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African Swine Fever Virus Proteinase Is Essential for Core Maturation and Infectivity

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African swine fever virus (ASFV) encodes two polyprotein precursors named pp220 and pp62 that are sequentially processed during viral infection, giving rise to six major structural proteins. These reside at the core shell, a matrix domain located between the endoplasmic reticulum-derived inner envelope and the DNA-containing nucleoid. Proteolytic processing of the polyprotein precursors is catalyzed by the viral proteinase pS273R, a cysteine proteinase that shares sequence similarity with the SUMO1-processing peptidases. We describe here the construction and characterization of an ASFV recombinant, vS273Ri, that inducibly expresses the ASFV proteinase. Using vS273Ri, we show that repression of proteinase expression inhibits polyprotein processing and strongly impairs infective virus production. Electron microscopic examination of vS273Ri-infected cells showed that inhibition of proteolytic processing leads to the assembly of defective icosahedral particles containing a noncentered electron-dense nucleoid surrounded by an abnormal core shell of irregular thickness. The analysis of purified extracellular defective particles revealed that they contain the unprocessed pp220 and pp62 precursors, as well as the major DNA-binding nucleoid proteins p10 and pA104R. Altogether, these results indicate that the proteolytic processing of the polyproteins is not required for their incorporation into the assembling particles nor for the incorporation of the DNA-containing nucleoid. Instead, the ASFV proteinase is involved in a late maturational step that is essential for proper core assembly and infectivity.

Virus-encoded proteinases are involved in a variety of processes, such as the control of viral protein expression and the assembly and maturation of the virus particles, as well as in the interaction of the virus with the host cell. Positive-strand RNA viruses and retroviruses encode polyproteins that are proteolytically cleaved by viral proteinases to yield the nonstructural and structural proteins required for their replication and morphogenesis (20, 27, 33). DNA viruses, such as adenoviruses and poxviruses, on the other hand, usually synthesize precursor proteins whose maturation by proteolytic removal of terminal peptides plays an essential role in virion formation (27). However, African swine fever virus (ASFV), which is also a deoxyvirus, uses polyprotein processing as a strategy to produce several major structural components (42, 43). The ASFV pS273R proteinase is a 30-kDa cysteine proteinase (4) that belongs to a recently identified family of enzymes, including the cellular SUMO-1-processing proteinases from eukaryotes (35), as well as the viral proteinases L3 of adenovirus (1) and I7 of vaccinia virus (VV) (11). All of the enzymes share a conserved homology domain of ca. 90 amino acids that contains the residues of the catalytic triad. Furthermore, all of them recognize similar cleavage sites in their substrates. Thus, the adenovirus L3 proteinase cleaves at the conserved (M,L,I)XGX-G or (M,L,I)XGG-X sites (46, 47) that are similar to the GG-X cleavage sites for SUMO-1-processing proteinases (30) and the ASFV proteinase (37) or the AG-X motif for the VV enzyme (44).

ASFV, the sole member of the *Asfarviridae* family (19), is an icosahedral, enveloped virus (17, 41, 45) that encodes more than 150 polypeptides, 30 to 50 of which are components of the virus particle (12, 23, 48). ASFV assembles within discrete cytoplasmic areas, which are close to the microtubule organizing center (29). Viral factories virtually exclude membrane organelles but are typically surrounded by a vimentin cage (13, 29) and recruit both mitochondria and cellular chaperones (29, 39). In a first step of the assembly process, endoplasmic reticulum cisternae are recruited and transformed to give rise to precursor viral membranes (2, 3, 40), which represent the first identifiable viral structures. Viral membranes become icosahedral particles by the gradual assembly of the outer capsid layer formed by protein p72 (2, 25) in an ATP- and calcium-dependent process (14). At the same time, the core shell is formed underneath the viral envelope and, subsequently, the viral DNA and nucleoproteins are packaged and condensed to form the electron-dense nucleoid (2, 6, 10). A fraction of the intracellular mature virions, which are infectious (5), is transported to the plasma membrane by a microtubule-mediated mechanism (18) that is dependent on the viral protein pE120R (5). These virions are then released by budding (9) to give rise to the infectious extracellular mature virions, which contain an additional lipid envelope.

Two polyproteins named pp220 and pp62, which are expressed late during infection, are sequentially processed by the ASFV proteinase, giving rise to six major structural proteins. Proteins p150, p37, p34, and p14, derived from the pp220 precursor (42), and proteins p35 and p15, derived from polyprotein pp62 (43), are present in equimolecular amounts in the mature viral particle, representing >30% of the total virion protein mass (2, 7). These six structural proteins, as well as the ASFV proteinase, are located at the same viral domain, the core shell, which functions like a matrix domain

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between the DNA-containing nucleoid and the inner envelope.

Proteolytic processing of the ASFV precursors is a tightly regulated process that is related to virus morphogenesis, as deduced from the analysis of several inducible ASFV recombinants. Thus, we have recently shown that the repression of precursor pp220 gene abrogates pp62 processing and leads to the assembly of core-less particles (6, 7). On the other hand, the repression of the major capsid protein p72 impairs proteolytic cleavage of both polyproteins pp220 and pp62 and leads to the assembly of aberrant "zipper-like" structures consisting of a core shell-like domain flanked by lipid membranes (25). Interestingly, similar structures are formed when both unprocessed precursors are coexpressed in transfected cells, which indicates that both polyproteins are capable of associating with each other (7).

In order to study the role of proteolytic processing during ASFV assembly, we constructed and characterized a recombinant virus that inducibly expresses the ASFV proteinase. We show that proteolytic processing is essential for virus infectivity and mediates a late maturation step of core assembly.

