe, posing a serious risk of further expansion [2]. More recently, the OIE (World Organization for Animal Health) was notified by 20

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countries with new or ongoing outbreaks: 12 in Europe, 6 in Asia and 2 in Africa during the period from January to July 2019 (OIE Report: January 15—July 18, 2019). With no vaccine or treatment, culling pigs is presently a desperate yet most effective move to contain the outbreaks, just in China and Vietnam alone, more than five million pigs have been culled, causing severe economic losses for swine production worldwide. Of the most serious concern are the rapid and efficient transmission amongst pigs, relative stability of the virus in the environment and the lack of an ASF vaccine. These factors present huge challenges for global ASF control.

ASF is caused by African Swine Fever virus (ASFV), the sole member of the Asfaviridae and the only known DNA arbovirus. Despite sharing structural, genomic, and replicative characteristics with other nucleocytoplasmic large DNA viruses (NCLDV) [3], ASFV is unique in that it possesses a multilayered structure and an overall icosahedral morphology. It has a genome-contained nucleoid surrounded by a thick protein layer referred to as the core shell, which is wrapped by an inner lipid envelope, an icosahedral protein capsid and an outer envelope layer, comprising over 50 proteins [4, 5]. Large gaps in knowledge concerning the composition and structure of the infectious virion and immune characteristics hinder vaccine development.

Natural target cells of ASFV are macrophages, monocytes mainly present in the blood and bone marrow [6]. Here we isolated porcine bone marrow (PBM) cells from 30—40-day-old specific pathogen-free (SPF) pigs, propagated ASFV (genotype II) in primary PBM cells and purified extracellular ASFV particles from the cell supernatants, then inactivated the viral particles with formaldehyde. The purified virions were examined by electron microscopy (EM). The extracellular ASFV particle is on average 260—300 nm in diameter, significantly larger than previous observations (~200 nm) [7].

2 Architecture of the ASFV virion

The 3D reconstruction of ASFV reported here clearly shows structures of all five layers, amongst which the capsid has a maximum diameter of 2 500 Å, while the middle layer is a 70-Å-thick lipid-bilayer membrane that envelopes a 1 800-Å-diameter core shell. All three aforementioned layers adopt an overall icosahedral morphology that roughly follows the contour defined by the capsid (Fig. 1A). However, the outermost envelope and innermost nucleoid present weak densities due to the loss of some structural features resulting from icosahedral averaging.

By using an optimized "block-based" reconstruction approach combined with the gradient defocus correction, the resolution of the capsid reconstruction was improved to 4.8 Å (Fig. 1). The capsid is constructed of 2 772 capsomers arranged in a $T=277$ icosahedral lattice ($h=7$, $k=12$) (Fig. 1B). In this lattice, there are 12 pentasymmetrons (containing 30 pseudo-hexameric capsomers and a pentameric capsomer) and 20 trisymmetrons (containing 120 capsomers). A similar organization has been observed in other NCLDVs [8—10]. Notably capsomers within a trisymmetron all pack in essentially the same orientation, rotating by ~60° from the capsomers in neighboring trisymmetrons, creating 30 zippers (cleavage lines) on the capsid. Additionally, the core shell separately reconstructed to 9 Å shows 180 6-blade propellers with a central channel (30 Å in diameter) and 12 starfish-like pentons surrounded by 10 antennae (Fig. 1B). Assuming that the 6-blade propeller represents one hexameric capsomer, the core shell would have $T=19$ quasi-icosahedral symmetry, with $h=2$ and $k=3$.

