African Swine Fever Virus Protease, a New Viral Member of the SUMO-1-specific Protease Family*

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African swine fever virus (ASFV) is a complex DNA virus that employs polyprotein processing at Gly-Gly-Xaa sites as a strategy to produce several major core components of the viral particle. The virus gene S273R encodes a 31-kDa protein that contains a “core domain” with the conserved catalytic residues characteristic of SUMO-1-specific proteases and the adenovirus protease. Using a COS cell expression system, it was found that protein pS273R is capable of cleaving the viral polyproteins pp62 and pp220 in a specific way giving rise to the same intermediates and mature products as those produced in ASFV-infected cells. Furthermore, protein pS273R, like adenovirus protease and SUMO-1-specific enzymes, is a cysteine protease, because its activity is abolished by mutation of the predicted catalytic histidine residues and is inhibited by sulfhydryl-blocking reagents. Protein pS273R is expressed late after infection and is localized in the cytoplasmic viral factories, where it is found associated with virus precursors and mature virions. In the virions, the protein is present in the core shell, a domain where the products of the viral polyproteins are also located. The identification of the ASFV protease will allow a better understanding of the role of polyprotein processing in virus assembly and may contribute to our knowledge of the emerging family of SUMO-1-specific proteases.

Positive strand RNA viruses and retroviruses encode polyproteins, which are proteolytically cleaved by viral proteases to yield the nonstructural and structural proteins required for replication and morphogenesis (1–3). On the other hand, DNA viruses, such as adenoviruses and poxviruses, synthesize precursor proteins whose maturation by proteolytic enzymes, is a cysteine protease, because its activity is dependent on this modification (20). Li and Hochstrasser (16) have also shown that a “core domain” of −90 amino acids of the yeast enzyme have been identified in vertebrates (17–19). These proteases would regulate the SUMO-1-modified state of certain proteins whose function in a variety of cellular processes, such as cell cycle progression and nuclear import, is dependent on this modification (20). Li and Hochstrasser (16) have also shown that a “core domain” of −90 amino acids of the yeast protease, containing the conserved catalytic cysteine and histidine residues, presents some similarity to gene products from several animal viruses, including the adenovirus L3 protease, the I7 products of fowlpox and vaccinia virus, and open reading frame (ORF) S273R of ASFV. This suggested that the S273R product might be the protease involved in the processing of the ASFV polyproteins. We show here, using an expression system in COS cells, the specific processing of polyproteins pp62 and pp220 in the presence of protein pS273R. Furthermore, inhibitor profiling and site-directed mutagenesis results indicate that this protein is a cysteine protease. Studies on the synthesis and localization of protein pS273R during ASFV infection are also presented. The results are consistent with a role for the virus protease in the assembly of the virus core.

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1 The abbreviations used are: ASFV, African swine fever virus; kb, kilobase(s); ORF, open reading frame; Z-LVG-CHN$_2$, benzoyloxycarbonyl-leucyl-valyl-glycyl-diazomethylketone; E-64, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane.

2 G. Andrés, unpublished results.
**EXPERIMENTAL PROCEDURES**

**Cells and Viruses**—Vero and COS-7 cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. The Vero-adapted ASFV strain BA71V was propagated and titrated as described previously (21). Highly purified ASFV was prepared as described (22).

The recombinant vaccinia virus vTF7-3 expressing bacteriophage T7 RNA polymerase (23) was kindly donated by Dr. B. Moss.

**Plasmids and Site-directed Mutagenesis**—Plasmid pRSET-S273R carrying the ASFV S273R gene as a N-terminal histidine fusion was obtained as follows. The S273R gene was amplified by polymerase chain reaction using oligonucleotides 273-PCR1 (5'-CCGCGTCG-GAGATGTCTATATTAGAAAAATTACGT) and 273-PCR2 (5'-GGCG-GCAGTTCTATGCGATGCGAAGACGATGCT, digested with XhoI and EcoRI and inserted into the pRSET vector (Invitrogen).