MATERIALS AND METHODS

Cells and viruses. Vero cells (ATCC CCL81) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS), which was reduced to 2% during viral infection. The Vero cell-adapted ASFV strain BA71V, as well as the vGUSREP recombinant virus, have already been described (21, 25). Highly purified extracellular virus was obtained by Percoll equilibrium centrifugation as described previously (12).

Antibodies. The monospecific rabbit polyclonal antisera to the structural proteins p150, p37/p14, p34, p35, p15, p72, and pS273R have been described previously (4, 5, 42, 43). Antibodies to proteins K78R and A104R have been described by Andrés et al. (6).

Plasmid construction. The S273R promoter together with a single copy of the *Escherichia coli lac* operator sequence were amplified by PCR with the oligonucleotides 5′priS273R (GCGCGGTACCCGGGCTATTAAAAAATAACAATAAAAACC) and 3′priS273R (GCGCGGATCCAATTGTTATCCGCTCACAATTCTATACATAGATTTCATAAGGTTTT). The amplified sequence contains a 44-bp sequence ranging from position −41 to +3 relative to the main transcription initiation site of the S273R gene (designated +1) (4), followed by a single copy of the 21-bp sequence of the *E. coli lac* operator sequence (8). The PCR product was digested with *Kpn*I and *Bam*HI and cloned into plasmid pUCS273R, generating plasmid ppriS273R. This plasmid contains the entire S273R open reading frame, preceded by the inducible promoter, with the 3′ end of the operator sequence situated 15 bp from the initiation codon. A left flanking region corresponding to a 475-bp sequence located immediately upstream of the initiation codon of the S273R open reading frame was amplified by PCR with the oligonucleotides 5′LFS273R (CGCGGTACCAAAGGCCTGATTTATACACG) and 3′LFS273R (CGCGGTACCTTTAGCGGCCACTATACATAG) and cloned into the *Kpn*I site of plasmid ppriS273R, generating plasmid pLFpriS273R. Finally, the cassette containing the strong ASFV late promoter of p72 followed by the *lacZ* reporter gene was obtained from plasmid p72gal10T (38) and cloned in both orientations into the single *Sma*I site of plasmid pLFpriS273R, located between both flanking regions. Thus, plasmids pIS273R(r) and pIS273R(l), from which the reporter gene is transcribed in the same or the opposite orientation as gene S273R, respectively, were obtained. All of the fragments obtained by PCR were checked for the absence of unwanted mutations by DNA sequencing.

Generation of recombinant vS273Ri virus. Recombinant viruses were obtained as described previously (38) with minor modifications. Briefly, Vero cells were infected with vGUSREP (25) and transfected with plasmid pIS273R(r) or pIS273R(l) in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). At 48 h postinfection (hpi), the cells were harvested, and recombinant viruses vS273Ri(r) and vS273Ri(l) were isolated by sequential rounds of plaque purification in the presence of 1 mM IPTG. The genomic structure of the recombinant viruses was confirmed by Southern blotting. The IPTG growth dependence of both viruses was determined, and the vS273Ri(r) virus was selected for further characterization and is referred to as vS273Ri.

Plaque assays. Preconfluent monolayers of Vero cells seeded on six-well plates were infected with 100 PFU of recombinant vS273Ri or BA71V. After 1 h, the inoculum was removed and the cells were overlaid with DMEM containing 0.6% Noble agar and 2% FCS in the absence or presence of 1 mM IPTG. Five days later, the medium was removed and the monolayers were stained with 1% crystal violet.

One-step virus growth curves. Vero cell monolayers in 24-well plates were infected with BA71V or recombinant vS273Ri at a multiplicity of infection of 5 PFU per cell. After a 1-h adsorption period, the cells were incubated in DMEM supplemented with 2% FCS, and 1 mM IPTG was added when needed. The infected cells with their medium were collected at different times postinfection and then sonicated, and the virus titers were determined by plaque assay in the presence of 1 mM IPTG.

Western blotting. Preconfluent Vero cell monolayers were either mock-infected or infected with BA71V or vS273Ri in the presence or absence of 1 mM IPTG at a multiplicity of infection of 10 PFU per cell. The cells were lysed at 18 hpi in Laemmli sample buffer, and equivalent amounts were electrophoresed in sodium dodecyl sulfate–12% polyacrylamide gels and transferred to nitrocellulose as described previously (2). Protein detection was carried out with peroxidase-conjugated antibodies and the ECL system (Amersham Pharmacia Biotech) according to the manufacturer's indications.

Electron microscopy. For conventional Epon section analysis, Vero cells were infected with 10 PFU of BA71V or vS273Ri per cell in the presence or absence of 1 mM IPTG. At 18 hpi, the cells were fixed with 2% glutaraldehyde in 200 mM HEPES (pH 7.4) for 1 h at room temperature. Postfixation was carried out with 1% OsO_4 and 1.5% $K_3Fe(CN)_6$ in H_2O at 4°C for 30 min. The samples were dehydrated with acetone and embedded in Epon according to standard procedures. Purified parental BA71V and recombinant vS273Ri particles were processed as described above. Specimens were examined in a JEOL 1010 electron microscope.

RESULTS

Generation of recombinant virus vS273Ri. To study the role of the ASFV proteinase pS273R during infection, we constructed the ASFV recombinant vS273Ri (Fig. 1A), in which the endogenous proteinase promoter was substituted by an IPTG-dependent version. This inducible promoter consists of a 42-bp sequence derived from the S273R promoter region that contains the transcription initiation site of gene S273R (4), as well as the predicted minimal promoter elements (26), followed by a single copy of the *E. coli lac* operator sequence O1. In the recombinant vS273Ri virus, the expression of the S273R gene is regulated by the *E. coli lac* repressor, which is encoded by a copy of the *lacI* gene inserted within the nonessential thymidine kinase locus (24, 25). The genomic structure of the resulting recombinant virus was confirmed by DNA hybridization analysis (data not shown).

