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African Swine Fever Virus IAP Homologue Inhibits Caspase Activation and Promotes Cell Survival in Mammalian Cells

MARÍA L. NOGAL,[1] GONZALO GONZÁLEZ DE BUITRAGO,[2] CLARA RODRÍGUEZ,[1] BEATRIZ CUBELOS,[1] ANGEL L. CARRASCOSA,[1] MARÍA L. SALAS,[1] AND YOLANDA REVILLA[1]*

Centro de Biología Molecular "Severo Ochoa" (C.S.I.C.-U.A.M.)[1] and Centro Nacional de Biotecnología (C.S.I.C.),[2] Universidad Autónoma de Madrid, 28049 Madrid, Spain

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African swine fever virus (ASFV) A224L is a member of the inhibitor of apoptosis protein (IAP) family. We have investigated the antiapoptotic function of the viral IAP both in stably transfected cells and in ASFV-infected cells. A224L was able to substantially inhibit caspase activity and cell death induced by treatment with tumor necrosis factor alpha and cycloheximide or staurosporine when overexpressed in Vero cells by gene transfection. We have also observed that ASFV infection induces caspase activation and apoptosis in Vero cells. Furthermore, using a deletion mutant of ASFV lacking the A224L gene, we have shown that the viral IAP modulates the proteolytic processing of the effector cell death protease caspase-3 and the apoptosis which are induced in the infected cells. Our findings indicate that A224L interacts with the proteolytic fragment of caspase-3 and inhibits the activity of this protease during ASFV infection. These observations could indicate a conserved mechanism of action for ASFV IAP and other IAP family members to suppress apoptosis.

The inhibitor of apoptosis protein (IAP) family was initially identified in baculovirus (5, 10) and more recently has been described in metazoans (14, 15, 23). The IAPs are able to prevent the apoptosis induced in cells by a variety of apoptotic triggers, their range of action being wider than that observed for any other family of apoptotic inhibitors, including the *bc1-2* family (9, 12, 41, 43, 49).

Although the biochemical mechanism by which IAP family proteins inhibits cell death has been elusive, it now appears that the majority of IAPs inhibit the cysteine proteases known as caspases (13, 22, 25, 28). The human IAPs (cIAP1, cIAP2, and XIAP) have been documented to bind to at least two members of the caspase family of cell death proteases, caspase -3 and caspase- 7, strongly suppressing their activity. In addition, they can prevent the proteolytic processing of procaspase-3, -6, and -7 by blocking the cytochrome *c*-induced activation of procaspase-9 (14, 46, 47).

The common structural feature of all IAP family members is an N-terminal 70-amino-acid motif termed the baculovirus internal repeat (BIR), which is present in one to three copies (3, 4, 24, 51). The BIRs are involved in all known interactions between IAPs and other proteins and are required for the antideath activity of IAPs (14, 20). For example, the interaction between mammalian IAPs and procaspase-6 and -9 and active caspase-3 and -7 occurs via the BIR domains. It has been shown that the second of the three BIR domains of XIAP is necessary and sufficient for inhibiting certain caspase-family cell death proteases, implying that a single BIR domain can possess antiapoptotic activity (48). The second defining motif found in baculoviral IAPs is the COOH terminus RING Zn finger (6). The presence of this motif appears to be critical for the baculovirus IAP antiapoptotic function, while it is dispensable for apoptotic inhibition by some cellular IAPs (14, 20, 49).

African swine fever virus (ASFV), the causative agent of African swine fever, is a large enveloped virus with a double-stranded DNA genome of about 170 kbp. The analysis of the complete nucleotide sequence of the genome of the BA71V strain (53), an avirulent cell culture-adapted European virus, has revealed the existence of several genes potentially capable of modulating the virus-host interaction (7, 36–38). Two of these genes encode proteins which are candidates to control the apoptosis in the infected cell. We have previously described the antiapoptotic function of protein pA179L, an early protein in the viral cycle, which contains two highly conserved domains, BH1 and BH2, characteristic of the antiapoptotic members of the *bc1-2* family (37).

The second gene, a BIR motif-containing gene with similarity to IAP genes, has been identified in a pathogenic African isolate and also in the BA71V strain (7, 33). The ASFV gene, designated A224L, encodes a protein containing a single BIR motif at the NH_2 terminus and a sequence that may constitute a zinc finger domain of the 4-cysteine type at the C-terminal region (7, 31). In contrast to this, the zinc finger sequences in baculovirus IAPs are RING fingers of the C3HC4 type (4).

Gene products of several viruses affect apoptosis by interacting directly with components of the conserved pathways that regulate cell death (19, 50). Certain viruses can block apoptosis to prevent death of the host cell, thus allowing the production of virus progeny or facilitating the establishment of a persistent infection, while other viruses promote apoptosis to allow the spreading of virus progeny and to evade the host immune response. Still other viruses, like adenovirus, might perform both functions (40).

To obtain further insight into the role of ASFV IAP in regulating apoptotic cell death, we examined the properties of the viral IAP in the context of both viral infection of Vero cells and in Vero cells transfected with the A224L gene. ASFV IAP

* Corresponding author. Mailing address: Centro de Biología Molecular "Severo Ochoa" (C.S.I.C.-U.A.M.), Universidad Autónoma, 28049 Madrid, Spain. Phone: 3491-397.84.86. Fax: 3491-397.47.99. E-mail: yrevilla@cbm.uam.es.

was found to protect cells from apoptosis induced both by ASFV infection and apoptotic stimuli such as staurosporine or tumor necrosis factor alpha (TNF-α) and cycloheximide (CHX). We also show that the viral IAP interacts with caspase-3 and inhibits the activity of this cell death protease, this probably being the mechanism by which ASFV IAP controls apoptosis in the infected cell.