The S273R gene was cloned from pRSET-S273R into the T7 promoter containing mammalian expression vector pcDNA 3.1 (Invitrogen), and the recombinant plasmid pcDNA-S273R was used for activity assays in transfection experiments. Site-directed mutagenesis of the S273R gene was performed by polymerase chain reaction (24) using as a template the pcDNA-S273R plasmid. The histidine residue at position 168 was mutated to arginine (S273R,H168R mutant) and cysteine 232 was mutated to serine (S273R,C232S mutant). Amplified mutant fragments corresponding to the complete S273R gene were cloned into the pcDNA 3.1 vector, and the presence of the desired mutation and absence of additional changes were confirmed by sequencing of the resulting plasmids. The recombinant plasmid KS-CP530R containing ORF CP530R, which encodes the p62 polyprotein of 530 amino acids under the control of the T7 polymerase promoter, was obtained as described (7).

The plasmid KS-p17C, used for the generation of antibodies against both polyprotein pp62 and its mature p53 product, was obtained as follows. The plasmid KS-CP530R was digested with PstI, blunted with Mung Bean exonuclease, and then digested with MluI and filled in with Klenow fragment. After ligation, this recombinant plasmid lacks the region encoding the polyprotein residues 1–167 of pp62 polyprotein. This plasmid was again digested with XhoI to remove the 3' end of ORF CP530R spanning amino acid residues 343–530.

Plasmid pGEM-CP2475L was constructed by cloning the 0.7-kb XhoI/XbaI fragment from plasmid pAR-pS273 (46) and the 6.9-kb PstI/XbaI fragment of the RC'-restriction fragment of the ASFV genome (5) into vector pGEM-4 (Promega). The resulting plasmid contained the complete CP2475L ORF, encoding polyprotein pp220, under the control of the T7 polymerase promoter.

**Antibodies**—To prepare antibodies against protein pS273R, *Escherichia coli* BL21(DE3) cells harboring the pRSET-S273R plasmid were induced for 1.5 h with 0.4 mM isopropyl-β-D-thiogalactopyranoside, and the recombinant protein was purified under denaturing conditions using single-step Ni²⁺-nitrilotriacetic acid affinity chromatography. Antibodies against the purified recombinant pS273R protein were raised in rabbits.

The mouse monoclonal antibody 18H.H7, which recognizes both the ASFV polyprotein pp62 and its mature product p50, was used in the immunofluorescence studies. This antibody, as well as the rabbit polyclonal anti-p150, anti-p34, and anti-p37/p14 sera, which also recognize polyprotein pp220, have been previously characterized (6, 8, 25).

The rabbit polyclonal serum against proteins pp62 and p53 was raised against the 17-kDa recombinant protein encoded by plasmid KS-p17C. For this, KS-p17C′ transformed *E. coli* BL21(DE3) cells were induced for 2 h with 1 mM isopropyl-β-D-thiogalactopyranoside and then incubated for 2 h with rifampicin (0.2 mg/ml). After lysis and centrifugation, the pellet containing the recombinant protein as the major component was used to generate antibodies in rabbits. The rabbit polyclonal serum against ASFV polyprotein pp62 and its mature product p15 has already been described (7).

**Coexpression Experiments**—COS-7 cells were transfected with 250 ng of DNA²⁰ cells of the plasmids indicated in each case using LipofectAMINE Plus reagent (Life Technologies, Inc.) following the manufacturer's indications for 1 h at 37 °C. In cotransfection experiments, the percentage of cells transfected with pS273R was ½ of the polyprotein gene-containing plasmid. Subsequently, the cells were infected with vTF7-3 at a multiplicity of infection of 5 plaque-forming units/cell, allowing the expression of T7 RNA polymerase and thus both, the polyprotein and S273R genes. The infection was carried out in the presence of 40 μg/ml cytosine arabinoside to inhibit vaccinia virus late protein synthesis. The expression and processing of the polyprotein was analyzed at 16 h post-infection by Western blot, as indicated below.