Figure 1B shows one-step growth curves of recombinant vS273Ri virus in the presence or in the absence of 1 mM IPTG. Under permissive conditions, the virus titers of recombinant vS273Ri, determined by plaque assay, were similar to those obtained with parental BA71V virus. In contrast, under restrictive conditions, vS273Ri titers were significantly lower. The maximal difference in infectious virus production was observed at 18 hpi and corresponded to ca. 2 log units. At later time points of infection, this difference decreased slowly, but it remained still greater than 1 log unit at 48 hpi. We also tested the ability of vS273Ri grown for 18 h under nonpermissive conditions to produce infectious virus upon IPTG addition. It was found that virus titers did not increase for an additional period of 8 h compared to a vS273Ri infection maintained in the absence of IPTG, indicating that the inhibition of infectious virus production in cells infected with vS273Ri under restrictive conditions is not reversible (data not shown). Plaque for-

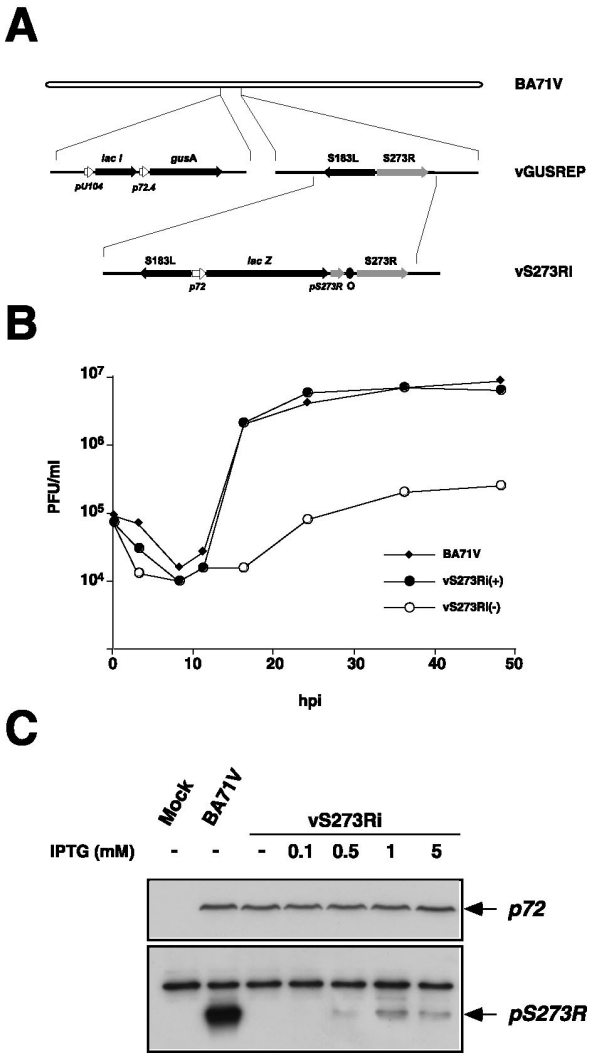

FIG. 1. (A) Genomic structure of the ASFV recombinant vS273Ri. The gene S273R is under the transcriptional control of an inducible promoter consisting of a minimal S273R promoter (*pS273R*) sequence, followed by the *lac* operator sequence (O). The inducible system is completed with the *lacI* gene encoding the *lac* repressor, which is inserted into the nonessential thymidine kinase locus. The reporter genes *lacZ* and *gusA*, used for selection of the recombinants, are also represented. (B) One-step growth curves of vS273Ri. Vero cells were infected with 5 PFU per cell of vS273Ri in the presence (+) or absence (−) of 1 mM IPTG. The samples were collected at the indicated times and titrated by plaque assay on fresh Vero cells in the presence of the inducer. Parental BA71V infectivity was also titrated as a control. (C) IPTG-dependent expression of protein pS273R. Vero cells were infected with BA71V or vS273Ri virus in the absence or presence of the indicated IPTG concentration. Samples were lysed at 18 hpi and analyzed by Western blotting with anti-p72 or anti-pS273R antibodies. Arrows indicate the position of p72 and pS273R proteins. The anti-pS273R antibody also recognizes a cellular protein present in similar amounts in mock-infected and infected cells.

mation by the recombinant vS273Ri was dependent on the presence of the inducer in the assay. Altogether, these results indicate that vS273Ri is a lethal conditional mutant.

pS273R expression is IPTG dependent in vS273Ri-infected cells. To evaluate the effect of IPTG on the synthesis of pS273R, Vero cells were mock infected or infected with parental BA71V or recombinant vS273Ri virus in the absence or in the presence of IPTG concentrations ranging from 0.1 to 5 mM and collected at 18 hpi. The samples were analyzed for pS273R expression by Western blotting (Fig. 1C). As a control, the expression of the major capsid protein p72 was examined on the same blot. In vS273Ri-infected cells, maximal pS273R expression was obtained at 1 mM IPTG. These maximal levels were, however, significantly lower than those observed in parental BA71V infections, thus suggesting that the IPTG-inducible promoter is weaker than the wild-type promoter. In spite of this, these proteinase levels were able to sustain a productive vS273Ri infection, as shown before (Fig. 1B). Importantly, the proteinase band was not detectable in the absence of IPTG, demonstrating that expression of pS273R can be strongly repressed in this inducible virus.

