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SHORT COMMUNICATION

MATILDE R. CHACÓN, FERNANDO ALMAZÁN, MARIA L. NOGAL, ELADIO VIÑUELA,[2] and JOSE F. RODRÍGUEZ[1]

Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Facultad de Ciencias, Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain

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The analysis of the complete nucleotide sequence of the African swine fever virus genome has revealed the existence of a number of genes potentially capable of modifying the host's response to the virus infection. In this report, we describe the results of the characterization of the A224L gene that encodes a novel member of the family of apoptosis inhibitors known as IAP proteins. A224L is expressed during the late phase of the infectious cycle, and its polypeptide product is assembled into virus particles.

African swine fever virus (ASFV), the causative agent of a severe and economically important disease of swine, is a large enveloped DNA virus with an icosahedral morphology (reviewed in Refs. *23* and *24*). The cell targets of ASFV infection are blood monocytes and resident macrophages. The ability of ASFV to replicate in these cell types *in vivo* appears to be a critical factor in determining the virulence of the virus (reviewed in Ref. *25*).

The failure of conventional methods in the development of a vaccine against ASFV has led to speculation that the virus is able to counteract the host's immune system. The analysis of the complete nucleotide sequence of the genome of the BA71V strain of ASFV, a single molecule of double-stranded DNA of 170 kb, has revealed the existence of several genes potentially capable of modulating the virus–host interaction (*27*). Two of these genes are strong candidates for the production of proteins capable of controlling the apoptotic response of the infected cell.

In baculoviruses two apparently unrelated genes capable of preventing virus-induced apoptosis have been described: p35 (*6*) and *iap* (*2, 8*). The ability of p35 to block programmed cell death was discovered during the characterization of a spontaneous mutant of the *Autographa californica* nuclear polyhedrosis virus (*6*) and the *iap* gene was detected as a result of its ability to functionally replace p35 in a genetic complementation assay (*8*). After these initial identifications, additional members of the *iap* family of inhibitors were found in two other baculoviruses: *Cydia pomonella* granulosis virus and *Orgyia pseudotsugata* nuclear polyhedrosis virus (*2, 8*). More recently, as a result of the sequencing of an incomplete ORF of the *Chilo iridescent* virus (an iridovirus), a non-baculovirus member of the *iap* family was identified (*12*).

Of the two ASFV genes encoding proteins potentially capable of controlling the apoptotic response, A179L, which was first found in the highly virulent Malawi strain (*19*), encodes a polypeptide that belongs to the bcl-2/bax family of apoptosis regulators (*26*), and A224L (the subject of the present report), which maps within the left hand side of the virus genome and is transcribed leftwards (see Fig. 1A), encodes a polypeptide with a predicted molecular mass of 27 kDa homologous to members of the IAP family of inhibitors of apoptosis.

IAPs are characterized by the presence of: (i) a C-terminal cysteine-rich region containing two zinc finger-like motifs; and (ii) two copies of the amino acid sequence $GX_2YX_4DX_3CX_2CX_6WX_9HX_{6\text{-}10}C$ (X indicates any residue) known as the BIR (baculovirus IAP repeat) motif. The spacing of cysteine and histidine residues within this motif has led to the suggestion that it may mediate interactions with DNA molecules (*2, 8*).

The zinc finger-like sequences in baculovirus IAPs are RING fingers of the C_3HC_4 type (*8*). In contrast to this, the C-terminal region of pA224L contains the sequence $CX_2CX_{11}CX_2C$ (residues 190 to 208), which may constitute a zinc finger domain of the C_4 type, akin to those found in glucocorticoid receptors (*13*). An additional difference between the baculovirus IAPs and pA224L is that the latter contains a single BIR-like motif. This matches the BIR consensus sequence (*2*) with one exception, the lack of a glutamic acid residue in the eighth position (Fig. 1B).

The characterization of the expression of A224L was initiated by analyzing its transcriptional regulation. For this, two complementary approaches, RNA hybridization

[1] Present address: Centro Nacional de Biotecnología (CSIC), Universidad Autónoma, Campus de Cantoblanco, 28049 Madrid, Spain.

