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Proteolytic Processing in African Swine Fever Virus: Evidence for a New Structural Polyprotein, pp62

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We have identified an open reading frame (ORF), CP530R, within the *Eco*RI C′ fragment of the African swine fever virus (ASFV) genome that encodes a polyprotein of 62 kDa (pp62). Antisera raised against different regions of ORF CP530R recognized a polypeptide of 62 kDa in ASFV-infected cells during the late phase of virus replication, after the onset of viral DNA synthesis. Pulse-chase experiments showed that polyprotein pp62 is posttranslationally processed to give rise to two proteins of 35 kDa (p35) and 15 kDa (p15). This proteolytic processing was found to take place at the consensus sequence Gly-Gly-X through an ordered cascade of proteolytic cleavages like that which also occurs with ASFV polyprotein pp220 (C. Simón-Mateo, G. Andrés, and E. Viñuela, EMBO J. 12:2977–2987, 1993). Immunofluorescence studies showed that polyprotein pp62 is localized in the viral factories. In addition, immunoprecipitation analysis of purified virus particles showed that mature products p35 and p15 are major structural proteins. According to these results, polyprotein processing represents an essential strategy for the maturation of ASFV structural proteins.

African swine fever virus (ASFV) is a large, DNA-containing virus responsible for a highly lethal disease of domestic pigs (for reviews, see references 6 and 27); it also infects soft ticks (*Ornithodorus* sp.), which act as vectors for the virus. Animal viruses with very large DNA genomes include families of icosahedral viruses (*Herpesviridae* and *Iridoviridae*) and brick-shaped viruses (*Poxviridae*). However, ASFV does not fit well into any of these groups since, although its genomic structure is similar to that of poxviruses (hairpin loop structures and terminal inverted repetitions at the DNA ends) (11, 24), its icosahedral morphology is similar to that of iridoviruses (5). It is the only member of the new genus "African swine fever-like virus" (8). The complexity of ASFV, with a genome length of 170 kbp, is evidenced by the large number (about 100) of virus-induced polypeptides detected in infected cells by two-dimensional (2-D) gel electrophoresis analysis (10, 21). In addition, the ASFV particle contains more than 50 proteins with molecular masses ranging from 10 to 150 kDa (4, 10), including the enzymatic machinery required for the synthesis and processing of early mRNA (20). Recently, the nucleotide sequence of the genome of ASFV strain BA71V has been reported and 151 open reading frames (ORFs) have been identified (28).

A striking feature of this complex DNA virus is the use of polyprotein processing as a mechanism of gene expression. Thus, we previously showed that ASFV encodes a polyprotein, named pp220, which after proteolytic processing gives rise to four major structural proteins: p150, p37, p34, and p14 (23). Interestingly, all of the proteolytic cleavages were shown to take place at the consensus sequence Gly-Gly-X (19, 23). This report extends the study of proteolytic processing in the maturation of some ASFV proteins by describing a new polyprotein, named pp62. Polyprotein pp62 was identified as a late protein which, after proteolytic processing at Gly-Gly-X sequences, produces two major structural proteins: p35 and p15. According to our results, polyprotein processing is an essential mechanism for the generation of major components of the virus particle, sharing this gene expression strategy with positive-strand RNA viruses and retroviruses (12, 15).

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MATERIALS AND METHODS

Cells and viruses. Vero cells (CCL 81), obtained from the American Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, the concentration of which was reduced to 2% during viral infection. ASFV strain BA71V, adapted to grow in Vero cells, has been previously described (9). Highly purified ASFV was obtained by Percoll equilibrium centrifugation as described by Carrascosa et al. (4).

Computer analysis. Computer analyses of DNA and protein sequences were performed by using the software package of the University of Wisconsin Genetics Computer Group (7).

Antisera. The complete CP530R ORF was cloned in plasmid KS (Stratagene) in two steps. The nucleotide sequence coding for the first 14 amino acids was obtained by synthesizing two complementary oligonucleotides containing a *Pst*I site, which were paired and cloned into the pGMT7 vector. This recombinant plasmid was digested with *Xba*I-*Pst*I to obtain a fragment (100 bp) which also contained the ϕ10 promoter and the Shine-Dalgarno sequence. The remainder of the CP530R ORF (2.5 kbp) was obtained from the restriction fragment SD/RC′ of the ASFV genome (strain BA71V) (18) by digestion with the restriction enzymes *Pst*I and *Hin*dIII. Both fragments were ligated into plasmid KS (Stratagene) to produce recombinant plasmid KS-CP530R.

From this plasmid, we obtained plasmids KS-p35C′ and KS-p15C′, containing different regions of ORF CP530R. Plasmid KS-p35C′ was obtained by cutting plasmid KS-CP530R with the restriction enzyme *Pst*I and then blunt ending it with the mung bean enzyme. Thereafter, the linearized plasmid was digested with *Mlu*I, blunt ended with Klenow, and finally religated to obtain plasmid KS-p35C′, which contains the 3′ region of ORF CP530R (see Fig. 4B). To obtain plasmid KS-p15C′, containing the 5′ region of ORF CP530R, plasmid KS-CP530R was digested with the restriction enzyme *Acc*I and religated (see Fig. 4B).