**Preparation of Polyprotein or Protease-containing Extracts and in Vitro Assays of Processing Activity**—COS-7 cells were transfected/infection as described before using the corresponding plasmids. At 16 h post-infection, the cells were resuspended at 10⁶ cells/ml in homogenization buffer containing 20 mM HEPES, pH 7.4, 0.28 m sucrose, 2 mM EDTA, and passed through a 25-gauge syringe 15 times. The homogenate was centrifuged at 700 × g for 5 min to sediment nuclei and unbroken cells, and the supernatant fraction was subsequently centrifuged at 100,000 × g for 30 min at 4 °C to separate the soluble cytoplasm from the membrane/particulate material. The activity assays in vitro were performed using the soluble cytoplasmic fraction as source of protein pS273R and polyprotein pp62 and the membrane/particulate cytoplasmic fraction as a source of polyprotein pp220. Reactions were performed at 30 °C in a final volume of 50 μl containing 5 μl of both of protease and polyprotein-containing extracts diluted in 20 mM HEPES, pH 7.4. After the indicated times, the samples were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting.

For inhibitor studies, the reactions were incubated for 6 h as above in the absence or in the presence of the following protease inhibitors: 10 μM pepstatin, 1 mM 1,10-phenanthroline, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM N-ethylmaleimide, 100 mM benzamidoxycarbonyl-leucyl-valine-glycyl-glycyl-diazomethylketone (Z-LVG-CH₃), or 100 μM t-trans-epoxy-oxysuccinyl-leucylamidomethylketone-(4- guanidino)-butane (E-64). All the inhibitors were freshly prepared as a 100-fold concentrated stock solution in methanol with the exception of Z-LVG-CH₃ and E-64, prepared in dimethyl sulfoxide and water, respectively.

**Western Blot Analysis**—For Western blots, equivalent amounts of whole cell extracts or cytosolic fractions were electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with a 1:1,000 dilution of the antibodies and then with a 1:10,000 dilution of peroxidase-labeled anti-rabbit serum (Amersham Pharmacia Biotech), and the proteins were detected with the ECL system (Amersham Pharmacia Biotech) according to the manufacturer's recommendations.

**Preparation and Analysis of RNA**—Vero cells were mock-infected or infected with ASFV (BA71V strain) at a multiplicity of 20 plaque-forming units/cell. To obtain RNA, the cells were infected in the presence of 100 μg/ml cycloheximide or 40 μg/ml cytosine arabinoside for 7 h. Late RNA was isolated from cells infected for 18 h in the absence of inhibitors. Whole cell RNA was prepared by the guanidinium isothiocyanate/cesium chloride extraction procedure (26), and poly (A)⁺ RNA was selected by oligo (dT)-cellulose chromatography as described by Sambrook et al. (27). Northern blot hybridization was carried out as reported elsewhere (28) with 2 μg of each class of poly (A)⁺ RNA, using a 32P-end-labeled oligonucleotide S273R (5′-TACTTAAAAGGCTATCTTTGTTTGTAAGGT), complementary to nucleotides +79 to +50 of gene S273R. Primer extension analysis was performed essentially as described by Sambrook et al. (27). After 5′ end-labeling with 32P, the oligonucleotide S273R was annealed at 30 °C to the different classes of RNA and extended with avian myeloblastosis virus reverse transcriptase for 2 h at 42 °C. The primer extension products were then electrophoresed in a 6% polyacrylamide gel alongside an irrelevant DNA sequence reaction.