[2] To whom correspondence and reprint requests should be addressed. Fax: 34-1-3974799.

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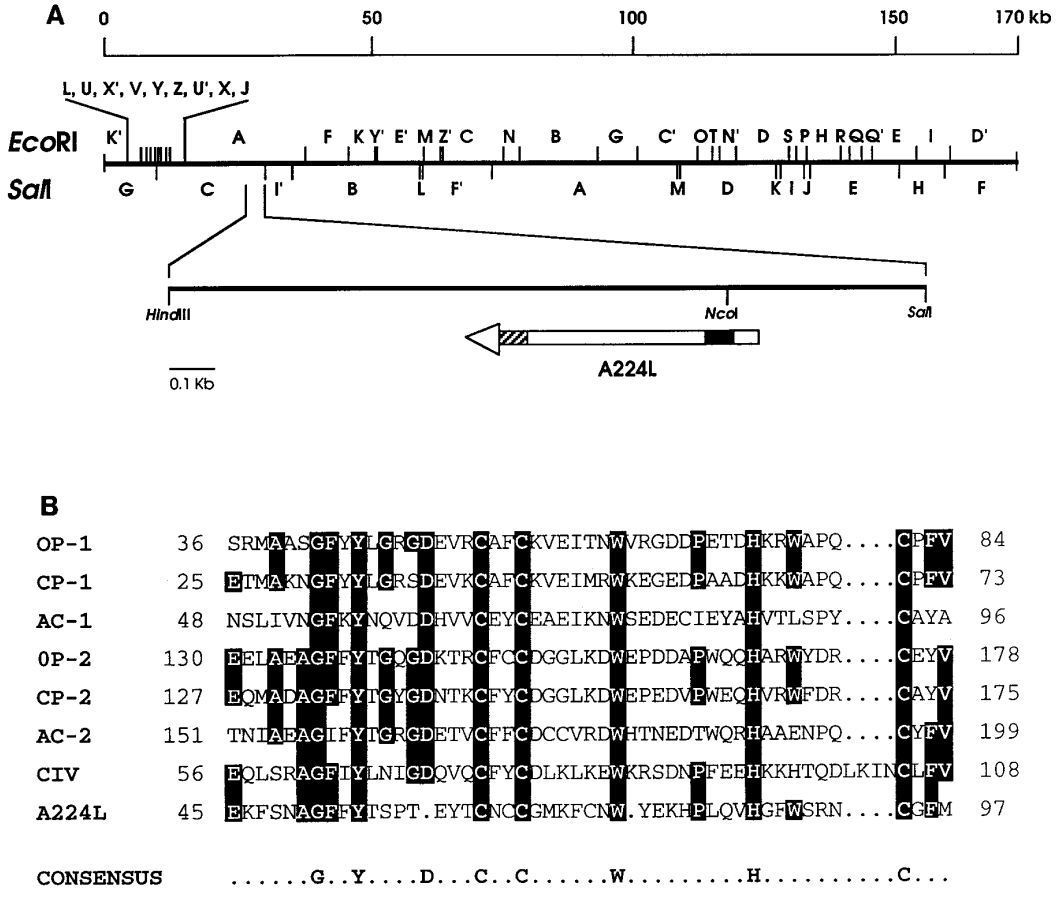

FIG. 1. (A) Location of A224L within the ASFV genome. The position and the nomenclature of the *Eco*RI and *Sal*I restriction fragments of the virus genome are shown. The relative position of A224L, represented as an arrow, within a *Hin*dIII/*Sal*I fragment of 1794 bp is indicated. The boxes within A224L indicate the positions of the sequences coding for the BIR (solid) and the zinc finger-like (hatched) motifs of pA224L. Also indicated is the location of the *Nco*I restriction site used to clone A224L as a *Hin*dIII/*Nco*I fragment of 1301 bp into the expression vector pRSET. (B) Amino acid alignment of pA224L BIR-like sequence, the first (−1) and second (−2) BIR motifs of IAP proteins from *Orgyia pseudotsugata* nuclear polyhedrosis virus (OP), *Cydia pomonella* granulosis virus (CP), *Autographa californica* nuclear polyhedrosis virus (AC), and the BIR motif found in the IAP homolog of *Chilo iridescent* virus (CIV). The alignment was generated using the program PILEUP (9). Residues identical in five or more sequences are boxed. The BIR consensus sequence (2) is also shown.