The cryo-EM maps for the pseudo-hexameric capsomer of capsid could be improved further by local averaging of equivalent copies present in the trisymmetron and a resolution of 4.1 Å was attained. The outer capsid shell is composed of 8 280 MCPs and 60 penton proteins, while many of the remaining uninterpreted densities decorating the inner capsid surface represent minor capsid proteins (Fig. 1C), presumably facilitating capsid assembly and maintaining the stability of the capsid shell. Minor capsid proteins should be among them. The combination of the proteomic analysis, protein abundance level in the ASFV particle and similarity of the cryo-EM map with predicted structural features of target proteins, including protein sequence, protein secondary structure and protein topology, led to the identification of

the penton protein (H240R) and three minor capsid proteins (p17, p49 and M1249L) (Fig. 1C). Each icosahedral asymmetric unit of the outer capsid shell contains 46 pseudo-hexameric capsomers with 6 of these in the pentasymmetron (a, b, c, d, e, and f) and 40 in the trisymmetron (A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X, Y, Z, A′, B′, C′ D′, E′, F′, G′, H′, I′, J′, K′, L′, M′ and N′) (Fig. 1D). The penton and minor capsid proteins (p17, p49 and M1249L) form a complicated network that is immediately below the outer capsid shell, stabilizing the whole capsid (Fig. 1C and D).

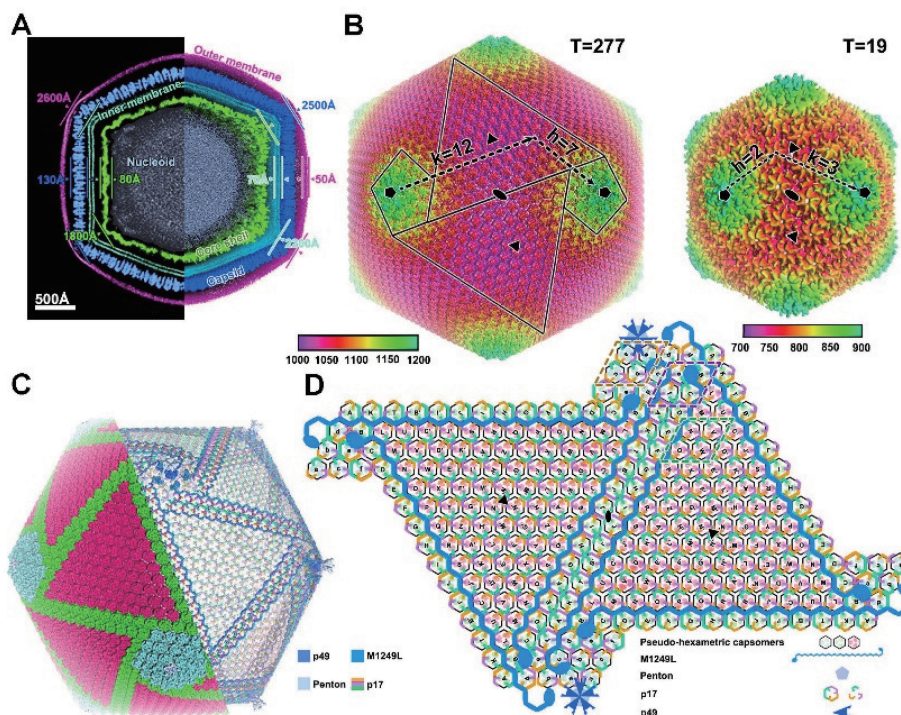

Figure 1 Architecture of the ASFV virion.

3 Structure and organization of p72

The p72, one of the key protective antigens, forms a homotrimer with each monomer adopting a double jelly-roll structure that makes up pseudo-hexameric capsomers [11,12]. Double jelly-roll fold can be found in many other viral capsid proteins, including adenovirus [13], the phage PRD-1 [14] and vaccinia virus [15], while the jelly-roll structure itself consisting of eight antiparallel β-strands (from B to I) widely exists in more viral capsid proteins, indicative of common ancestry. As is the case for double jelly-roll capsid proteins, four insertions (named ER, exposed region) within D1E1, D2E2, F1G1 and H1I1 loops, being the most exposed, together with the N-terminal base domain, determine the specific differences between the virus types. These four ERs probably acting as the neutralizing epitopes, could be used to guide ASF vaccine design. Deep profiling of human or animal B cells often reveals potent neutralizing antibodies that emerge from natural infection, but these specificities are generally subdominant (i. e., are present in low titers). Recently developed epitope-focused immunogen vaccination strategies have demonstrated great success in boosting subdominant neutralizing antibody responses against HIV or respiratory syncytial virus (RSV) infection [16, 17], which might be good solutions for ASF vaccine development.