**Immunofluorescence Microscopy**—Vero cells grown on coverslips were mock-infected or infected with ASFV at a multiplicity of infection of 1 plaque-forming unit/cell and fixed at 18 h post-infection with methanol at −20 °C for 5 min. The cells were incubated with a 1:250 dilution of rabbit polyclonal anti-pS273R serum and a 1:10 dilution of mouse monoclonal (18H.H7) anti-p3220 antibody as indicated in the legend to Fig. 5 and incubated for 45 min at 37 °C with a 1:500 dilution of Alexa 488 goat anti-rabbit IgG and a 1:100 dilution of Alexa 594 goat anti-mouse IgG antibodies (Molecular Probes, Inc.). As a control, each antibody was individually tested. Cells were examined with an Axiovert fluorescence microscope (Carl Zeiss, Inc.).

**Immunoelectron Microscopy**—Vero cells were infected with ASFV at a multiplicity of infection of 10 plaque-forming units/cell and fixed, at 20 h post-infection, with 4% formaldehyde and 0.1% glutaraldehyde in 200 mM HEPES, pH 7.2, for 1 h at room temperature. After fixation, the cells were processed for cryosectioning as detailed by André et al. (29). Ultrathin thawed cryosections were incubated for 45 min at room temperature with a 1:10 dilution of the anti-pS273R antibody followed by an incubation of 30 min at room temperature with a 1:40 dilution of protein A-gold (diameter, 10 nm; Biocell Research Laboratories). Specimens were examined with a Jeol 1010 microscope.

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**RESULTS**

**Polyprotein Processing Activity of Protein pS273R**—To investigate the putative protease activity of protein pS273R, we have used a system of COS-7 cells transiently coexpressing the S273R gene and the genes coding for polyprotein pp62 and polyprotein pp220, which served as substrates. During ASFV infection, polyprotein pp62 is first cleaved at a Gly-Gly-Gly sequence at positions 157–159 to produce the 15-kDa structural protein p15 and an intermediate precursor of 46 kDa (pp46). A second cleavage at the Gly-Gly-Asn site at positions 462–464 gives rise to the mature structural protein p35 of 35 kDa and to an 8-kDa polypeptide that has not been detected in the infected cells (7) (Fig. 1A, upper diagram). An alternative, although minor, processing pathway, described in the lower diagram of Fig. 1A, has also been found. In this case, processing first takes place at the most C-terminal cleavage site, producing an intermediate pp52 precursor of 52 kDa. Cleavage of this intermediate at the Gly-Gly-Gly sequence at position 157–159 then produces the two structural proteins p15 and p35.

As shown in Fig. 1B (lanes 3 of top and middle panels), a specific band corresponding to the unprocessed pp62 polyprotein was detected by Western blot using both anti-p35 and anti-p15 antibodies in lysates from cells transfected with the polyprotein gene and infected with vTF7-3. When the polyprotein was coexpressed with the S273R gene (Fig. 1B, top and middle panels, lanes 4), the pp62 band strongly decreased coinciding with the appearance of the proteolytic products p35 (upper panel) and p15 (middle panel), which had the same electrophoretic mobility as the structural proteins p35 and p15 detected in the samples of highly purified ASFV (Fig. 1B, top and middle panels, lanes 5). In addition, the pp46 intermediate could be detected with the anti-p35 serum, whereas the antibody against protein p15 revealed a specific band of 52 kDa, which probably corresponds to pp52 precursor. The expression of gene S273R in the transfected cells is shown in the lower panel of Fig. 1B. The protein had a size of 31 kDa, which is consistent with its predicted molecular weight of 31,550 (30).