Repression of pS273R expression severely impairs proteolytic processing. To analyze the effect of pS273R repression on the processing of polyproteins pp220 and pp62, Vero cells were infected with the parental BA71V virus or with the recombinant vS273Ri virus in the presence or absence of IPTG. The cells were harvested at 18 hpi and analyzed by Western blotting with a panel of antibodies against the different polyprotein-derived products. As shown in Fig. 2, the processing of both polyproteins was severely impaired under restrictive condi-

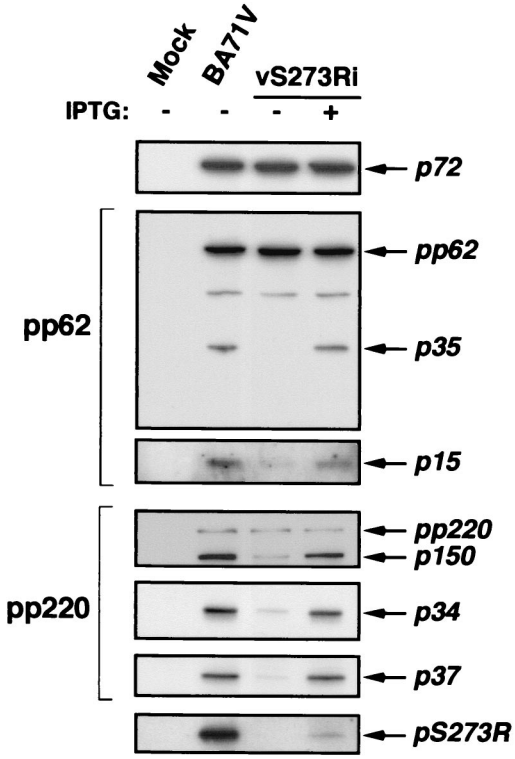

FIG. 2. Polyprotein processing in vS273Ri-infected cells. Vero cells were infected with BA71V in the absence of IPTG or with vS273Ri virus in the absence or presence of 1 mM IPTG and lysed at 18 hpi. The samples were analyzed by Western blotting with antibodies against the polyprotein pp62-derived products, p35 and p15, and against the polyprotein pp220-derived proteins p150, p34, and p37. Additionally, proteins p72 and pS273R were analyzed. Arrows indicate the different products of polyprotein processing and their precursors, as well as proteins p72 and pS273R.