Escherichia coli BL21DE3 (25), which contains in its genome the T7 RNA polymerase gene under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, was transformed with the recombinant plasmids. The 16-kDa (KS-p15C′, p15 region) and 46-kDa (KS-p35C′, p35 region) proteins which were used as immunogens were obtained by inducing the cultures for 2 h with 1 mM IPTG (data not shown). The cultures were further incubated for 2 h with rifampin (0.2 mg/ml). The bacterial pellet (from a 100-ml culture) was lysed in 5 ml of phosphate-buffered saline (PBS) by freezing at −20°C and thawing and sonicated twice for 2 min each time. Bacterial lysates were subjected to centrifugation (10 min at 12,000 × *g*). In all cases, the recombinant proteins were the main component present in the pellet. About 100 μg of each protein was injected subcutaneously into rabbits in complete Freund's adjuvant. At intervals of 2 weeks, rabbits were boosted subcutaneously three times, once in incomplete

Freund's adjuvant and twice in PBS. After the fourth injection, the animals were bled and the sera were tested by immunoprecipitation.

Radiolabeling and immunoprecipitation. Preconfluent monolayers of Vero cells, cultured in 24-well plates, were infected with ASFV at 20 PFU/cell. At 12 h postinfection (hpi), cells were pulse-labeled for 2 h with 300-μCi/ml [^{35}S]methionine-cysteine (ICN Biomedicals) in methionine- and cysteine-free DMEM. Before and after the pulse, the medium was replaced with methionine- and cysteine-free DMEM for 30 min to remove any residual methionine and cysteine. DMEM containing 2 mM methionine and cysteine was added, and the mixture was incubated for different chase periods. After removal of the medium, the cells were washed with PBS and lysed in dissociation buffer (2% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 1% sodium deoxycholate, 100 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM tosyl-L-lysine chloromethyl ketone, 0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone in PBS). The extracellular virus present in the medium was recovered by centrifugation in a Beckman Airfuge at 133,000 × *g* for 20 min. Metabolic labeling of highly purified ASFV particles with [^{35}S]methionine-cysteine was performed as described by Carrascosa et al. (4).

For immunoprecipitation assays, cell extracts in dissociation buffer were diluted at least 10-fold in radioimmunoprecipitation assay buffer (0.01 M Tris-HCl [pH 7.5], 0.15 M NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS) and incubated with a 1:20 dilution of the specific antisera for 2 h at 4°C. A 25% suspension of protein A-coated Sepharose CL-4B (Sigma Chemical Co., St. Louis, Mo.) was then added, and incubation was continued for 30 min at 4°C. The immune complexes were isolated by centrifugation, washed four times with cold radioimmunoprecipitation assay buffer, and finally solubilized by boiling in Laemmli sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 100 mM dithiothreitol, 10% glycerol) for 3 min.

Gel electrophoresis. High-resolution 2-D gel electrophoresis was performed as described by Simón-Mateo et al. (23). SDS-polyacrylamide gradient (7 to 20%) gels (16) were used for both 2-D and conventional gels.

Protein sequencing. To determine the N-terminal sequence of protein p35, ASFV proteins from Percoll-purified virions were resolved by 2-D gel electrophoresis and then transferred to a polyvinylidene difluoride membrane. After Coomassie blue staining, protein p35 was directly subjected to N-terminal sequencing by using an ABI 473A pulse-liquid phase protein sequencer (Applied Biosystems) and the modified cycles described in the manufacturer's instructions.

Indirect immunofluorescence. Vero cells were infected at a multiplicity of infection of 1 PFU/cell. At 12 hpi, the cells were rinsed with PBS and fixed with methanol at −20°C for 5 min. For staining, fixed cells were incubated with a 1:100 dilution of the different antisera and then with a 1:200 dilution of Texas red-conjugated goat anti-rabbit antibodies (Amersham). Nuclear and viral DNAs were visualized by staining with 5 μg of bis-benzimide (Hoechst 33258; Sigma) per ml of PBS for 5 min. Cells were examined under an Axiovert fluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany) and photographed with Kodak TMAX (ASA 400) film.

RESULTS

Identification of ORF CP530R. One strategy used in our laboratory to identify genes coding for viral proteins has been the screening of an ASFV expression library (pEX) in which viral proteins are synthesized as fusion products with β-galactosidase (1). To identify the gene encoding structural protein p35, a serum raised against the 35-kDa proteins of purified virus particles isolated from SDS-polyacrylamide gels was used. Nine different clones were selected with this serum and divided by DNA hybridization into two groups, one with six clones (group I) and the other with three clones (group II). A representative clone of each group was hybridized to a genomic library of viral DNA to localize the corresponding genes in the viral genome of the BA71V strain. Both groups were found to hybridize specifically with *Eco*RI fragment C′ (RC′) (data not shown).

The sequencing at the insertion point of the viral DNA of the clones from group I confirmed that all of them expressed part of ORF CP2475L as a fusion protein with β-galactosidase (data not shown). This ORF has been reported to encode polyprotein pp220, which is posttranslationally processed to give rise to structural proteins p150, p37, p34, and p14 (23). Specifically, all clones contained the region corresponding to protein p37.