MATERIALS AND METHODS

Cells and viruses. Vero cells were cultured at 37°C in DMEM supplemented with 5% newborn calf serum. Jurkat cells were cultured at 37°C in RPMI supplemented with 10% fetal calf serum. The Vero-adapted ASFV strain BA71V was propagated and titrated by plaque assay on Vero cells as described (16). The ASFV deletion mutant ΔIAP was obtained as described below.

ASFV ΔIAP deletion mutant construction. The IAP-defective mutant ΔIAP virus was obtained by insertion of the *Escherichia coli* β-glucuronidase (β-gus) gene into the A224L open reading frame. For this, an 844-bp left-flanking DNA fragment was generated as follows. A 1.3-kbp intermediate fragment obtained by digestion with *Hin*dIII of the *Eco*RI A/*Sal*IC (RA/SC) restriction fragment of the BA71V genome (29) was digested with *Afl*III and treated with Klenow enzyme. The 844-bp fragment was then cloned into plasmid p72 GUS10T (17) digested with *Hin*dIII-*Afl*III and Klenow enzyme treated. The resulting plasmid was digested with *Sma*I to generate deletion plasmid pΔA224L by cloning a second right-flanking 480-bp DNA fragment obtained by digestion of the RA/SC restriction fragment with *Hin*dIII and *Sal*I and treatment with Klenow enzyme. The deletion removed all but 62 and 98 nucleotides of the 5′ end and 3′ end of open reading frame A224L, respectively.

The recombinant ΔIAP virus was obtained as previously described (17). Briefly, Vero cells were infected with BA71V and transfected by lipofection with pΔA224L. After 48 h of infection, the cells were harvested, and diluted samples were used to infect Vero cell monolayers. The infected cells were covered with agar (Gibco), and 4 days later, the β-gus substrate X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) was added to the culture medium. The blue-stained plaques were selected and used to infect fresh monolayers of Vero cells. The recombinant virus was purified after successive rounds of plaque isolation.

The lack of gene A224L in the recombinant ΔIAP virus was assessed by Southern blot hybridization. Briefly, DNA samples from the genome of previously purified virus BA71V and ΔIAP were digested with the restriction endonuclease *Eco*RI, subjected to electrophoresis in agarose gels, and transferred to nylon membranes following standard procedures (45). The DNA probes, specific for the β-gus and A224L genes, were labeled with a DIG DNA labeling kit (Boehringer Mannhein), and hybridizations were done as described elsewhere (1).

Assay of virus production. To assay ASFV production, Vero cell cultures were infected with either BA71V or ΔIAP at a multiplicity of infection (MOI) of 10 PFU per cell. After virus adsorption the inoculum was removed, and cell cultures were washed twice with warm medium and then incubated at 37°C. Total virus was collected at different times after infection and titrated by plaque assay on Vero cells as indicated (16).

Transfections. The viral IAP was subcloned into the pcDNA3 mammalian expression vector (Invitrogen). Vero cells at 10^5 per 6-cm-diameter dish were plated 24 h before transfection in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum. Nearly confluent cells were transfected with 0.5 μg of pcDNA3 or 0.5 μg of pcDNA3-A224L, using 3 μl of Lipofectamine (GIBCO/BRL) per dish.

For G418 selection, cells were transfected as described above. After washing, the cells were trypsinized and plated at 1:10 dilution in 10-cm-diameter dishes. The next day, antibiotic selection was applied starting at 500 μg of G418 (Sigma) per ml. Cells were refed with medium with fresh antibiotic every 3 days until colonies were apparent (2 to 3 weeks).

Western blot analysis. Cells were washed twice with phosphate-buffered saline (PBS) and lysed in radio immunoprecipitation buffer. Protein concentration was determined by the Bradford protein assay (Bio-Rad). Proteins (10 to 50 μg) were electrophoresed in a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel, blotted onto an Inmobilon extra membrane (Amersham), and reacted with specific antibodies.

Specific antiviral IAP antibody was raised in rabbits after immunization with specific peptides corresponding to regions in the A224L sequence not conserved in others IAPs. Rabbit polyclonal anti-caspase-3 and anti-poly (ADP-ribose) polymerase (anti-PARP) antibodies were purchased from PharMingen International and were used diluted 1:1,000 and 1:300, respectively.

After incubation with the specific primary antibody, membranes were exposed to horseradish peroxidase-conjugated secondary anti-rabbit antibody (Dako), followed by visualization of positive bands with the Amersham Life Science enhanced chemiluminescence (ECL) procedure by using Kodak film.

Caspase activity assays. (i) Preparation of cytosols. All steps were performed at 4°C. Vero cells were sedimented at 250 × *g* for 10 min, washed twice in PBS, and resuspended in extraction buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.5 mM EDTA, 10 mM Na_2HPO_4, 1% Nonidet P-40), supplemented immediately before use with 0.4 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, aprotinin (10 μg/ml), and leupeptin (10 μg/ml). After 30 min of incubation in ice, the cell lysate was centrifuged at 20,000 × *g* for 30 min, and the supernatant was used as cytosolic extract.