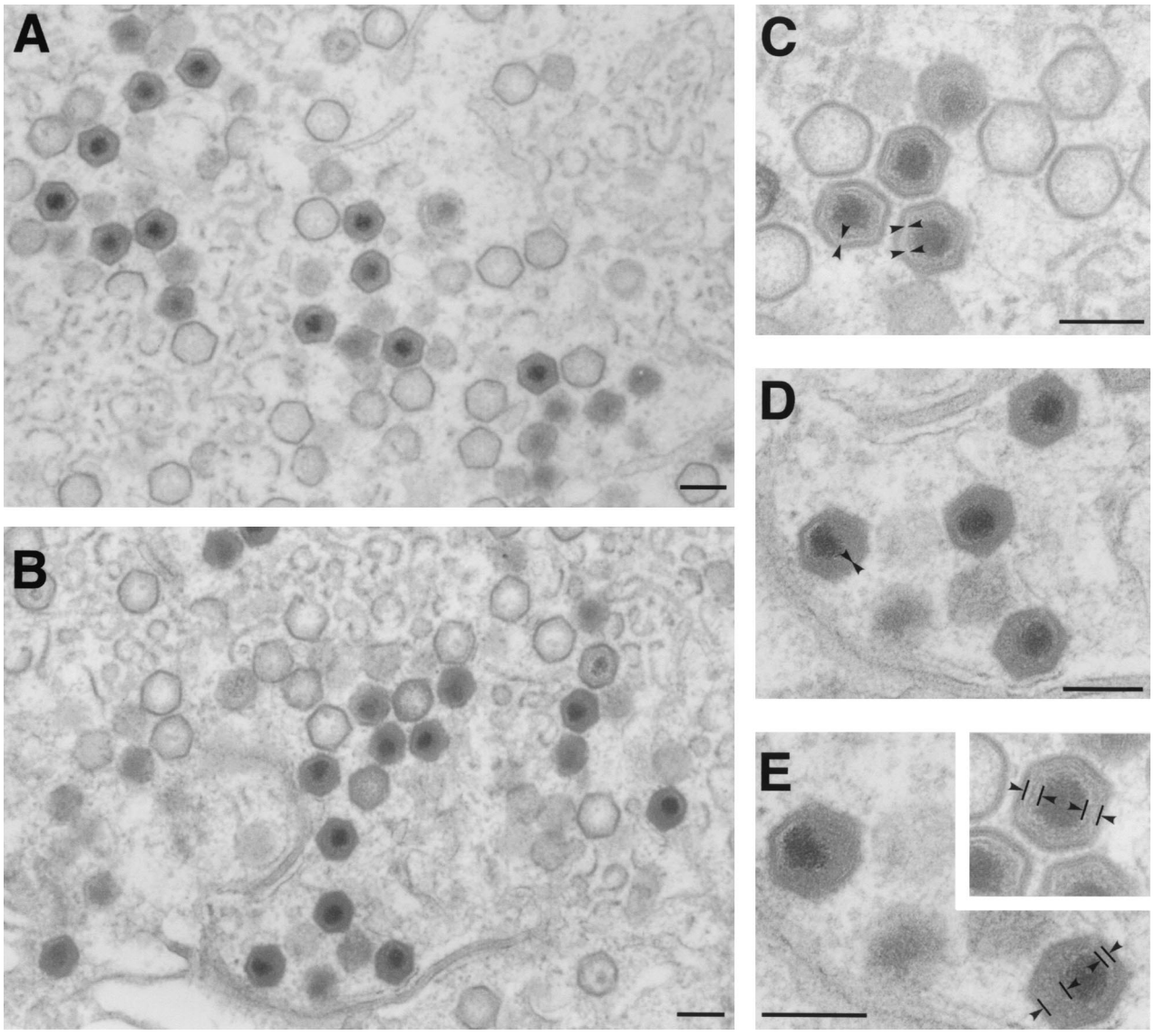

FIG. 3. Effect of proteinase repression on ASFV assembly. Vero cells were infected with parental BA71V (A, C, and E [inset]) or vS273Ri (B, D, and E) in the absence of IPTG and processed at 18 hpi for conventional Epon embedding. Note that the overall appearance of the viral factories is similar in both situations (A and B). However, when compared in detail with mature BA71V virions (C and inset in panel E), the full vS273Ri particles (D and E) clearly exhibit an abnormal core structure. Whereas the normal mature viruses contain a centered nucleoid surrounded by a regular core shell (delimited by arrows in the inset in panel E), the defective particles contain a noncentered nucleoid of irregular shape surrounded by a disorganized core shell (delimited by arrows in panel E). Note also the tight apposition of the aberrant core to the inner envelope in the vS273Ri particles (arrowheads in panel D) in contrast to the partial retraction of the core from the inner envelope in the parental BA71V virions (arrowheads in panel C). Bars, 200 nm.

tions. Thus, proteins p35 and p15, derived from pp62, were nearly undetectable when no IPTG was added during the vS273Ri infection. Similarly, the polyprotein pp220 products p150, p34, and p37 were weakly detected under these conditions. In the presence of IPTG, however, the amount of processed products was similar to that found during infection with the parental virus. The residual processing of the polyproteins observed in the absence of IPTG is probably due to the presence of very low levels of the proteinase, which are undetectable by Western blot (see Fig. 1 and 2). As also shown in Fig. 1, under permissive conditions, the proteinase levels were significantly lower than those observed in the BA71V infection. This low-level expression of the proteinase under permissive conditions was, however, sufficient to process polyproteins pp220 and pp62 efficiently. As can be seen, the level of protein p72 expression determined on the same blots showed no appreciable variation.

Polyprotein processing is required for proper core maturation. To determine the effect of the inhibition of polyprotein processing on virus assembly, we performed electron microscopy on Epon sections of Vero cells infected with BA71V or with vS273Ri in the presence or in the absence of IPTG and fixed at 18 hpi.

As shown in Fig. 3, the viral factories of parental BA71V

TABLE 1. Quantification of mature and defective virions in vS273Ri-infected Vero cells[a]

Virus	Mean TV/μm^2 ± SD	% EV	% FV	% MV	% DV
BA71V	4.4 ± 0.7	69	31	86	14
vS273Ri + IPTG	4.0 ± 0.7	64	36	70	30
vS273Ri − IPTG	3.7 ± 0.6	58	42	12	88

[a] Thin sections of BA71V-infected Vero cells or vS273Ri-infected cells in the presence or absence of IPTG and fixed at 18 hpi were examined under the electron microscope. In each case, the number of total icosahedral virus particles (TV) per factory was counted, and the average surface area of the factory was estimated. Among total virus particles, full particles (FV), which were considered either mature (MV) or defective (DV) particles based on the appearance of their core, and empty particles (EV) were counted. The percentage of each class of particle is indicated. More than 200 total particles were counted for each situation in at least four different viral factories.

(Fig. 3A) or nonpermissive vS273Ri (Fig. 3B) infections had a similar overall appearance, showing a similar distribution of assembly intermediates, as well as apparently "full" icosahedral virions. When examined in detail, however, evident differences were observed in the core structure of the full particles. As previously described (2), the core region of a normal virion consists of a 30-nm core shell (see Fig. 3C and E [inset]), which is composed by the polyprotein products and an 80-nm central electron-dense nucleoid that contains the viral genome. In contrast, and as shown in detail in Fig. 3D and E, the vast majority of the full vS273Ri particles obtained under restrictive conditions contained a disorganized core shell of uneven thickness and a noncentered nucleoid with a high degree of condensation and that frequently presents an irregular shape. Moreover, whereas the mature BA71V particles exhibited an apparent retraction of the core (Fig. 3C), the defective vS273Ri particles showed a close association between the viral core and the surrounding envelope (Fig. 3D).

To quantitatively assess this ultrastructural phenotype, we estimated the percentage of aberrant and normal full icosahedral virions present in the virus factories at 18 hpi in the presence or in the absence of the inducer. As shown in Table 1, the total density of apparently closed icosahedral particles was similar in vS273Ri factories, either in the presence or in the absence of IPTG, and in the assembly sites of parental BA71V infections. However, whereas the proportion of particles containing an aberrant core was 30% of the total full virions under permissive conditions or 14% in BA71V-infected cells, it was close to 90% in vS273Ri infections performed under restrictive conditions.

In a further approach, extracellular vS273Ri particles obtained in the absence of IPTG at 36 hpi were purified by Percoll gradient centrifugation to analyze their protein composition and morphology. The mutant vS273Ri particles were found to sediment at the same position in the gradient as BA71V particles (data not shown). Purified mutant vS273Ri particles and the parental BA71V virions were further processed for electron microscopy. Compared to the mature parental virions (Fig. 4A), the mutant extracellular vS273Ri particles (Fig. 4B) presented the abnormal core, with the defect in nucleoid localization and core shell structure that is observed in the intracellular vS273Ri virions. This indicates that the core-defective full virions represent final particles produced in the absence of polyprotein processing.

Next, equivalent particle amounts of mutant and parental virions, as judged by their p72 protein content, were analyzed on Western blots for the presence of the polyprotein products. As shown in Fig. 4C, the processing products p34 and p35, derived from pp220 and pp62 polyproteins, respectively, were essentially absent from the defective virions; instead, the pp220 and pp62 unprocessed precursors were clearly present. Finally, we analyzed the defective vS273Ri particles for their content in the major DNA-binding proteins p10 and pA104R, which reside at the nucleoid. As is apparent, both proteins are present in amounts similar to those of the parental BA71V particles. Altogether, these results indicate that the two polyproteins can be incorporated into the virus particles as unprocessed precursors and that proteolytic processing represents a late maturational step necessary for the correct organization of the internal virus domains.