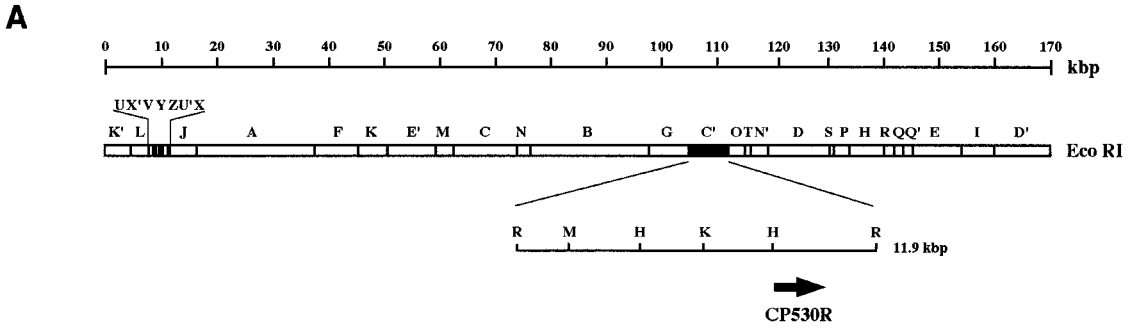
The sequencing of the clones from group II revealed that they contained part of ORF CP530R (27) (Fig. 1). This ORF codes for a protein of 530 amino acids with a predicted molecular mass of 60.5 kDa. This result suggests that protein p35 is synthesized as a precursor with a higher molecular weight which would be proteolytically processed. Interestingly, two overlapping Gly-Gly-X sequences are located at positions 157 to 160 (see Fig. 4B). This sequence has previously been reported as a cleavage site for the maturation of ASFV and adenovirus structural proteins and polyubiquitin (19, 23). The cleavage at that position could produce two proteins with predicted molecular masses of 18 and 43 kDa, the latter possibly corresponding to protein p35. In agreement with this hypothesis is the fact that all of the clones selected for protein p35 expressed proteins in which the insertion point is downstream of the putative cleavage site at positions 157 to 160. In addition to the two Gly-Gly-X sequences mentioned above, another is present at positions 462 to 464 in ORF CP530R (Fig. 1B). The cleavage at this position could give rise to a protein with a predicted molecular mass of 8 kDa.

A computer search of the databases for ORF CP530R (7) showed similarity with an ASFV ORF, PIG1, reported by Hingamp et al. (13). ORF PIG1 is located in the RC′ restriction fragment of the BA71V isolate, like ORF CP530R, and encodes a putative polypeptide of 225 amino acids. Despite its similarity, the PIG1 sequence significantly differs from that reported here. Thus, several nucleotide changes and frameshifts in ORF PIG1 would produce amino acid changes and, what is more important, a striking difference in the length of this ORF. The results presented below support the conclusion that our sequence is correct and indeed codes for a protein of 530 amino acids.

ORF CP530R codes for a protein of 62 kDa. To characterize the protein coded for by ORF CP530R, two polyclonal rabbit antisera against different regions of its sequence were obtained. The different polypeptides used as immunogens were expressed in bacteria from the appropriate recombinant plasmids as described in Materials and Methods. For a schematic representation of the localization of the CP530R regions used as immunogens, see Fig. 4B.

Both sera were used in immunoprecipitation experiments with extracts of infected Vero cells metabolically labeled with [^{35}S]methionine-cysteine along the infection cycle. Figure 2 shows the results obtained after immunoprecipitation of uninfected (U) and infected (I) cells labeled for 2 h at 12 hpi. Only one protein with an apparent molecular mass of 62 kDa, named pp62, was specifically detected with both sera in the extract of ASFV-infected cells (lanes I−). The molecular mass of this protein was similar to that predicted from the amino acid sequence of ORF CP530R (60.5 kDa). It should be noted that protein pp62 was not detected in extracts obtained in the presence of cytosine arabinoside (AraC), an inhibitor of viral DNA replication (Fig. 2, lanes I+). This result indicates that protein pp62 is expressed after the onset of viral DNA synthesis and therefore belongs to the class of late ASFV proteins.

Characterization of the mature proteins produced by proteolytic processing of protein pp62. To identify the proteins that might be derived from protein pp62 by proteolytic processing, infected cells were pulse-labeled with [^{35}S]methionine-cysteine for 30 min at 12 hpi and chased for 1, 3, 6, 12, and 24 h in the presence of a 10-fold excess of methionine-cysteine. Figure 3 shows an immunoprecipitation analysis with the different antisera raised against protein pp62. In the pulse, the antiserum specific for the p35 region recognized protein pp62, the level of which decreased during the chases, coinciding with the accumulation of a 35-kDa protein (named p35) (Fig. 3, anti-p35). The cleavage to produce protein p35 was detected 3 h after the pulse and continued in the following chase periods.