While surrounding the penton to fill the pentasymmetron, p72 capsomers also pack themselves together to form the trisymmetron and zipper (Fig. 1C and D). Given the fact that p72 capsomers exhibit three distinct assembly patterns according to their locations, they have to adopt different arrangements to allow three distinct interactions: "head to back", "head to head" and "back to back". The "head to head" interactions in the zipper are straight and tight with a four-stranded β-sheet contributed from two adjacent

ER1s, while the counterpart interactions in the pentasymmetron significantly decrease due to the relative rotation of p72 capsomers caused by higher curvature in the pentasymmetron. Overall, the "back to back" contacts in the zipper and all three modes of contacts in the pentasymmetron seem insufficient to facilitate the assembly of higher-order structures, which suggests that minor capsid proteins are needed to build up the networks, triggering the assembly of the whole capsid.

4 Extensive intermolecular networks from minor capsid proteins underpin capsid stability

Correlated with fewer contacts among capsomers, all three minor capsid proteins and the penton are included to strengthen the interactions in the pentasymmetron. The density that forms the penton at the fivefold vertices of the outer capsid exhibits a single jelly-roll and a globular cap. This indicates the penton protein is different from yet homologous with the p72, which is consistent with structural observations in some known large dsDNA viruses (e. g. , PBCV-1, Mavirus, PRD-1 and more). Coincidentally, H240R, 240-residues in length enriched with β strands, is predicted to possess a single jelly-roll fold and an ~70-amino-acid N-terminal extension, which further verifies the identity of the penton in ASFV. Underneath the pentons, weak lantern-like densities (~9 Å) that connect the penton to inner membrane and associate with five neighboring capsomers (a) are observed, presumably playing roles in the assembly of the vertices. Previous studies reported that the putative capsid protein p49 (B438L) is required for the formation of the capsid vertices [18] and is located in close proximity to the capsid vertices (5). Additionally, p49 behaving as an integral membrane protein, is not involved in particle transportation from the virus assembly sites to the plasma membrane [18], suggesting that p49 might be positioned at the inner shell of capsid. Together with functional investigations and our structural analysis, it allows us to propose that lantern-like densities are five copies of p49.

Inner membrane protein p17 (D117L) is an essential and abundant protein, required for the assembly of the capsid and icosahedral morphogenesis [19]. The snake-shaped structure consisting of three continuous alpha-helices matches well with the secondary structure prediction of the ectodomain of p17, together with protein abundance level analysis, suggesting its identity to be p17. Interestingly, tight interdigitations of three p17s from three adjacent p72 capsomers mediate interactions amongst three capsomers under the type 1 microenvironment, along with spontaneous interactions among three capsomers under the type 1′ microenvironment, guiding an ordered packing of the capsomers for most parts on the capsid, mainly within the trisymmetron.

Microenvironments within pentasymmetrons and zippers are more complicated, where the skeleton protein M1249L emerges to hold 34 capsomers together, not only fixing one pentasymmetron, but also linking two neighboring pentasymmetrons (Fig. 1D). The 1249-amino acid—long M1249L, predicted to be full of coils, essentially exists in the virion and exhibits a fiber-like configuration with two terminal lobes (~150 residues/lobe), the fiber part is about 100 nm in length and possesses 30 extended helices (~30 residues/helix) (Fig. 1D). The capsomer-pairs (b and c, B and C), which normally show the weakest interactions (~150 $Å^2$) in the mode of "back to back" (types 3′ and 3), are now held together by the two lobes of M1249L, which function as two boat anchors.

5 The proposed assembly pathway for the ASFV capsid

ASFV assembly begins with the appearance of viral inner membrane precursors, which presumably derive from the endoplasmic reticulum (ER) and then evolve into icosahedral intermediates and icosahedral particles by the progressive assembly of the capsid layer [19, 20]. Taking together previous observations on viral morphogenesis process and our structural analysis, we could propose a capsid assembly model for ASFV. Firstly, the ability of p49 to associate with membrane mediates the docking of the penton complex to the inner membrane, then the penton complex recruits capsomers (a) to form the penton core (Fig. 2), initiating the assembly. Secondly, the skeleton unit M1249L with two capsomer pairs (b-c and B-C)