and primer extension, were used. The RNA samples were obtained from cultures of Vero cells either mock-infected or infected with ASFV (BA71V strain) at a m.o.i. of 10 PFU/cell. ASFV immediate-early RNA was obtained from cells infected for 8 hr in the presence of the inhibitor of protein synthesis cycloheximide (150 μg/ml), early RNA was obtained after 8 hr in the presence of the inhibitor of DNA replication cytosine arabinoside (40 μg/ml), and late RNA was isolated from cells infected for 18 hr in the absence of metabolic inhibitors. Both the Northern blot and primer extension analyses were performed as previously reported (*21*), using a ^{32}P-end-labeled oligonucleotide complementary to nucleotides 25 to 55 of the coding strand of the A224L gene. For the Northern blot, 20 μg of total RNA were fractionated on formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized with the ^{32}P-labeled probe.

The results from the RNA hybridization (Fig. 2A) reveal the existence of two virus-induced late mRNA species of 0.75 and 1.3 kb which are specifically recognized by the radioactive probe. Fainter bands corresponding to a RNA species of more than 1.4 kb are also detected in the lanes from the early and immediate early RNA samples.

The results from the primer extension analysis (conducted as described previously; Ref. *21*) revealed several primer-extended products with sizes ranging from 76 to 79 nucleotides in samples corresponding to late virus-induced RNA (Fig. 2B), thus demonstrating the existence of a late transcriptional initiation site at a short distance, 21 to 24 nucleotides, upstream of the initiation codon of the A224L ORF (see Fig. 2C). In view of these results, it is feasible that the abundant late mRNAs species detected by the RNA hybridization initiate at the detected A224L late transcriptional initiation site. In ASFV, the signal for 3′ end mRNA formation is formed by a stretch of seven or more consecutive thymidylate residues within the coding strand of the DNA template (*1*). Such polythymidylate tracts are located at a distance of 38 and