B

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                                M  P  S  N  M  K  Q  F  C  K  I  S  V  W  L  Q  Q  H  D  P  D  L  L    23
AAATTATAAAATAATAAGAAGATGCCCTCTAATATGAAACAGTTTTGCAAGATTTCTGTATGGCTGCAGCAGCACGATCCAGATTTATTA

E  I  I  N  N  L  C  M  L  G  N  L  S  A  A  K  Y  K  H  G  V  T  F  I  Y  P  K  Q  A  K    53
GAAATTATCAACAACTTATGTATGCTTGGCAATTTATCCGCGGCAAAGTACAAACACGGAGTTACCTTCATTTATCCCAAACAGGCAAAG

I  R  D  E  I  K  K  H  A  Y  S  N  D  P  S  Q  A  I  K  T  L  E  S  L  I  L  P  F  Y  I    83
ATCCGCGATGAAATAAAAAAACATGCCTACTCCAATGACCCCTCACAGGCCATAAAGACCTTAGAATCACTCATCCTTCCATTTTACATT

P  T  P  A  E  F  T  G  E  I  G  S  Y  T  G  V  K  L  E  V  E  K  T  E  A  N  K  V  I  L   113
CCCACTCCAGCGGAGTTCACCGGGGAAATCGGCTCCTACACCGGAGTGAAATTAGAGGTCGAAAAAACGGAGGCGAATAAAGTTATTTTG

K  N  G  E  A  V  L  V  P  A  A  D  F  K  P  F  P  D  R  R  L  A  V  W  I  M  E  S  G  S   143
AAAAATGGAGAAGCAGTCCTGGTTCCGGCGGCCGATTTTAAGCCCTTTCCTGATCGCCGACTAGCGGTCTGGATCATGGAGTCAGGCTCT

M  P  L  E  G  P  P  Y  K  R  K  K  E  G  G  G  N  D  P  P  V  P  K  H  I  S  P  Y  T  P   173
ATGCCCCTGGAGGGTCCCCCCTATAAGCGGAAAAAGGAGGGTGGGGGGAATGACCCGCCGGTTCCTAAGCATATCTCGCCGTATACTCCG

R  T  R  I  A  I  E  V  E  K  A  F  D  D  C  M  R  Q  N  W  C  S  V  N  N  P  Y  L  A  K   203
CGCACGCGTATTGCCATTGAGGTGGAAAAGGCCTTTGATGACTGTATGCGTCAAAACTGGTGTAGTGTCAATAATCCCTATCTTGCCAAA

S  V  S  L  L  S  F  L  S  L  N  H  P  T  E  F  I  K  V  L  P  L  I  D  F  D  P  L  V  T   233
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F  Y  L  L  L  E  P  Y  K  T  H  G  D  D  F  L  I  P  E  T  I  L  F  G  P  T  G  W  N  G   263
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T  D  L  Y  Q  S  A  M  L  E  F  K  K  F  F  T  Q  I  T  R  Q  T  F  M  D  I  A  D  S  A   293
ACAGATCTGTATCAAAGTGCTATGCTGGAATTTAAAAAGTTTTTTACCCAGATTACTCGCCAAACCTTTATGGACATAGCCGATTCGGCT

T  K  E  V  D  V  P  I  C  Y  S  D  P  E  T  V  H  S  Y  T  N  H  V  R  T  E  I  L  H  H   323
ACTAAGGAGGTGGATGTTCCTATATGTTATTCGGATCCCGAAACCGTACATTCCTATACCAATCACGTGCGTACTGAAATTTTGCATCAC

N  A  V  N  K  V  T  T  P  N  L  V  V  Q  A  Y  N  E  L  E  Q  T  N  T  I  R  H  Y  G  P   353
AATGCCGTCAATAAGGTTACAACACCTAACCTAGTCGTGCAGGCCTATAATGAGCTCGAGCAAACCAATACCATACGACATTACGGCCCT

I  F  P  E  S  T  I  N  A  L  R  F  W  K  K  L  W  Q  D  E  Q  R  F  V  I  H  G  L  H  R   383
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T  L  M  D  Q  P  T  Y  E  T  S  E  F  A  E  I  V  R  N  L  R  F  S  R  P  G  N  N  Y  I   413
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N  E  L  N  I  T  S  P  A  M  Y  G  D  K  H  T  T  G  D  I  A  P  N  D  R  F  A  M  L  V   443
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A  F  I  N  S  T  D  F  L  Y  T  A  I  P  E  E  K  V  G  G  N  E  T  Q  T  S  S  L  T  D   473
GCCTTTATCAACAGTACTGACTTTTTATACACCGCCATTCCCGAAGAAAAGGTAGGGGGGAATGAAACCCAAACCAGTAGCCTTACAGAC

L  V  P  T  R  L  H  S  F  L  N  H  N  L  S  K  L  K  I  L  N  R  A  Q  Q  T  V  R  N  I   503
CTAGTTCCAACACGGCTACACTCTTTTTTAAATCATAATCTAAGCAAACTTAAAATCTTAAACCGCGCGCAGCAAACGGTTAGAAACATT

L  S  N  D  C  L  N  Q  L  K  H  Y  V  K  H  T  G  K  N  E  I  L  K  L  L  Q  E  *         530
CTTTCAAATGATTGTCTTAATCAACTGAAACATTATGTTAAACACACGGGAAAAAATGAAATACTAAAGTTACTTCAAGAATAACTATGT
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FIG. 1. Nucleotide and predicted amino acid sequences of ORF CP530R. (A) Localization in the ASFV genome of restriction fragment RC′ containing ORF CP530R. The position and orientation of ORF CP530R are indicated by the arrow. (B) Sequence of ORF CP530R. The numbers on the right are amino acid positions. Potential cleavage sites (Gly-Gly-X) are indicated by open boxes. The arrows indicate the sites of insertion into the pEX vector of the different clones selected in the screening with anti-p35 serum. The NH_2-terminal sequence determined for protein p35 is indicated by shadowed letters.