To assay the activity of protein pS273R in vitro, cytosolic extracts from COS-7 cells expressing protein pS273R or polyprotein pp62, prepared as described under "Experimental Procedures," were mixed and incubated for varying time periods, as indicated in Fig. 1C. After incubation, the samples were analyzed by Western blot using antibodies against proteins p35 (upper panel) or p15 (lower panel). Fig. 1C shows that in the presence of pS273R polyprotein pp62 is processed in a time-dependent and cascade fashion, with the formation of pp52 and pp46 intermediates, which are converted to the mature p35 and p15 products. As can also be seen in this figure, the p15 product, detected at the earliest time examined (15 min), increased at 1 h, and remained constant at later times, whereas the amount of the mature protein p35 increased considerably from 1 to 3 h of incubation. These results suggest that, as it occurs during ASFV infection (7), the main initial event in the in vitro...
processing of pp62 by protein pS273R is the recognition of the cleavage site at position 158–159, with the formation of the mature p15 protein and preprotein pp46. This latter protein would then be cleaved at positions 463–464 to produce the mature protein p35.

A similar in vitro assay was performed to analyze whether protein pS273R was also able to cleave polyprotein pp220 into its mature products. During ASFV infection, polyprotein pp220 is firstly cleaved at the Gly-Gly-Gly sequence at positions 43–45 and the Gly-Gly-Ala sequence at positions 892–894 to give rise to the mature product pp150 and the intermediate precursor protein pp90 (6) (Fig. 2A). Preprotein pp90 is subsequently cleaved at a Gly-Gly-Asp sequence at positions 367–369 to generate the mature protein p34 and the intermediate precursor pp55, which is finally processed at a Gly-Gly-Ala site at positions 521–523 to produce the mature products pp14 and p37. As shown in Fig. 2B (lanes 1), the antibodies against products pp150 (top panel), p34 (middle panel), and p37 and p14 (bottom panel) detected the unprocessed pp220 polyprotein in pp220 containing extracts incubated for 6 h in the absence of protein pS273R. Additionally, lower molecular weight bands, probably corresponding to incomplete forms of the pp220 polyprotein produced in the transfected cells, were observed. After incubation with pS273R containing extracts for 1 h (lanes 2), 3 h (lanes 3), and 6 h (lanes 4), the levels of polyprotein pp220 decreased in a time-dependent fashion coinciding with the appearance and accumulation of proteins of 150 kDa (upper panel), 34 kDa (middle panel), and, more weakly, of 37 kDa, which was only visualized after a longer exposure time (lower panel). These proteolytic products comigrated with the mature proteins p150, p34, and p37 present in samples of purified ASFV particles (lanes 5). Interestingly, a 90-kDa protein was also specifically detected by the antibodies against protein p34 (middle panel, lanes 2–4) and proteins p37/p14 (lower panel, lanes 2–4), whereas a 55-kDa protein was recognized only by the anti-p37/p14 serum (lower panel, lanes 2–4). The detection of these proteins is consistent with the formation of the intermediate precursors pp90 and pp55 (Fig. 2A). The other mature product p14 could not be detected in the in vitro assays because of the poor recognition of protein p14 by Western blot analysis (see lane 5 in the lower panel) and, possibly, because of a low efficiency of processing of precursor pp55. These results indicate that the two ASFV polyproteins pp220 and pp62 are accurately cleaved in the presence of protein pS273R to give rise to the same mature products and intermediate precursors as those produced during ASFV infection.

Characterization of Protein pS273R as a Cysteine Protease—As reported by Li and Hochstrasser (16) and shown in the alignment of Fig. 3A, the ASFV protein pS273R conserves near its C terminus the catalytic core domain of the SUMO-1-specific proteases and the adenovirus protease containing the four key catalytic residues, His, Glu/Asp (Asn in the case of the adenovirus protease), Gln, and Cys, which are characteristic of cysteine proteases. To investigate whether the ASFV protein was also a cysteine protease, a number of inhibitors specific for the cysteine proteases (pepstatin), metallo-proteases (1,10-phenanthroline), or serine proteases (phenylmethanesulfonyl fluoride) had no effect on pp62 processing. On the other hand, two cysteine protease inhibitors, N-ethylmaleimide and the peptidyl drazomethane Z-Leu-Val-Gly-CHN₂, completely or partially blocked, respectively, the cleavage of the polyprotein. It is of interest to note that E-64, which is also a specific inhibitor of cysteine proteases, did not inhibit the processing activity of protein pS273R. In this connection, it should be mentioned that the adenovirus protease is also relatively insensitive to this compound (31, 32).