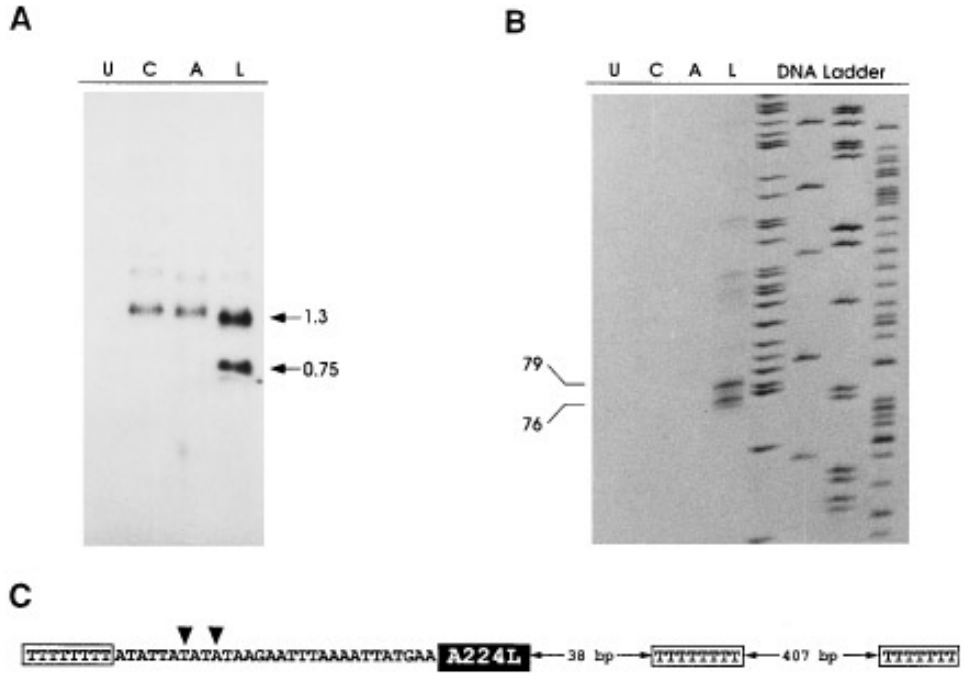

FIG. 2. Transcriptional analysis of the A224L gene. Autoradiographs of RNA hybridization (A) and primer extension (B) of uninfected Vero cell RNA (U) and ASFV-induced immediate-early (C), early (A), and late (L) RNA are shown. Numbers to the right side of A indicate the size (in kb) of the most abundant detected mRNAs. Primer extension samples (B) were electrophoresed alongside an irrelevant DNA-sequencing reaction (DNA ladder) used as markers, the sizes (in nucleotides) of the relevant DNA fragments are shown on the left. (C) Schematic representation of the transcriptional initiation sites of A224L. The clear boxes enclose the polythymidylate signals in proximity of the A224L ORF (depicted as a solid box). Arrows above the sequence indicate the location of the A224L transcriptional initiation sites detected by primer extension. The distance between the A224L termination codon and the first two polythymidylate signals downstream of the ORF is indicated in bp.

407 nucleotides from the termination codon of the A224L ORF (Fig. 2C) and the sizes of the two late mRNA species detected by Northern blot match exactly the expected sizes of transcripts initiating at the A224L transcriptional initiation site and terminating at either the first or the second polythymidylate signal. The origin of the radioactive bands in samples from early and immediate-early virus-induced RNA in the Northern blot is unknown. However, the lack of a specific early transcriptional initiation site along with the data concerning the expression of pA224L in infected cells described below suggest that these bands may reflect hybridization of the labeled oligonucleotide with A224L-unrelated mRNAs.

To facilitate the identification of pA224L a specific polyclonal antiserum was raised against this protein. For this, an A224L expression plasmid, pRSET-A224L, was constructed by cloning an A224L-containing *Hin*dIII/*Nco*I fragment (see Fig. 1A) from plasmid pRASC (*18*) into the expression vector pRSETB (*16*) digested with *Hin*dIII/*Nco*I. pRSET-A224L was used to transform the BL21 strain of *E. coli* (*22*). After induction, the transformed cells express a fusion protein that contains the 186 C-terminal amino acid residues of pA224L (data not shown). The fusion protein was used to immunize rabbits after purification under denaturing conditions using a Ni^{+2} affinity chromatography column (Quiagen) according to the manufacturer's instructions.

The expression of pA224L during infection was analyzed by Western blot. The protein extracts used for these experiments were generated from Vero cells either mock-infected or infected with ASFV at m.o.i. of 20 PFU/cell. The cultures were maintained either for 8 hr in cytosine arabinoside-supplemented medium (early proteins) or for 20 hr in normal medium (late proteins). After dissociation in sample buffer (40 m*M* Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue), 20 μg of protein from the different cell extracts were run on SDS–PAGE gels (*17*) and transferred to nitrocellulose filters.

The presence of pA224L within ASFV virions was also analyzed. For this, 15 μg of total protein from ASFV particles purified using percoll gradients (*4*) were separated by SDS–PAGE electrophoresis and transferred to nitrocellulose.