(ii) Fluorogenic assays. The cytosolic extract (aliquots containing 1 mg of cytosolic protein per ml) was diluted five times with assay buffer (25 mM HEPES [pH 7.5], 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS], 10% sucrose, 10 mM dithiothreitol and 0.1 mg/ml ovalbumin) and incubated at 37°C for 2 h with a 10 μM concentration of fluorescent substrate for specific caspases. The reaction was stopped by the addition of HPLC buffer (H_2O-acetonitrile $^{75}/_{25}$, 0.1% trifluoroacetic acid). Cleaved fluorescent substrate was determined by C_{18} reverse-phase high-performance liquid chromatography using fluorescent detection (338 nm [excitation] and 455 nm [emission]). Control experiments (not shown) confirmed that the release of substrate was linear with time and with protein concentration under the conditions specified.

Biochemical induction of apoptosis. Cell apoptosis was induced by the addition of staurosporine (Calbiochem) to the culture medium at a final concentration of 1 μM. Cells were incubated in the presence of staurosporine for 24 h and were then washed twice in PBS and analyzed by fluorescence-activated cell sorting (FACS). In separate experiments, Vero cells were treated with CHX (5 μg/ml; Sigma) and TNF-α (100 ng/ml) for 16 h. Cells were collected and measured for caspase activity.

FACS analysis. After ASFV infection or incubation with apoptotic stimuli, approximately 10^6 cells were washed twice with PBS and fixed in 50% ethanol in PBS at 4°C for 1 h. Fixed cells were washed and resuspended in propidium iodide (50 mg/ml) containing 125 U of RNase A per ml. They were analyzed using a Becton Dickinson FACScan to determine the percentage of apoptotic cells in the cellular cycle.

Coimmunoprecipitation assay. 10^6 Vero cells were mock infected or infected with 10 PFU of BA71V or ΔIAP per cell and labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine (Amersham Corp.) (50 μCi/10^6 cells). After 24 h, the cells were washed twice with PBS and lysed in 300 μl of TNT buffer (1% Triton X-100, 20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 0.4 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, and 10 μg of leupeptin per ml). Cellular debris was removed by centrifugation at 10,000 × *g* for 5 min, and the supernatants were then incubated with 20 μg of specific anti-caspase-3 antiserum, coupled to protein A-Sepharose beads, at 4°C for at least 2 h. Immunoprecipitates were washed five times with 5 ml of washing buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1% Triton X-100), eluted by boiling in electrophoresis sample buffer, and electrophoresed in an SDS–10% polyacrylamide gel. For autoradiography, the gel was exposed on a Fuji-Film BAS-MP 20405 imaging plate. The exposed imaging plate was analyzed with a Fuji BAS 1500 reader.

RESULTS

ASFV IAP can protect transfected cells from apoptosis. Recent studies have demonstrated that several members of the human IAPs (XIAP, c-IAP1 and c-IAP2) inhibit caspases directly when overexpressed in cells derived from multiple species (14, 21, 27). Considering the similarity in overall organization and primary amino acid sequence between ASFV IAP and cellular IAP proteins, we have investigated the ability of the A224L gene to inhibit caspase activity and to protect cells from apoptosis.

To this end, the full-length A224L gene was stably transfected in Vero cells by lipofection using the eukaryotic expression pcDNA3 plasmid. The synthesis of RNA specific for the A224L gene was determined by reverse transcription-PCR using the appropriate complementary primers. As expected, an A224L-specific band with the predicted size was found in the

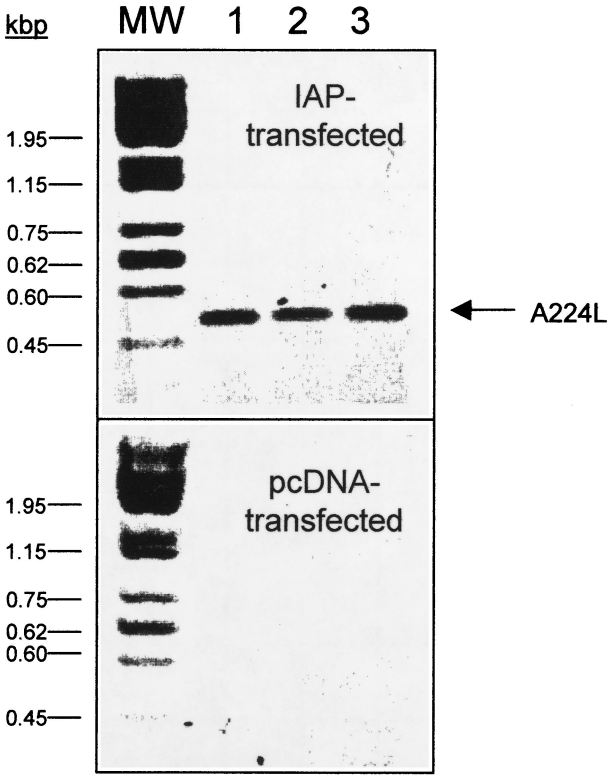

FIG. 1. Transcription of ASFV IAP gene in A224L stably transfected Vero cells. RNAs were isolated from three different clones (lanes 1, 2, and 3) of IAP-transfected or pcDNA-transfected Vero cells. PCR products were generated using A224L-specific primers, and cDNAs were synthesized from RNAs and resolved in a 1.5% agarose gel. The sizes in kilobase pairs of molecular weight markers (lane MW) are indicated on the left.

Vero cell clones transfected with the viral IAP gene, while no band could be detected in clones transfected with the control plasmid (Fig. 1).