DISCUSSION

In this study we have analyzed the role of polyprotein processing in ASFV replication by means of a recombinant virus that inducibly expresses the proteinase pS273R. To construct this virus, we modified the endogenous S273R promoter to obtain an IPTG-regulated version that allows a tight repression of proteinase expression. Using the recombinant vS273Ri virus we have shown that the ASFV proteinase is essential for the production of infectious virus particles. Thus, under repressive conditions, the yield of infectious virus in one-step growth curves was ca. 2 log units lower than that obtained in the presence of IPTG or with the parental virus. This finding correlates with a strong inhibition of the proteolytic processing of polyproteins pp220 and pp62. Despite the low levels of proteinase expression under permissive conditions, both virus

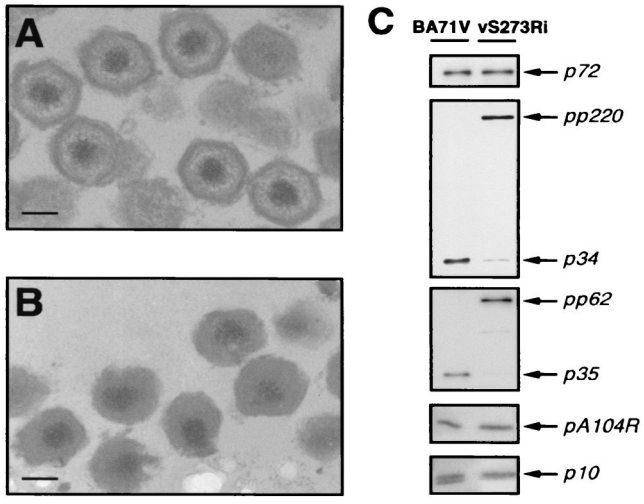

FIG. 4. Analysis of Percoll-purified defective vS273Ri particles. Parental BA71V (A) and defective vS273Ri (B) viruses were collected from the culture medium at 36 hpi and purified through a Percoll density gradient. Virions were fixed with glutaraldehyde and processed for conventional electron microscopy. Bars, 100 nm. (C) Equivalent amounts of vS273Ri and BA71V particles were analyzed by Western blotting with antibodies against the proteolytic processing products of polyproteins pp220 and pp62 (p34 and p35, respectively), the major DNA-binding proteins pA104R and p10 or the capsid protein p72.

yield and proteolytic processing were found to occur as efficiently as in control BA71V infections. This can probably be explained by the fact that the regulated gene product is an enzyme.

Electron microscopic examination of vS273Ri-infected cells demonstrated that the impairment of proteolytic processing results in the assembly at the viral factories of full, icosahedral virions with an abnormal core structure. This deficient core consists of a misplaced electron-dense nucleoid with irregular shape, which is surrounded by a disorganized core shell of uneven thickness. Also, unlike mature virions, whose core structure seems to be somewhat detached from the surrounding inner envelope, the defective cores appear to be tightly associated with it. This mutant phenotype seems to be irreversible, since the induction of pS273R expression in vS273Ri-infected cells previously maintained under restrictive conditions did not increase virus yield. This suggests that newly synthesized proteinase has no access to the previously assembled mutant particles. Analysis of the purified mutant vS273Ri particles showed that they contain the unprocessed pp220 and pp62 precursors, as well as the major DNA-binding proteins p10 and pA104R. Therefore, the incorporation of the polyprotein precursors and of some of the major components of the genome-containing nucleoid is independent of polyprotein processing. These findings are consistent with previous results showing that the two unprocessed precursors are capable of associating with each other to form a core shell-like structure when coexpressed in transfected cells (7). Moreover, pulse-chase experiments suggest that the proteolytic cleavage of pp220 and pp62 precursors occurs after their incorporation into the assembling particles (7). Collectively, these results strongly indicate that polyprotein processing is involved in a later maturational step that would be essential for the organization of the core shell and the DNA-containing nucleoid. This process could be accomplished by the presentation of motifs that are masked on the unprocessed precursors and that mediate a stable interaction with one or several components of the internal nucleoid.

Intriguingly, infection with an ASFV Malawi strain recombinant lacking gene 9GL, homologous to yeast ERV1 (36), leads to the formation of particles with a phenotype characterized by the presence of an irregular and acentric nucleoid (34), very similar to the one described for the defective vS273Ri particles. This raises the interesting question of whether polyprotein processing is also prevented in cells infected with the 9GL deletion mutant.

VV, which shares a number of properties with ASFV (41), encodes, as mentioned above, a proteinase with sequence similarity to the SUMO-1-processing proteinases. It has been shown that proteolytic processing of the VV core proteins P4a, P4b, and VP8 is tightly coupled to particle assembly, and its absence has been extensively used as an indicator of a defect in viral morphogenesis. A thermosensitive vaccinia mutant, *ts*16, that maps to the proteinase gene I7 (15, 16), shows, when grown under restrictive conditions, a blockage in virus morphogenesis with the generation of spherical, not fully sealed particles. These particles contain the unprocessed P4a, P4b, and P28 precursors, a noncentered, dense nucleoid and essentially the same protein composition than that of intracellular mature virus particles obtained under permissive conditions (22, 31). Thus, a feature shared by VV and ASFV is that proteolytic processing appears to be involved in a maturational step of the core that allows the proper packaging of the nucleoid and formation of an infectious particle.