The protein blots were incubated with the rabbit anti-pA224L antiserum (at a dilution 1:100), and the antigen–antibody complexes were detected by incubation with peroxidase-labeled goat anti-rabbit IgG followed by a further incubation with peroxidase buffer (0.02% 1-chloro-4-naphthol, 0.006% hydrogen peroxide in PBS (pH 7.2)) using standard procedures (*14*). As shown in Fig. 3, a protein with an electrophoretic mobility of approximately 27 kDa is strongly and specifically recognized by the anti-pA224L antiserum in the lanes corresponding to ASFV-

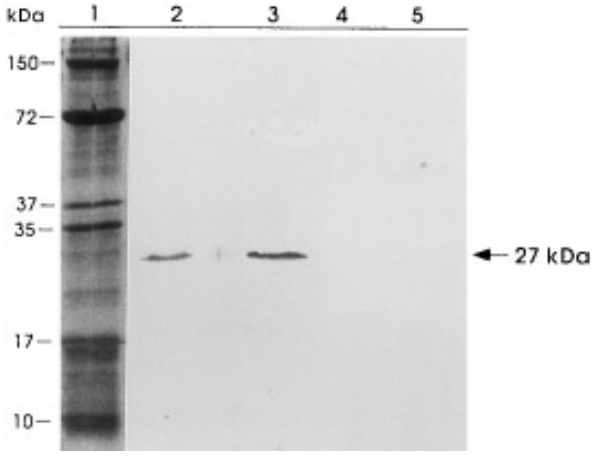

FIG. 3. Identification of pA224L protein by Western blot analysis. Protein extracts from purified ASFV particles (lane 2), ASFV-infected cultures harvested at 20 hr postinfection (lane 3), ASFV-infected cultures maintained in the presence of cytosine arabinoside (lane 4), and mock-infected Vero cells (lane 5) were subjected to SDS–PAGE electrophoresis and blotted onto nitrocellulose paper. The blot was incubated with rabbit anti-pA224L antiserum, and bound antibody was detected using a second antibody labeled with peroxidase. Lane 1 corresponds to a Coomassie blue-stained gel from purified ASFV particles. The molecular mass of the most abundant ASFV structural proteins and the immunoreactive polypeptide are indicated.

induced late proteins and purified ASFV particles. This corresponds with the predicted molecular mass of pA224L (*27*). The specific detection of pA224L during the late phase of the infection correlates with the results of the transcriptional analysis, demonstrating that pA224L is a genuine ASFV late protein. Similar results were obtained by immunoprecipitation of samples from [^{35}S]-methionine-labeled ASFV-infected cells (data not shown).

The subcellular distribution of pA224L within ASFV-infected cells was analyzed by immunofluorescence. Infected cells were washed four times with staining buffer (10 m*M* EDTA in PBS (pH 7.2)) at 4°C and incubated for 30 min at 4°C with 15 μg/ml of total immunoglobulins purified from the anti-A224L antiserum (as described in Ref. *5*). The cells were then washed twice with staining buffer and incubated with rhodamine-conjugated goat anti-rabbit IgG for 30 min at 4°C. Subsequently, cells were incubated with the mAb 17LD3, specific for the ASFV capsid protein p72 (*5*), for 30 min at 4°C, washed with staining buffer, reacted with fluorescein-conjugated rabbit anti-mouse IgG for 30 min at 4°C, and were then observed using a Zeiss Axiovert microscope. Immunofluorescence produced after staining with pA224L-specific antibodies accumulates in large spots within the cytoplasm of the infected cells (Fig. 3A, panel 2), matching the fluorescence pattern produced by mAbs against p72 (Fig. 3A, panel 4). This strongly suggests that, as it is the case with p72 (*5*), the ASFV IAP homolog preferentially accumulates within virus factories, the site of ASFV morphogenesis. Furthermore, the almost complete lack of fluorescence in samples from cultures infected for less than 10 hr (not shown) corroborates the results from the Western blots showing that the accumulation of pA224L is a late event of the ASFV replication cycle.