attaches to the penton core, meanwhile, skeleton units, penton cores and p17 could move on the inner membranes, making it possible to form high order assemblies (Fig. 2). Under the guide of p17, capsomers, skeleton proteins M1249L and p17 contribute to the formation of the zippers, which connect neighboring penton cores and gradually construct a polyhedral framework (Fig. 2). Accompanying the formation of the polyhedral framework, capsomers fill in the trisymmetrons to complete the capsid assembly (Fig. 2). In our model, skeleton protein M1249L serves as the backbone for the construction of the capsid framework and determines the size of capsid.

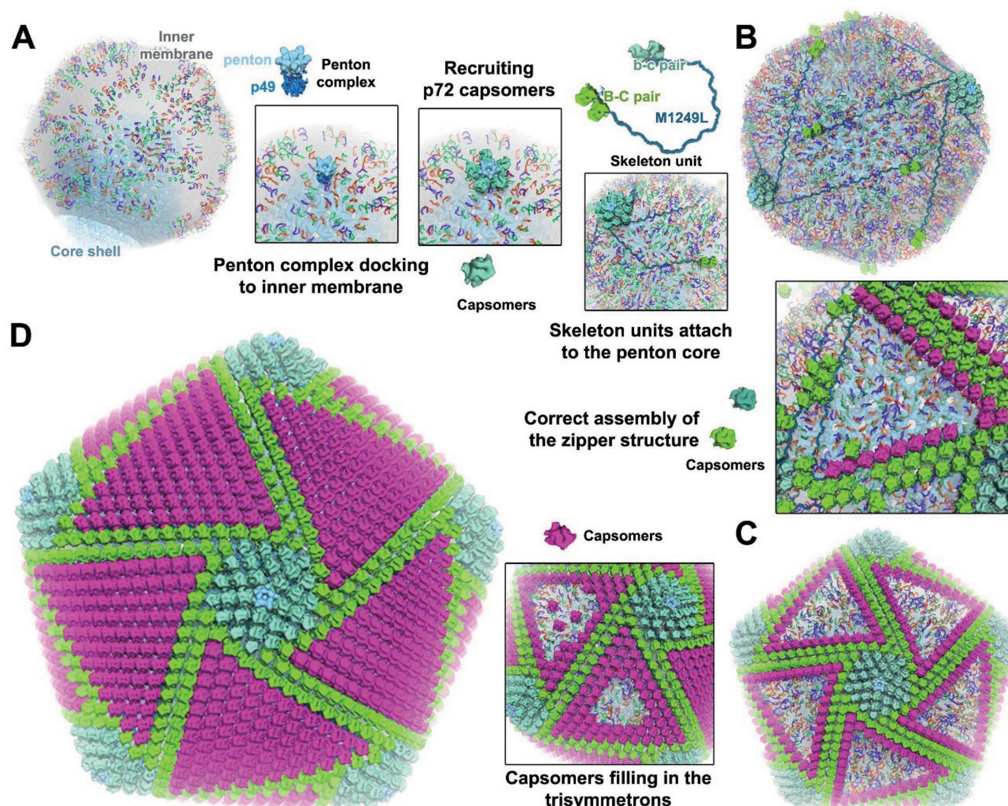

Figure 2 The proposed assembly pathway for the ASFV capsid.

6 Conclusions and perspectives

The first ASFV architecture at near-atomic resolution reported here allows the first steps toward understanding what drives the assembly of the capsid and the basis for its stability. In addition, the structural details, p72 atomic structure in particular, can guide the rational design of an epitope-focused immunogen, which will have profound impacts on the development of new strategies for vaccine intervention against ASFV infections. The study highlights the ability of the subunits and the role of their intermolecular interactions in overriding constraints imposed by symmetry during formation of the capsid. Local perturbations in symmetry seem to be well tolerated and probably play a role in the assembly of the capsids. The capsid acting as a research platform together with the atomic models can be utilized to further explore the molecular mechanisms underlying the infective cycle, unveiling the space-time complexity in the ASFV life cycle and may ultimately inform future prevention and therapeutic invention.

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