The antiserum against the NH_2-terminal region (Fig. 4B) of ORF CP530R also recognized protein pp62 during the pulse, and the level of this protein declined from 3 h of chase in parallel with an increase of a 15-kDa protein (named p15) (Fig. 3, anti-p15). Interestingly, immunoprecipitation with the antiserum against the protein p35 region detected a faint 46-kDa protein (named pp46) during the chase periods (Fig. 3, anti-p35). This protein could be an intermediate precursor in which

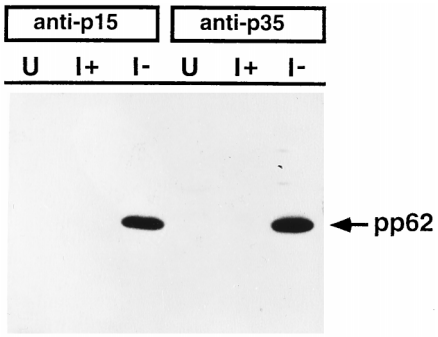

FIG. 2. Immunoprecipitation of the protein encoded by ORF CP530R. Vero cells were uninfected (U) or infected with ASFV in the absence (I−) or in the presence (I+) of 40 μg of the DNA replication inhibitor cytosine arabinoside per ml and labeled with [^{35}S]methionine-cysteine for 2 h at 12 hpi. Cell extracts were immunoprecipitated with anti-p15 and anti-p35 sera and then analyzed by SDS-polyacrylamide gel electrophoresis. Protein pp62 is indicated.

the Gly-Gly-X at positions 463 and 464 has not yet been cleaved to produce the mature protein p35. The processing at this putative cleavage site could give rise to an 8-kDa protein besides protein p35. However, this protein could not be detected in this experiment.

Considering that the 8-kDa protein has 13 leucines and only one cysteine (Fig. 1B), an attempt was made to detect it by pulse-labeling with [^{3}H]leucine. However, no protein with the expected molecular weight was detected after immunoprecipitation or immunoblotting with antiserum against the p35 region (data not shown). One possible explanation for these results might be the nonimmunogenic properties of the protein, since a specific antiserum against polyprotein pp62 using the region spanning amino acids 464 to 530 as an antigen could not be obtained. Another possible explanation is that the protein is rapidly degraded after the cleavage, since the NH_2 terminus consists of an Asn residue. This amino acid is considered destabilizing and would confer on this protein a half-life of 3 min (26).

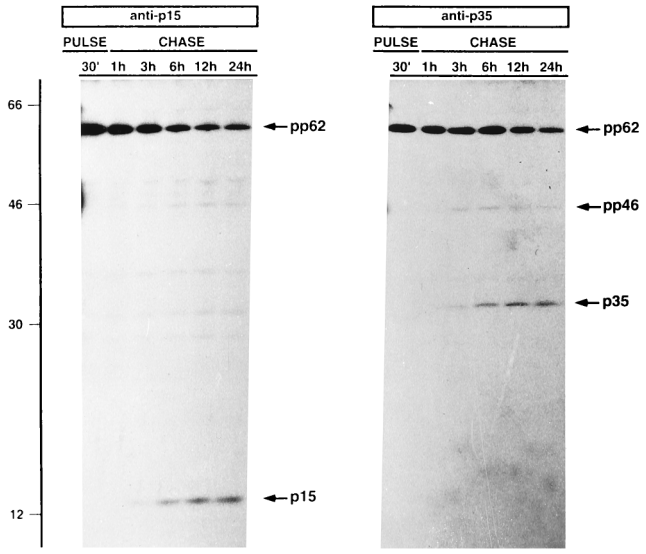

FIG. 3. Proteins derived from ASFV polyprotein pp62 by proteolytic processing. Infected Vero cells were pulse-labeled with [^{35}S]methionine-cysteine for 30 min (PULSE) at 12 hpi and then chased for 1, 3, 6, 12, and 24 h (CHASE). The cell extracts were immunoprecipitated with anti-p15 and anti-p35 sera and analyzed by SDS-polyacrylamide gel electrophoresis. The proteins detected in both pulses and chases, as well as the positions of molecular weight markers (10^3), are indicated.