Positive cells were then treated either with TNF-CHX or staurosporine as described under Materials and Methods. CHX is often required for apoptosis induced by TNF, probably due to the CHX-induced decrease in the amount of some short-lived proteins that otherwise protect cells from apoptosis.

The cleavage of specific caspase-3 substrate was analyzed in extracts from cells treated for 16 h with TNF-CHX, revealing a marked reduction in TNF-CHX-induced caspase-3 activation in the IAP-transfected Vero cells (Fig. 2A).

Flow cytometric analysis of the staurosporine-treated cells for DNA content showed that, compared with cells transfected with control pcDNA, there was a considerable decrease in the apoptotic fraction of A224L-transfected cells, from 46.3% of death in control cells to 12.4% in cells expressing the A224L gene (Fig. 2B).

A224L modulates caspase activity during ASFV infection. To obtain further insight into the role of the viral IAP in regulating apoptotic cell death, we examined the properties of A224L in the context of an ASFV infection of Vero cells. It is known that apoptosis is induced in ASFV-infected cells (33), although the mechanisms involved have not been described. Since caspases have been shown to play a key role in programmed cell death (2, 42), we have analyzed whether ASFV infection induces caspase activity in Vero cells. Caspase activity in cell extracts prepared at different times after infection was assayed by in vitro hydrolysis of the specific caspase-3, -6, and -8 substrates. Figure 3A shows the kinetics of induction of caspase activity in ASFV-infected cells. An increase of caspase activity is observed at 16 hpi and later, this increase being greater for caspase-3 than for caspase-6 and -8. The increase in caspase activity probably reflects that ASFV induces the processing of caspases in the infected cell (see below), and, consequently, the activation of the apoptotic pathway. To confirm this, we have explored the activity of caspase on biological substrates such as PARP. PARP is a 112-kDa nuclear protein that is specifically cleaved by caspase-3 and caspase-6 into an 85-kDa apoptotic fragment (34). As shown in Fig. 3B, the infection of Vero cells induces the cleavage of PARP, with a maximun at 36 hours postinfection (hpi), supporting the argument that the infected cells have undergone apoptosis.

Since, as described above, the ASFV IAP can prevent caspase activation and apoptosis when overexpressed in transfected cells, it was possible that the viral gene might also modulate in some way caspase activity and cell death during ASFV infection. To explore this possibility, an ASFV A224L deletion mutant, designated ΔIAP, was constructed from the BA71V isolate by homologous recombination between the parental genome and the deletion plasmid pΔA224L in Vero cells, as described under Materials and Methods. Recombinant virus expressing the β-gus gene was purified, and genomic DNA from wild-type and ΔIAP virus was analyzed by Southern blotting, using digoxigenine-labeled DNA probes. As shown in Fig. 4, pUC118 plasmid probe failed to hybridize with genomic DNA from both parental and ΔIAP virus, ruling out the possibility of a single crossover event. DNA fragments of predicted size were observed in both viruses when probed with the parental DNA fragment RA/SC. A β-gus gene probe hybridized only with DNA from ΔIAP. As expected, a A224L gene probe failed to hybridize with DNA from ΔIAP.

The production of infective virus in the infection with the deletion mutant was similar to that with the parental virus, as determined by analyzing the virus yield 24 and 48 h after infection. At 24 h virus production of BA71V and ΔIAP were 3.2×10^6 and 2.6×10^6 PFU/ml, respectively; at 48 h, the production was 1.5×10^7 and 1.0×10^7, respectively. This result indicates that viral production is not affected by the lack of the A224L gene.

We next examined the role of the viral IAP in modulating the caspase activity by comparing the levels of this activity in Vero cells infected with the deletion mutant ΔIAP and the parental virus BA71V, using a caspase-specific assay. Vero cell cultures were infected with either BA71V or ΔIAP (MOI = 5), and cytosolic extracts from infected cells at different times after infection were obtained and analyzed. As shown in Fig. 5A, the induction of caspase-3 activity is greater in cells infected with ΔIAP than in those infected with wild-type virus. In contrast, no significant differences have been found in the induction of caspases-6 and -8 between both viruses.

The caspases exist as inactive procaspases which require proteolytic activation (46, 47). To explore the mechanism of action of the viral IAP and whether a correlation exists be-