To further characterize the pS273R protein as a cysteine protease, the conserved cysteine and histidine residues of the putative catalytic domain were mutated to serine and arginine,
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Expression of Gene S273R in ASFV-infected Vero Cells—To study the transcription of gene S273R during ASFV infection, RNA hybridization and primer extension analysis were carried out with the ³²P-labeled oligonucleotide S273R (see “Experimental Procedures”) specific for ORF S273R. Hybridization of this oligonucleotide to Northern blots containing RNA from mock-infected cells and early (cycloheximide and cytosine arabinoside) and late RNA from cells infected with ASFV showed a main band of 4.5 kb, as well as other species, in the late RNA from cells infected with ASFV and late RNA from mock-infected cells and early (cycloheximide and cytosine arabinoside) and late (cycloheximide and arabinoside) RNA from cells infected with ASFV showed a main band of 4.5 kb, as well as other species, in the late RNA from cells infected with ASFV. Residues conserved or identical in at least 80% of the 15 sequences aligned are in bold type over a gray background. The invariant residues in the 15 sequences are indicated with an asterisk. Full triangles mark the amino acid residues of the catalytic triad (His, Asn/Asp/Glu, and Cys) and the Gln residue involved in the formation of the oxyanion hole in the active site. Sequences: ASFV S273R, protein pS273R of ASFV (Q00946); VACCV I7, vaccinia virus I7 protein (P12926); ADE2 VPRT, human adenovirus 2 protease (P03252); SENP1 Hs, human sentrin-specific protease (AF149770); Ulp1 Sc, Saccharomyces cerevisiae Ulp1 protease (S63462). B, effect of protease inhibitors on the processing of pp62. Cytosolic extracts containing polyprotein pp62 or protein pS273R were mixed and incubated for 6 h at 30 °C in the absence (lanes) or presence of 10 μM peptatin (Peps), 1 mM 1,10-phenanthroline (Phen), 1 mM phenylmethanesulfonyl fluoride (PMSF), 2.5 mM N-ethylmaleimide (NEM), 100 μM Z-LVG-CH₂N₂ (LVG), or 100 μM E-64. A control reaction containing the proteolytic substrate pp62 alone is also presented (−). The samples were then analyzed by Western blot using an anti-p35 antibody. The pp62 and p35 bands are indicated.

Localization of Protein pS273R in Infected Cells—To study the intracellular localization of protein pS273R, mock-infected and ASFV-infected Vero cells were fixed at 18 h after infection and analyzed by immunofluorescence with the anti-pS273R serum. As shown in Fig. 5A, a low background signal was observed in the mock-infected cells used as a control. In contrast, the antibody against protein pS273R stained discrete cytoplasmic areas of the virus-infected cells (Fig. 5B), which were identified as virus factories by immunolabeling with a monoclonal antibody that recognizes the polyprotein precursor pp220 and its mature product p150 (Fig. 5C) (8). As previously reported, the antibody to pp220/p150 proteins also labeled virus particles scattered throughout the cytoplasm (8). Although more weakly, the anti-pS273R serum also stained cytoplasmic clusters of virions outside the assembly areas (Fig. 5B), suggesting that protein pS273R is incorporated into the virus particles, in agreement with the immunoblotting experiments performed with purified virions.
To examine in more detail the localization of ASFV protease within the virus factories, we carried out an immunoelectron microscopic analysis of Vero cells infected with ASFV for 20 h. For this, thawed cryosections were incubated with the anti-pS273R antibody followed by protein A-gold. As shown in Fig. 6A, the antibody labeled the region of the viral factories, in keeping with the immunofluorescence results. Within the assembly sites, labeling was essentially associated with both intermediate virus structures (Fig. 6B) and mature virions (Fig. 6C). Interestingly, gold grains were localized within the core region of immature icosahedral particles that lack the electron dense DNA-containing nucleoid. Within mature particles, the signal was associated with the core shell (Fig. 6C), the protein domain surrounding the electron dense nucleoid and containing the polyprotein products (8).