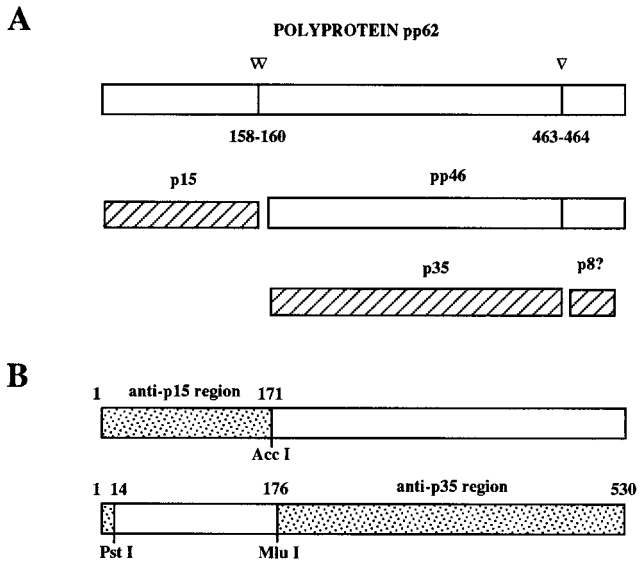

FIG. 4. Schematic representation of the cascade of proteolytic cleavages occurring during ASFV polyprotein pp62 processing. (A) Triangles indicate the positions of the Gly-Gly-X cleavage sites in polyprotein pp62. Precursors (pp62 and pp46) are represented by empty boxes. Mature products (p15, p35, and p8) are represented by hatched boxes. (B) Scheme of polyprotein pp62 with the regions used to obtain the different antisera (anti-p15 and anti-p35) against the polyprotein. The numbers of the amino acids spanning the regions and the restriction sites used in the cloning of these regions are indicated.

Despite the inability to detect the 8-kDa protein, there are several reasons to believe that the 46-kDa protein is an intermediate precursor. First, immunoprecipitation with the antiserum against the protein p35 region detected a 46-kDa protein which was not detected with antiserum against the protein p15 region. Second, the pp46 intermediate precursor appeared 3 h after the pulse, at the same time as mature protein p35. Finally, the size of the polypeptide expressed in bacteria spanning amino acid residues 176 to 530 and that of the 46-kDa protein coincided (data not shown), as did the 35-kDa protein detected during the chase periods and that predicted from the region including amino acids 160 to 463.

In summary, protein pp62 is a polyprotein that after proteolytic processing gives rise to two mature proteins, p15 and p35, and most likely to an 8-kDa protein. Interestingly, the presence of the intermediate precursor pp46 indicates that polyprotein pp62 is processed through an ordered cascade of cleavages like that which occurs with ASFV polyprotein pp220 (23). Figure 4A shows a scheme of the proteolytic cleavages involved in the generation of the mature proteins. The initial event in the proteolytic cascade would be recognition of one of the two overlapping cleavage sites located at positions 158 to 160. This cleavage takes place 2 to 3 h after the synthesis of polyprotein pp62, giving rise to mature protein p15 and preprotein pp46. The last processing step would be cleavage at positions 463 and 464 within preprotein pp46, producing mature protein p35 and an 8-kDa protein corresponding to the COOH-terminal fragment of preprotein pp46.

Polyprotein pp62 processing gives rise to two major structural proteins. To determine whether the proteins generated from polyprotein pp62 are structural, immunoprecipitation analyses of ^{35}S-labeled, highly purified ASFV particles were

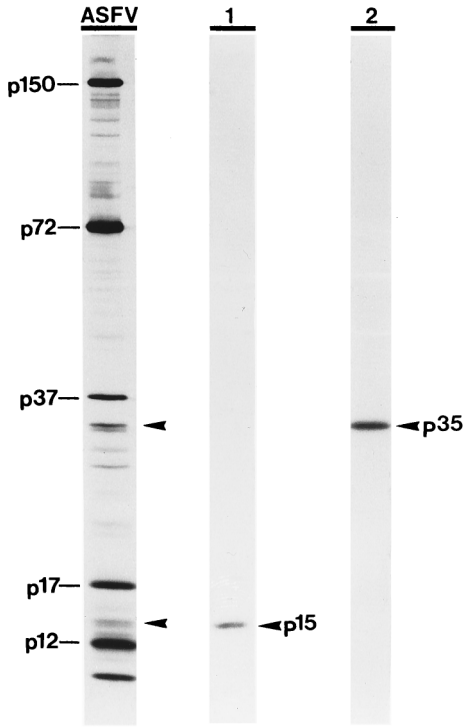

FIG. 5. Proteins p35 and p15 are major structural proteins. Highly purified virus particles labeled with [^{35}S]methionine-cysteine were analyzed by SDS-polyacrylamide gel electrophoresis before (lane ASFV) or after immunoprecipitation with anti-p15 (lane 1) or anti-p35 (lane 2) serum. The positions of p15 and p35, as well as other major ASFV structural proteins, are indicated.

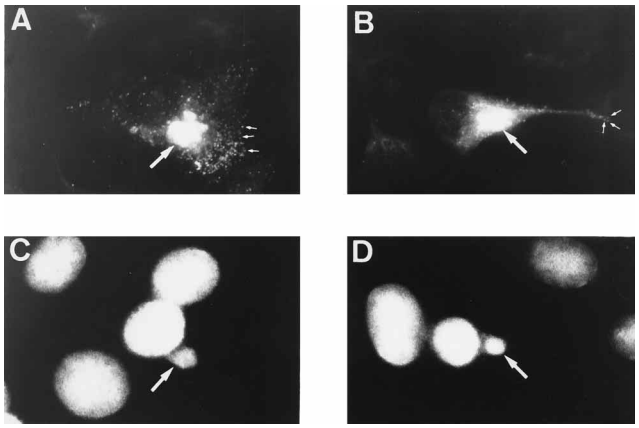

FIG. 6. Immunofluorescence detection of ASFV polyprotein pp62 in infected cells. ASFV-infected Vero cells were fixed at 16 hpi and incubated with anti-p35 (A) or anti-p15 (B) serum and then stained with Texas red-conjugated goat anti-rabbit antibodies and a fluorescent DNA dye (Hoechst 33258). Both sera labeled the viral factories (large arrows in A and B) as defined by DNA staining (arrows in C and D). Additionally, strong labeling of punctate structures spread throughout the cytoplasm (small arrows in A and B), which most likely correspond to virus particles, was detected.