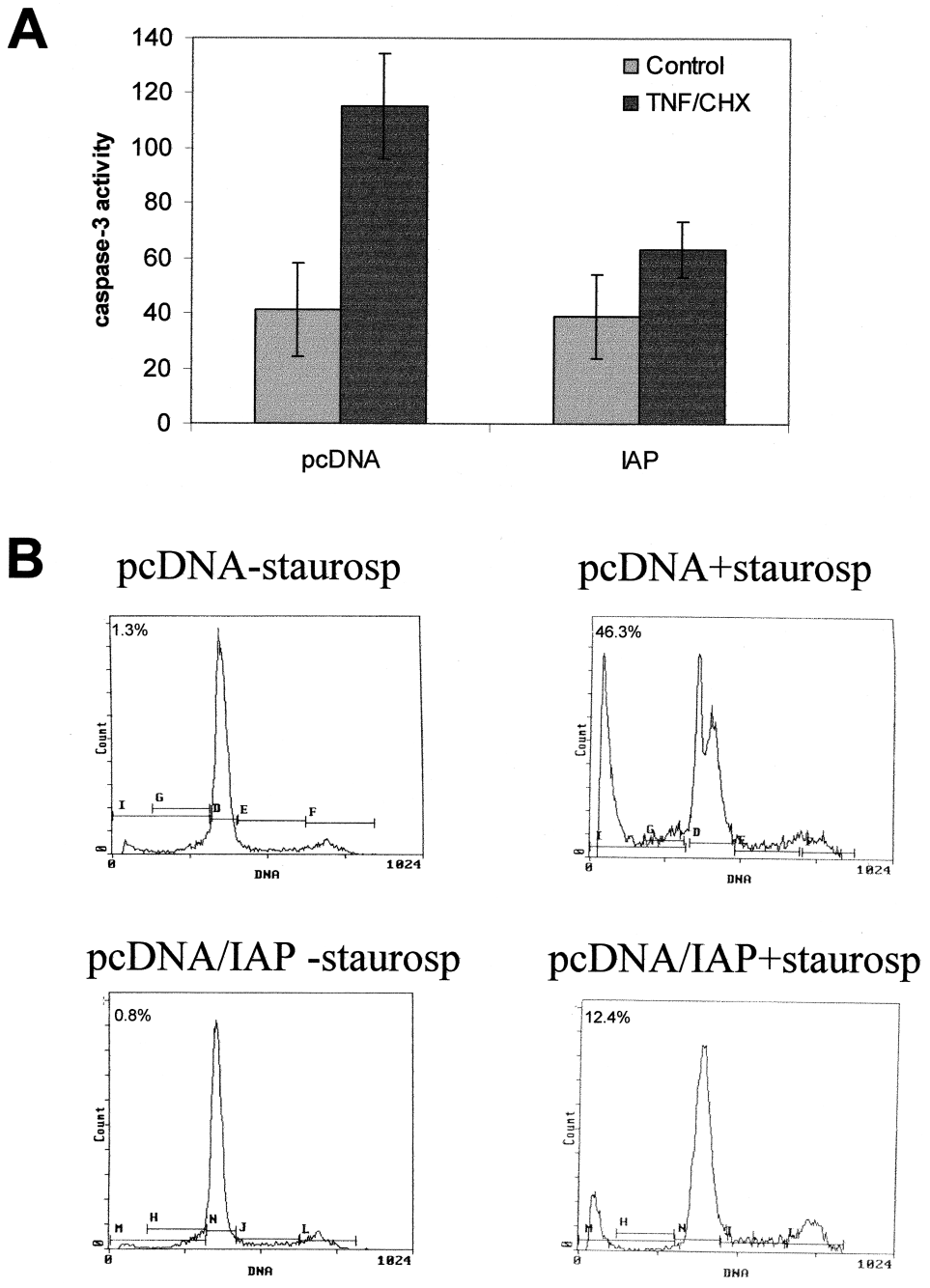

FIG. 2. ASFV IAP protects Vero cells from TNF-CHX or staurosporine-induced apoptosis. Cells were stably transfected with control pcDNA vector or vector containing the A224L gene. (A) DEVD cleavage activity was measured using extracts from transfected Vero cells incubated or not with TNF-α (100 ng/ml) and CHX (5 μg/ml) for 16 h. The results are the average of three experiments (error bars, standard deviation). (B) Transfected Vero cells were incubated with medium or medium plus 1 μM staurosporine for 24 h, and the DNA content of the cells was determined by flow cytometry after propidium iodide staining.

tween induction of caspase-3 activity and the processing of proenzyme to the proteolytic fragment, we have examined in parallel samples from cells infected with ΔIAP and BA71V either for caspase-3 activity (Fig. 5B) or cleavage (Fig. 5C). Cellular extracts obtained at the indicated times after infection were standardized for protein concentration (30 μg per lane) and analyzed by Western blotting using a specific serum anti-caspase-3. This antiserum recognizes both the unprocessed (32-kDa) and cleaved (17-kDa) forms of caspase-3. The figure shows that infection with BA71V causes proteolytic processing of caspase-3 from its 32-kDa zymogen precursor into active subunit from 15 h postinfection (hpi) on. On the other hand, the infection with ΔIAP induces the processing of caspase-3 at an earlier time (13 hpi) and markedly increases the amount of this active subunit from 15 until 24 hpi. No processing of the enzyme can be found in mock-infected cells. This result indi-