An interesting question is the reason for the lack of infectivity of the defective vS273Ri particles. One possibility is that the defective particles fail to incorporate one or more essential structural components. Alternatively, the aberrant formation of the core might preclude some crucial early step in infection, such as the uncoating process itself. Thus, in the absence of processing, the entire core could remain bound to the inner envelope through the N-terminal myristic moiety of unprocessed polyprotein pp220 (6), whereas, after proteolytic processing, only the N-terminal 44-amino-acid fragment of pp220 would remain bound to the envelope. In support of this view is the observation of a close association between the viral core and the surrounding envelope in the defective vS273Ri particles and the core retraction in the normal mature particles. This anomaly in the defective particles might hinder the disassembly process, as has been postulated for the uncoating of the incoming particle during human immunodeficiency virus infection (32, 49). In addition, the presence of the ASFV proteinase in the mature virions (4) raises the possibility that the proteinase activity itself is needed for the disassembly process of the incoming virion, as has been demonstrated for the adenovirus proteinase (28). Exploration of these possibilities may further contribute to our understanding of the role of polyprotein processing in virus morphogenesis and infectivity.

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REFERENCES

1. **Anderson, C. W.** 1990. The proteinase polypeptide of adenovirus serotype 2 virions. Virology **177:**259–272.
2. **Andrés, G., C. Simón-Mateo, and E. Viñuela.** 1997. Assembly of African swine fever virus: role of polyprotein pp220. J. Virol. **71:**2331–2341.
3. **Andrés, G., R. García-Escudero, C. Simón-Mateo, and E. Viñuela.** 1998. African swine fever virus is enveloped by a two-membraned collapsed cisterna derived from the endoplasmic reticulum. J. Virol. **72:**8988–9001.
4. **Andrés, G., A. Alejo, C. Simón-Mateo, and M. L. Salas.** 2001. African swine fever virus protease, a new viral member of the SUMO-1-specific protease family. J. Biol. Chem. **276:**780–787.
5. **Andrés, G., R. García-Escudero, E. Viñuela, M. L. Salas, and J. M. Rodríguez.** 2001. African swine fever virus structural protein pE120R is essential for virus transport from assembly sites to plasma membrane but not for infectivity. J. Virol. **75:**6758–6768.
6. **Andrés, G., R. García-Escudero, M. L. Salas, and J. M. Rodríguez.** 2002. Repression of African swine fever virus polyprotein pp220-encoding gene leads to the assembly of icosahedral core-less particles. J. Virol. **76:**2654–2666.
7. **Andrés, G., A. Alejo, J. Salas, and M. L. Salas.** 2002. African swine fever virus polyproteins pp220 and pp62 assemble into the core shell. J. Virol. **76:**12473–12482.
8. **Betz, J. L., and J. R. Sadler.** 1981. Variants of a cloned synthetic operator. I. A palindromic dimer lactose operator derived from one strand of the cloned 40-base-pair operator. Gene **13:**1–12.
9. **Breese, S. S., Jr., and C. J. DeBoer.** 1966. Electron microscope observation of African swine fever virus in tissue culture cells. Virology **28:**420–428.
10. **Brookes, S. M., A. D. Hyatt, T. Wise, and R. M. Parkhouse.** 1998. Intracellular virus DNA distribution and the acquisition of the nucleoprotein core during African swine fever virus particle assembly: ultrastructural in situ hybridisation and DNase-gold labelling. Virology **249:**175–188.