performed with antisera against the different regions of polyprotein pp62. Both antisera recognized the mature proteins derived from polyprotein pp62 in ASFV particles (Fig. 5), indicating that they are incorporated into the viral particle. Analysis of highly purified virions by 2-D gel electrophoresis revealed that proteins p35 and p15 are among the major structural proteins (data not shown).

To ascertain that protein p35 was produced after the cleavage of one of the two overlapping Gly-Gly-X sequences at positions 157 to 160 (Fig. 1B), we analyzed the amino-terminal sequence of structural protein p35 recovered from 2-D gels of purified virions. The sequence obtained, GNDPPVXT, demonstrates that the Gly-Gly-Gly sequence at positions 157 to 159 is the cleavage signal that gives rise to structural protein p35.

Next, we investigated the subcellular localization of polyprotein pp62 and its derived mature proteins. For this, indirect immunofluorescence analyses were carried out with ASFV-infected cells processed at late times of infection by using antisera against polyprotein pp62. Specific labeling was detected with both antisera in discrete cytoplasmic areas close to the nucleus (large arrows in Fig. 6A and B), which were identified by bis-benzimide staining as viral factories (arrows in Fig. 6C and D). Additionally, we detected a punctate labeling spreading throughout the cytoplasm (small arrows in Fig. 6A and B), which most probably represents single virus particles.

DISCUSSION

Proteolytic processing of viral proteins is a common event during the replication of many viruses and can be considered to play two general roles: (i) a mechanism for gene expression in which a viral polyprotein is proteolytically processed to yield the mature proteins and (ii) protein processing involved in the maturation of virus particles during viral morphogenesis (for reviews, see references 12 and 15). A previous study revealed that four of the major ASFV structural proteins (p150, p37, p34, and p14) were primarily synthesized as a polyprotein (pp220) and then proteolytically processed to produce the mature proteins (23). In this report, we have extended the study of ASFV structural proteins by showing that proteins p35 and p15 are also synthesized as a polyprotein (pp62) which, after two proteolytic cleavages, gives rise to the mature proteins.

Both ASFV polyproteins are cleaved after the second Gly residue of the consensus sequence Gly-Gly-X that is also recognized in adenovirus structural proteins and in some cellular proteins such as polyubiquitin (19). A similar cleavage site, Ala-Gly-X, is used in the maturation of some core protein precursors of poxviruses such as vaccinia virus and fowlpox virus (17). The similarity in the cleavage motifs of all of these viruses might indicate that they use similar mechanisms for the processing of their structural protein precursors. In the case of ASFV, only some of the putative cleavage sites (Gly-Gly-X) present in the polyproteins are cleaved, suggesting that additional residues are involved in determining the secondary and tertiary structures of the precursor proteins necessary for the cleavage to occur. Comparison of the amino acid sequences surrounding the sites cleaved in pp220 and pp62 showed no homology. In general, it has been suggested that residues located in the negative positions (N terminal to a cleavage site) are usually the important ones in determining the specificity of the proteolytic processing of viral polyproteins (15). This could explain the strict Gly-Gly conservation found at the -2 and -1 positions of the polyprotein pp220 and pp62 cleavage sites.

Polyprotein pp62 is processed by an ordered cascade of proteolytic cleavages like that which occurs in the case of polyprotein pp220 (23). The initial event in the proteolytic cascade would be the cleavage at positions 158 and 159 by which structural protein p15 is separated from preprotein pp46. This proteolytic maturation does not occur immediately after the synthesis of the precursor, but rather there is a delay of about 3 h before any appreciable proteolysis is observed. This delay might be due to a requirement for conformational changes resulting from any posttranslational modification, ac-

tivation of the proteinase involved or entry of the precursor into a compartment containing the proteinase responsible. A second cleavage very likely occurs in protein pp46 at positions 463 and 464 to produce mature protein p35. Usually, the order of cutting of viral polyproteins at different sites is specifically regulated.

It is noteworthy that the second set of cleavages in polyprotein pp220 also occurs 3 h after the synthesis of the precursor (22), which could indicate that after their synthesis, both polyproteins follow similar pathways before proteolytic maturation is initiated to give rise to the mature proteins.

The finding that polyprotein pp62 is localized in viral factories where virus morphogenesis takes place and the fact that the mature proteins are major structural components suggest that polyprotein pp62 processing might be linked with some step of ASFV assembly like that which occurs with polyprotein pp220 (2). This has been shown to be the case for the maturation of structural polyproteins in RNA viruses (12) and of single proteins in some complex DNA viruses (3, 14). In this context, the order of the recognition of the different cleavage sites in both polyproteins could be a way to regulate viral assembly; i.e., a certain cleavage can only take place after another cleavage has been carried out. These ordered series of proteolytic events may ensure that only correctly folded viral proteins enter the assembly pathway and may provide a mechanism for rendering the process irreversible.