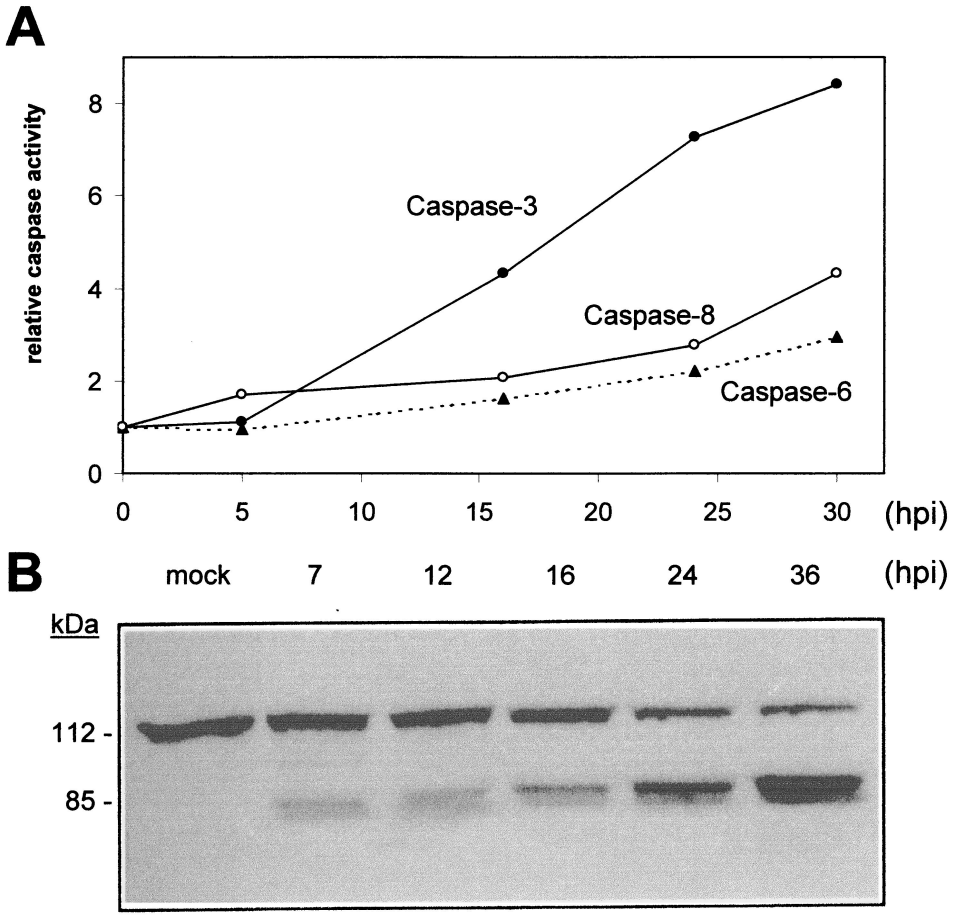

FIG. 3. ASFV induction of caspase-3, 6, and 8 activities and PARP fragmentation in Vero cells. BA71V- or ΔIAP-infected cells (MOI = 10) were collected at the indicated times after infection. (A) Protease activity was measured using specific substrates (Ac-DEVD-AMC, Ac-VEID -AMC, and Ac-IETD-AMC for caspase-3, -6, and -8, respectively) and extracts that were normalized for total protein content (1 mg/ml). Data represent relative caspase activity. Values for zero time were 32, 176, and 59 arbitrary fluorescent units for caspase-3, -6, and -8, respectively. (B) Cell lysates (30 μg) were subjected to SDS–10% polyacrylamide gel electrophoresis. Immunoblot analysis with anti-PARP antibody diluted 1:300 was used for detection using an ECL-based method. The positions of unprocessed and processed PARP are indicated.

cates that A224L inhibits the proteolytic processing of caspase-3 and decreases the level of this protease during the infective cycle of ASFV, showing a temporal correlation between the cleavage of this caspase and its enzymatic activity compared to the data on activity presented in Fig. 5B. It is noteworthy that the differences observed at 24 hpi between the wild-type and mutant virus in the cleavage of the procaspase to the active fragment are not reflected in a corresponding difference in caspase activity. The reasons for this are not clear at present. It is possible that, in the absence of the viral protein, the cellular IAPs might interact with the processed caspase inhibiting its activity to some extent.

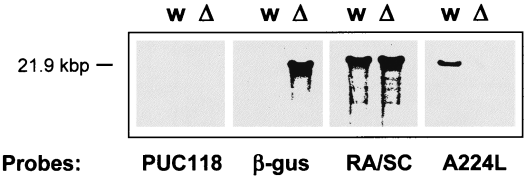

FIG. 4. Characterization of the BA71V A224L deletion mutant, ΔIAP. Southern blot analysis of parental BA71V virus (w) and ΔIAP (Δ). Purified viral DNA digested with *Eco*RI was electrophoresed, blotted, and hybridized with the indicated probes.

To confirm the role of the viral IAP in the control of caspase-3 processing, we infected with BA71V or ΔIAP (MOI = 5) the Vero cell line stably transfected with A224L. Extracts from infected cells were processed as previously described, electrophoresed and analyzed for the formation of the proteolytic fragment of caspase-3 by Western blotting. As shown in Fig. 6, and in contrast with the results obtained in nontransfected Vero cells, no differences in the kinetics or in the level of expression of the active fragment of caspase-3 could be found between the infection with the parental virus and with the A224L deletion mutant, strongly suggesting that the constitutive expression of A224L in Vero cells is able to restore the function of the viral IAP in these cells. It is of interest that the "extra" copy provided by the cell line does not appear to afford aditional protection against caspase-3 processing in the BA71V infected cells.

ASFV IAP interacts with the proteolytic fragment of caspase-3. Besides inhibiting the proteolytic activity of caspase-3 and -7,