11. **Byrd, C. M., T. C. Bolken, and D. E. Hruby.** 2002. The vaccinia virus I7L gene product is the core protein proteinase. J. Virol. **76:**8973–8976.
12. **Carrascosa, A. L., M. del Val, J. F. Santarén, and E. Viñuela.** 1985. Purification and properties of African swine fever virus. J. Virol. **54:**337–344.
13. **Carvalho, Z. G., A. P. De Matos, and C. Rodrigues-Pousada.** 1988. Association of African swine fever virus with the cytoskeleton. Virus Res. **11:**175–192.
14. **Cobbold, C., S. M. Brookes, and T. Wileman.** 2000. Biochemical requirements of virus wrapping by the endoplasmic reticulum: involvement of ATP and endoplasmic reticulum calcium store during envelopment of African swine fever virus. J. Virol. **74:**2151–2160.
15. **Condit, R. C., and A. Motyczka.** 1981. Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. Virology **113:**224–241.
16. **Condit, R. C., A. Motyczka, and G. Spizz.** 1983. Isolation, characterization, and physical mapping of temperature-sensitive mutants of vaccinia virus. Virology **128:**429–443.
17. **Costa, J. V.** 1990. African swine fever virus, p. 247–270. *In* G. Darai (ed.), Molecular biology of iridoviruses. Kluwer Academic Publishers, Boston, Mass.
18. **de Matos, A. P., and Z. G. Carvalho.** 1993. African swine fever virus interaction with microtubules. Biol. Cell **78:**229–234.
19. **Dixon, L. K., J. V. Costa, J. M. Escribano, D. L. Rock, E. Viñuela, and P. J. Wilkinson.** 2000. The *Asfarviridae*, p. 159–165. *In* M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner (ed.), Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses. Academic Press, Inc., San Diego, Calif.
20. **Dougherty, W. G., and B. L. Semler.** 1993. Expression of virus-encoded proteinases: functional and structural similarities with cellular enzymes. Microbiol. Rev. **57:**781–822.
21. **Enjuanes, L., A. L. Carrascosa, M. A. Moreno, and E. Viñuela.** 1976. Titration of African swine fever (ASF) virus. J. Gen. Virol. **32:**471–477.
22. **Ericsson, M., S. Cudmore, S. Shuman, R. C. Condit, G. Griffiths, and J. Krijnse Locker.** 1995. Characterization of *ts*16, a temperature-sensitive mutant of vaccinia virus. J. Virol. **69:**7072–7086.
23. **Esteves, A., M. I. Marques, and J. V. Costa.** 1986. Two-dimensional analysis of African swine fever virus proteins and proteins induced in infected cells. Virology **152:**192–206.
24. **García, R., F. Almazán, J. M. Rodríguez, M. Alonso, E. Viñuela, and J. F. Rodríguez.** 1995. Vectors for the genetic manipulation of African swine fever virus. J. Biotechnol. **40:**121–131.
25. **García-Escudero, R., G. Andrés, F. Almazán, and E. Viñuela.** 1998. Inducible gene expression from African swine fever virus recombinants: analysis of the major capsid protein p72. J. Virol. **72:**3185–3195.
26. **García-Escudero, R., and E. Viñuela.** 2000. Structure of African swine fever virus late promoters: requirement of a TATA sequence at the initiation region. J. Virol. **74:**8176–8182.
27. **Gorbalenya, A. E., and E. J. Snijder.** 1996. Viral cysteine proteinases. Persp. Drug Discov. Design **6:**64–86.
28. **Greber, U. F., P. Webster, J. Weber, and A. Helenius.** 1996. The role of the adenovirus proteinase on virus entry into cells. EMBO J. **15:**1766–1777.
29. **Heath, C. M., M. Windsor, and T. Wileman.** 2001. Aggresomes resemble sites specialized for virus assembly. J. Cell Biol. **153:**449–455.
30. **Kamitani, T., H. P. Nguyen, and E. T. Yeh.** 1997. Preferential modification of nuclear proteins by a novel ubiquitin-like molecule. J. Biol. Chem. **272:**14001–14004.
31. **Kane, E. M., and S. Shuman.** 1993. Vaccinia virus morphogenesis is blocked by a temperature-sensitive mutation in the I7 gene that encodes a virion component. J. Virol. **67:**2689–2698.
32. **Kiernan, R. E., A. Ono, G. Englund, and E. O. Freed.** 1998. Role of matrix in an early postentry step in the human immunodeficiency virus type 1 life cycle. J. Virol. **72:**4116–4126.
33. **Kräusslich, H.-G., and E. Wimmer.** 1988. Viral proteinases. Annu. Rev. Biochem. **57:**701–754.
34. **Lewis, T., L. Zsak, T. G. Burrage, Z. Lu, G. F. Kutish, J. G. Neilan, and D. L. Rock.** 2000. An African swine fever virus *ERV1-ALR* homologue, *9GL*, affects virion maturation and viral growth in macrophages and viral virulence in swine. J. Virol. **74:** 1275–1285.
35. **Li, S.-J., and M. Hochstrasser.** 1999. A new protease required for cell cycle progression in yeast. Nature **398:**246–251.
36. **Lisowski, T.** 1992. Dual function of a new nuclear gene for oxidative phosphorylation and vegetative growth in yeast. Mol. Gen. Genet. **232:**58–64.
37. **López-Otín, C., C. Simón-Mateo, L. Martínez, and E. Viñuela.** 1989. Gly-Gly-X, a novel consensus sequence for the proteolytic processing of viral and cellular proteins. J. Biol. Chem. **264:**9107–9110.
38. **Rodríguez, J. M., F. Almazán, E. Viñuela, and J. F. Rodríguez.** 1992. Genetic manipulation of African swine fever virus: construction of recombinant viruses expressing the β-galactosidase gene. Virology **188:**67–76.
39. **Rojo, G., M. Chamorro, M. L. Salas, E. Viñuela, J. M. Cuezva, and J. Salas.** 1998. Migration of mitochondria to viral assembly sites in African swine fever virus-infected cells. J. Virol. **72:**7583–7588.
40. **Rouiller, I., S. M. Brookes, A. D. Hyatt, M. Windsor, and T. Wileman.** 1998. African swine fever virus is wrapped by the endoplasmic reticulum. J. Virol. **72:**2373–2387.
41. **Salas, J., M. L. Salas, and E. Viñuela.** 1999. African swine fever virus: a missing link between poxviruses and iridoviruses?, p. 467–480. *In* E. Domingo, R. G. Webster, and J. J. Holland (ed.), Origin and evolution of viruses. Academic Press, Ltd., London, United Kingdom.
42. **Simón-Mateo, C., G. Andrés, and E. Viñuela.** 1993. Polyprotein processing in African swine fever virus: a novel gene expression strategy for a DNA virus. EMBO J. **12:**2977–2987.
43. **Simón-Mateo, C., G. Andrés, F. Almazán, and E. Viñuela.** 1997. Proteolytic processing in African swine fever virus: evidence for a new structural polyprotein, pp62. J. Virol. **71:**5799–5804.
44. **Vanslyke, J. K., S. S. Whitehead, E. M. Wilson, and D. E. Hruby.** 1991. The multistep proteolytic maturation pathway utilized by vaccinia virus P4a protein: a degenerate conserved cleavage motif within core proteins. Virology **183:**467–478.
45. **Viñuela, E.** 1987. Molecular biology of African swine fever virus, p. 31–49. *In* Y. Becker (ed.), African swine fever. Nijhoff, Boston, Mass.
46. **Webster, A., W. C. Russell, and G. D. Kemp.** 1989. Characterization of the adenovirus proteinase: development and use of a specific peptide assay. J. Gen. Virol. **70:**3215–3223.
47. **Webster, A., S. Russell, P. Talbot, W. C. Russell, and G. D. Kemp.** 1989. Characterization of the adenovirus proteinase: substrate specificity. J. Gen. Virol. **70:**3225–3234.
48. **Yáñez, R. J., J. M. Rodríguez, M. L. Nogal, L. Yuste, C. Enríquez, J. F. Rodríguez, and E. Viñuela.** 1995. Analysis of the complete nucleotide sequence of African swine fever virus. Virology **208:**249–278.
49. **Zhou, W., and M. D. Resh.** 1996. Differential membrane binding of the human immunodeficiency virus type 1 matrix protein. J. Virol. **70:**8540–8548.