The synthesis of at least six ASFV major structural proteins as polyproteins could serve to cluster genes whose products are somehow related, allowing them to be synthesized as the result of a single transcription-translation event and, if necessary, allowing their transport to a specific subcellular and subviral site. Furthermore, their initial synthesis as a polyprotein precursor may ensure a 1:1 molar ratio of the mature gene products, which could be necessary for establishing a certain ordered structure. This is the case for the four nonidentical polypeptides produced by the proteolytic processing of polyprotein P1 in picornavirus (12). In relation to this, we have recently shown that ASFV structural proteins p150, p37, p34, and p14, produced after the proteolytic processing of polyprotein pp220, are present in the virus particle in essentially equimolar amounts (2). Furthermore, all of these proteins share the same location in the virus particle, the "core shell," a thick protein layer surrounding the DNA-containing nucleoid.

In summary, we have provided evidence that polyprotein processing in ASFV-infected cells is an essential mechanism for the maturation of a significant proportion of the major ASFV structural proteins.

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REFERENCES

1. **Almendral, J. M., S. Baars, and E. Viñuela.** Unpublished results.
2. **Andrés, G., C. Simón-Mateo, and E. Viñuela.** Assembly of African swine fever virus: role of polyprotein pp220. J. Virol. **71:**2331–2341.
3. **Bhatti, A. R., and J. Weber.** 1979. Protease of adenovirus type 2: subcellular localization. J. Biol. Chem. **254:**12265–12268.
4. **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.
5. **Carrascosa, J. L., J. M. Carazo, A. L. Carrascosa, N. García, A. Santiesteban, and E. Viñuela.** 1984. General morphology and capsid fine structure of African swine fever virus particles. Virology **132:**160–172.
6. **Costa, J. V.** 1990. African swine fever virus, p. 247–270. *In* G. Darai (ed.), Molecular biology of iridoviruses. Kluwer Academic Publishers, Boston, Mass.
7. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
8. **Dixon, L. K., D. Rock, and E. Viñuela.** 1995. African swine fever-like particles. Arch. Virol. **10**(Suppl.)**:**92–94.
9. **Enjuanes, L., A. L. Carrascosa, M. A. Moreno, and E. Viñuela.** 1976. Titration of African swine fever virus. J. Gen. Virol. **32:**471–477.
10. **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:**181–191.
11. **González, A., A. Talavera, J. M. Almendral, and E. Viñuela.** 1986. Hairpin loop structure of African swine fever virus DNA. Nucleic Acids Res. **14:** 6835–6844.
12. **Hellen, C. U. T., and E. Wimmer.** 1992. The role of proteolytic processing in the morphogenesis of virus particles. Experientia **48:**201–215.
13. **Hingamp, P. M., M. L. Leyland, J. Webb, S. Twigger, R. J. Mayer, and L. K. Dixon.** 1995. Characterization of a ubiquitinated protein which is externally located in African swine fever virions. J. Virol. **69:**1785–1793.
14. **Katz, E., and B. Moss.** 1970. Formation of a vaccinia virus structural polypeptide derived from a high molecular weight precursor: inhibition by rifampicin. Proc. Natl. Acad. Sci. USA **66:**677–684.
15. **Kräusslich, H. G., and E. Wimmer.** 1988. Viral proteinases. Annu. Rev. Biochem. **57:**701–754.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
17. **Lee, P., and D. E. Hruby.** 1995. Analysis of the role of the amino-terminal peptide of vaccinia virus structural protein precursors during proteolytic processing. Virology **207:**229–233.
18. **Ley, V., J. M. Almendral, P. Carbonero, A. Beloso, E. Viñuela, and A. Talavera.** 1984. Molecular cloning of African swine fever virus DNA. Virology **133:**249–257.
19. **López-Otín, C., C. Simón-Mateo, L. Martinez, 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.
20. **Salas, M. L., J. Kuznar, and E. Viñuela.** 1981. Polyadenylation, methylation and capping of the RNA synthesis *in vitro* by African swine fever virus. Virology **101:**169–175.
21. **Santarén, J. F., and E. Viñuela.** 1986. African swine fever virus-induced polypeptides in Vero cells. Virus Res. **5:**391–405.
22. **Sanz, A., B. García-Barreno, M. L. Nogal, E. Viñuela, and L. Enjuanes.** 1985. Monoclonal antibodies specific for African swine fever virus proteins. J. Virol. **54:**199–206.
23. **Simón-Mateo, C., G. Andrés, and E. Viñuela.** 1993. Polyprotein processing in African swine fever virus: a novel strategy of gene expression for a DNA virus. EMBO J. **12:**2977–2987.
24. **Sogo, J. M., J. M. Almendral, A. Talavera, and E. Viñuela.** 1984. Terminal and internal inverted repetitions in African swine fever virus DNA. Virology **133:**271–275.
25. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of the T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
26. **Varshavsky, A.** 1992. The end rule of protein turnover. Cell **69:**725–735.
27. **Viñuela, E.** 1987. African swine fever virus, p. 31–49. *In* Y. Becker (ed.), African swine fever. Martinus Nijhoff Publishing, Boston, Mass.
28. **Yañez, R., 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.