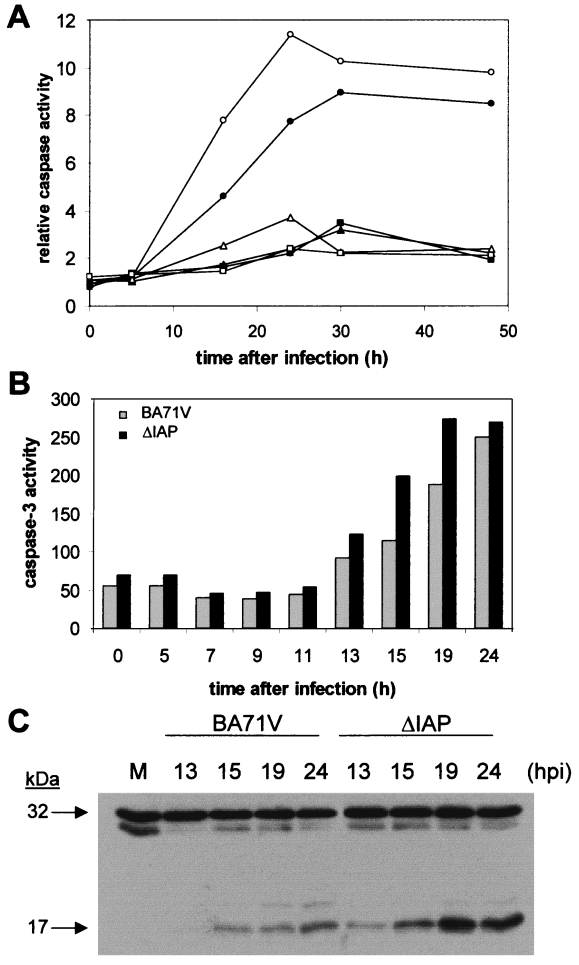

FIG. 5. ASFV IAP controls caspase-3 activity and processing during ASFV infection. (A) Caspase activity was measured using specific substrates (Ac-DEVD-AMC, Ac-VEID-AMC, and Ac-IETD-AMC for caspase-3, -6, and -8, respectively) and extracts from Vero cells infected (MOI = 5) with BA71V or ΔIAP at the indicates times after infection. The extracts were normalized for total protein content (1 mg/ml). Data represent relative caspase activity. Symbols: ●, caspase-3, BA71V; ○, caspase-3, ΔIAP; ▲, caspase-6, BA71V; △, caspase-6, ΔIAP; ■, caspase-8, BA71V; □, caspase-8, ΔIAP. (B) DEVD-cleavage activity was measured using extracts from Vero cells infected (MOI = 5) with BA71V or ΔIAP at the indicated times after infection. Extracts were normalized for total protein content. (C) Cell lysates (30 μg) from Vero cells infected under the same conditions were subjected to SDS–10% polyacrylamide gel electrophoresis. Immunoblot analysis with anti-caspase-3 antibody was used for detection of the protein using an ECL-based method. The upper and lower arrows indicate the proform and the catalytically active subunit of caspase-3, respectively. Lane M, mock-infected cells.

XIAP and some other IAP family proteins appear to bind directly to these proteases in vitro (8, 12, 52). XIAP, however, does not bind efficiently to the unprocessed proenzymes (14). The BIR regions of human IAPs were found to be necessary and sufficient to bind and inhibit caspases, and subsequently it was shown that survivin, which contains a single BIR domain, can be coimmunoprecipitated with caspase-3, -7 and -9 and suppresses apoptosis induced by overexpression of these caspases (49). On the other hand, the conservation of both sequence and function between baculoviral and cellular IAPs

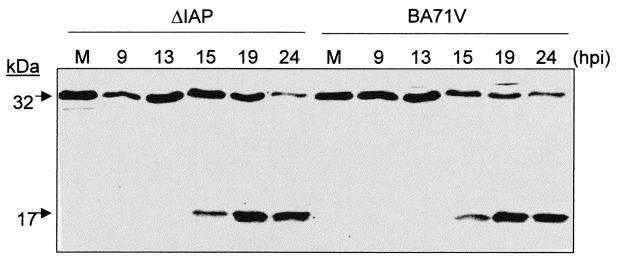

FIG. 6. A224L transfection rescues the phenotype of caspase-3 processing in ΔIAP-infected Vero cells. Vero cells stably transfected with A224L were mock-infected (M) or infected with BA71V or ΔIAP virus. At the indicated times of infection, cell extracts (30 μg) were subjected to SDS–10% polyacrylamide gel electrophoresis and immunoblot analysis with anti-caspase-3 antibody was performed. The respective mobilities of the proenzyme and the proteolytic fragment of caspase-3 are indicated by arrows.

suggests that they interact with conserved components of the apoptotic pathway (27, 44).

We examined the ability of ASFV IAP to bind caspase-3 during ASFV infection in Vero cells. Using lysates from Vero cells obtained at 16 h after infection with parental BA71V or recombinant BA71V-ΔIAP, caspase-3 was immunoprecipitated with specific anti-caspase-3 antibody. As shown in Fig. 7, the antibody immunoprecipitated a band of 17 kDa, corresponding to the proteolytic fragment of caspase-3, in lysates from cells infected either with BA71V or BA71V-ΔIAP. In addition to this, a band migrating at a position around 23 kDa was coprecipitated in extracts from cells infected with the parental BA71V but not in extracts from cells infected with the recombinant BA71V-ΔIAP. Since the only difference between these two viruses is the absence of the gene encoding IAP in the deletion mutant, this result strongly suggests that the band of 23 kDa represents the viral IAP. This indicates that, as has been described for several members of the IAP family, the ASFV IAP interacts with the active fragment of caspase-3, this probably being one of the mechanisms responsible for the inhibition of caspase-3 activity observed during ASFV infection.

A224L controls the survival of ASFV-infected cells. Although apoptosis clearly requires the participation of caspases, the particular caspases vary according to cell type and the stimulus triggering cell death, and, therefore, the ability of each IAP to control apoptosis may also vary.