**DISCUSSION**

A new family of structurally related cysteine proteases that process SUMO-1-modified proteins at consensus Gly-Gly-Xaa sequences has been recently described (16). The sequence similarity between the members of this family is largely confined to a C-terminal region of about 200 amino acids that contains a 90-amino acid core domain that is also present in the adenovirus protease, as well as in the poxvirus I7 protein and protein pS273R of ASFV. This core domain contains the active site triad of the adenovirus protease, composed of residues His54, Glu71, and Cys122, which in the crystal structure of this enzyme have a spatial disposition identical to that of the catalytic triad of the classical cysteine protease papain (35), although the sequential order of the residues in the polypeptide chain of this enzyme is Cys25, His159, and Asn175. In addition, a fourth residue, Gln115, of the adenovirus protease is in an equivalent spatial position to the Gln19 of papain involved in the formation of the oxyanion hole in the active site. The ASFV protein pS273R conserves the His, Cys, and Gln residues, and, as in the case of papain, has an Asn at the position of the adenovirus protease Glu71 residue. This sequence similarity strongly ar-
cascade processing of the viral polyproteins. It is also possible that the viral enzyme might be able to cleave cellular or viral ubiquitin-like modified proteins. This is an interesting possibility that may provide insight into the structural and functional aspects of this emerging family of cysteine proteases.

The studies on the expression and localization of protein pS273R in cells infected with ASFV further support a role for this protein in the processing of the viral polyproteins during the virus morphogenesis. Thus, protein pS273R, as its polyprotein substrates, is synthesized at late times of infection and is localized within the cytoplasmic viral factories where morphogenesis occurs.

We have previously shown that the mature proteins p150, p37, p34, and p14, products of polyprotein pp220, are major components of the core shell, representing about 25% of the total protein mass of the virus particle (8). The same location has been found for the structural proteins p35 and p15, which are derived from polyprotein pp62. These findings, together with the observed localization of protein pS273R in the virus morphogenetic intermediates, suggest a role for the ASFV protease in the assembly of the viral core. Thus, proteolytic processing might be an essential mechanism for the spatio-temporal control of the interactions between core components, in such a way that only preassembled and properly processed substrates could enter the assembly pathway. In connection with this, it should be mentioned that other complex DNA-containing viruses, such as adenoviruses and poxviruses, use proteolytic processing at similar or identical cleavage sequences for the maturation of major core proteins. In the case of poxviruses, the cleavage of core precursors is crucial for the formation of virus particles, which does not take place if the processing is blocked (36). In contrast, the adenovirus protease acts in newly assembled virions, being needed for their maturation into infectious virions (37). To ensure that processing of the viral precursor proteins takes place only after virion assembly, this enzyme employs a unique and complex regulatory mechanism, involving stimulation of its activity by peptide and DNA cofactors (13, 14). Further studies will be needed to ascertain whether similar mechanisms operate to control the temporal and spatial activity of the ASFV protease.

As has also been described for the adenovirus protease and the 17 protein of poxviruses (38, 39), the ASFV protein pS273R is present in the core of mature virus particles. This finding could merely reflect a residual presence of an enzymatic activity needed during the final steps of ASFV morphogenesis. Another possibility is that protein pS273R is involved in some early event of viral infection. This is the case for the adenovirus protease that has been implicated in virus entry into the host cell and disassembly of the incoming virus particles (40, 41). Whether the ASFV protease, besides a morphogenetic role late in the virus replicative cycle, might also cleave other viral or even cellular substrates early in infection is a question that remains to be addressed.

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