The presence of pA224L in virus factories and the detection of a strong pA224L-specific signal in protein extracts from samples of highly purified virus particles (Fig. 3) strongly suggests that pA224L might be integrated into ASFV virions. This was further assessed by immunoelectron microscopy. For this, cells infected with 20 PFU/cell for 24 hr were fixed with 0.5% glutaraldehyde in PBS containing 2% tannic acid. After the samples were dehydrated with ethanol and embedded in lowicryl K4M (*3*), thin sections were immunogold-labeled with 14 nm protein A–gold complexes as described previously (*10*), and examined using a Jeol 1010 electron microscope. As expected from the results obtained by immunofluorescence, most of the detected gold particles were found within electron-dense areas located in close proximity to the cell nuclei (not shown). As illustrated in Fig. 4B, a large proportion (over 50% from a total of more than 1000 observed particles) of the virions were immunodecorated and as a general rule the gold particles localized within the periphery of the nucleoide. Although a more detailed analysis will be required to define its precise topology,

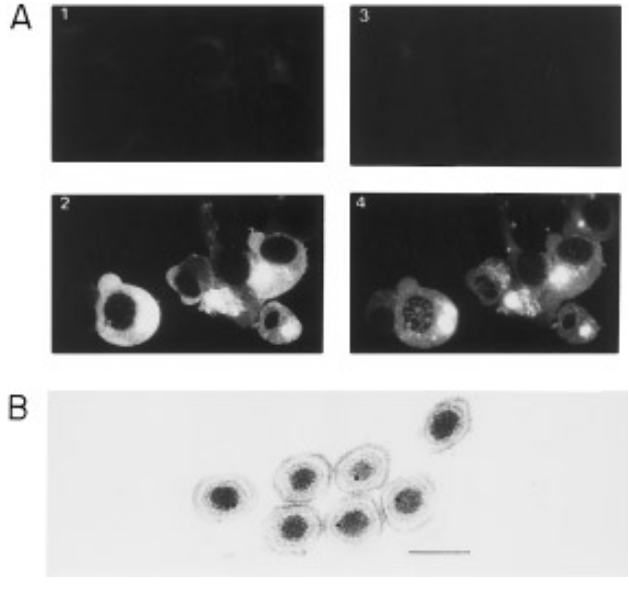

FIG. 4. Localization of pA224L in ASFV-infected cells. (A) Immunofluorescence analysis. Cultures of Vero cells either mock infected (1 and 3) or ASFV infected (2, 4) were fixed and processed for immunofluorescence using rabbit anti-pA224L antiserum and mouse mAb anti-p72. Bound antibodies were detected using rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated rabbit anti-mouse IgG. Samples were observed with using filters for either rhodamine (1 and 2) or fluorescein (2 and 4). (B) Immunoelectron microscopy of ASFV-infected cells. Cells infected for 24 hr were harvested, fixed, thin sectioned, and labeled with specific anti-pA224L polyclonal antiserum followed by incubation with protein A-gold complexes. The samples were visualized by electron microscopy. Bar, 200 nm.

the data presented here clearly demonstrate that pA224L is present within ASFV particles.

While expression of A224L is restricted to the late phase of the ASFV infectious cycle, the resultant ASFV-IAP homolog is incorporated into the virus particles as is the case with the apoptotic suppressor protein p35 of baculovirus (*15*). Consequently, it seems possible that the pA224L molecules carried by the infecting ASFV particles might efficiently prevent the triggering of programmed cell death during the initial steps of the infection. In addition, the expression of the A224L gene after virus DNA replication would ensure that cell integrity was maintained until the virus progeny were mature. The use of techniques to manipulate the ASFV genome (*11, 20*) may allow the specific role of A224L to be assessed: the construction of vectors for the generation A224L deletion mutants is currently underway.

The fact that the two potential ASFV apoptosis control proteins, pA179L and pA224L, are of different kinds, i.e., members of the bcl-2/bax and IAP families, respectively, when considered in the light of the ability of ASFV to replicate in both mammalian and arthropod hosts (several species of suids and soft ticks of the *Ornithodoros* genus, respectively; see Ref. *25*), leads to the speculation that A179L and A224L may function as host-range genes. Thus, pA224L could be specifically responsible for the blockade of apoptosis in tick cells while pA179L could prevent programmed cell death in the mammalian cells. The recent demonstration that expression of the human Bcl-2(alpha) gene in baculovirus-infected insect cells does not prevent apoptosis (*7*) supports this hypothesis.

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