We have shown before that A224L inhibits caspase activation and apoptosis induced by TNF-CHX or staurosporine when overexpressed in Vero cells. To further study the possible role of A224L in modulating apoptosis induced by ASFV infection in Vero cells, we have examined the cell viability of Vero cells infected with the deletion mutant ΔIAP or the parental BA71V virus. Vero cells were infected with either ΔIAP or BA71V (MOI = 10), and cell survival was assessed at 0, 13, 16, 18, and 24 hpi by analyzing the DNA content by FACS analysis. As shown in Fig. 8, an increase in the level of cellular death can be observed in cells infected with the deletion mutant from 13 hpi when compared to cells infected with the parental virus. This result confirms the antiapoptotic function of the viral IAP during the ASFV infection essentially at late times of the infective cycle, and strongly suggests that

A224L, a late protein in the infective cycle (7) controls the survival of the infected cell during the morphogenesis of the viral particle.

DISCUSSION

Upon viral infection, cell death limits the virus replication. Consequently, viruses that can modulate the apoptotic process have a selective advantage. Several viruses have acquired mechanisms to suppress or delay the apoptosis long enough for the production of sufficient amounts of progeny. These viruses carry genes that encode proteins that interfere with the host's apoptotic machinery or resemble host antiapoptotic genes. Some of these viral proteins have been described to block apoptosis by interacting directly with elements of the highly conserved biochemical pathways that regulate cell death (50). On the other hand, apoptosis induction represents a means by which several viruses can induce cell death and disseminate progeny while limiting the induction of inflammatory and immune responses (11, 26, 40).

Although less information is available on the process of ASFV-induced apoptosis (18) in terms of cellular pathways and specific proteins involved, it can be speculated, in light of previous results, that the virus might be able to delay the apoptosis induced during the infection to prevent premature death of the host cell and thus maximize virus progeny production or facilitate a persistent infection. It has been described that ASFV encodes a Bcl-2 homologue which has been found to inhibit apoptosis (37), and a BIR-containing gene, A224L, with similarity to viral and cellular IAPs (7, 33). Therefore, ASFV might encode a Bcl-2-homologue, which is an early protein in the infective cycle, to inhibit apoptosis at early times, and A224L, a late protein, to modulate apoptosis in the late steps of the infection, when viral morphogenesis occurs, thus facilitating the formation of the viral particles. To explore this hypothesis, we have studied the function of the ASFV IAP in the control of apoptosis in cells transfected with the viral gene, as well as in ASFV-infected cells.

Activation of effector caspases, such as caspase-3, is a general event associated with the apoptosis induced by multiple stimuli. These terminal effector enzymes account for most of the DEVD-cleaving activity which accumulates in cells undergoing apoptosis. In this study, we have observed that ASFV infection induces the activation of caspase-3, -6, and -8 from 13 to 16 hpi in Vero cells and the proteolytic processing of caspase-3 to produce the active form of this enzyme. In connection with this, the proteolytic processing of PARP, one of the direct targets of caspase-3 activity, can be clearly observed at these times of the ASFV infection. Caspase activation could, therefore, be a mechanism for the induction of apoptosis in the infected cell.

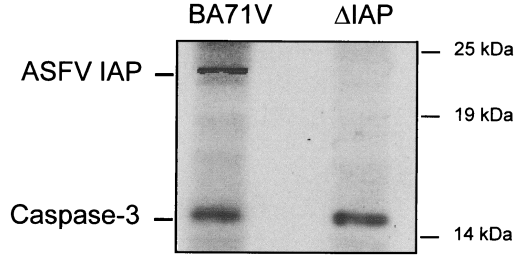

FIG. 7. A224L coprecipitates with the active subunit of caspase-3 during ASFV infection. Vero cells (10^6) were infected (MOI = 10) with BA71V or ΔIAP and labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine. After 24 h, cell extracts were incubated with serum anti-caspase-3 coupled to protein A-Sepharose and electrophoresed. The gel was exposed on a Fuji Film imaging plate and analyzed with a Fuji BAS reader. The positions of the bands corresponding to the ASFV IAP and the proteolytic subunit of caspase-3 are indicated. The positions of molecular mass markers are indicated on the right.

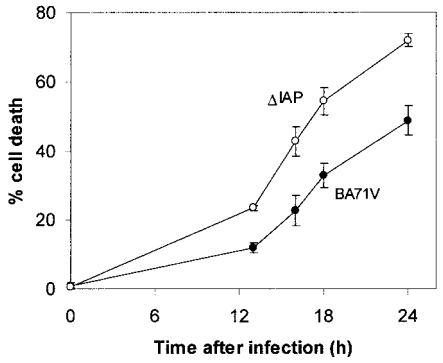

FIG. 8. Antiapoptotic function of viral IAP during the infection of ASFV. Vero cells were infected at an MOI of 10 with either BA71V or BA71V-ΔIAP, and cell survival was assessed at 0, 13, 16, 18, and 24 hpi by analyzing the DNA content of cells by flow cytometry after propidium iodide staining. Data represent the average of three independent experiments (error bars, standard deviations).

Several members of the IAP family proteins have been shown to modulate apoptosis by inhibiting caspase-3 activity (12, 14, 41). The IAPs were first identified in a genetic screen for baculovirus genes that can complement the loss of gene p35 and block apoptosis (15). Baculovirus infection activates the processing of Sf-caspase-1, which, like mammalian caspase-3, is expressed in its inactive proform in Sf-21 cells. The baculovirus IAPs are able to block the activation of procaspase, but cannot prevent the apoptosis induced by the active form of this protease (43).

In this work, we show that ASFV IAP modulates the apoptosis in ASFV infection in a similar way to that described for baculovirus IAP in baculovirus infection. Thus, A224L has been shown to control both the amount and the kinetics of formation of processed caspase-3, in ASFV-infected Vero cells, as deduced from data obtained with the recombinant lacking A224L. In contrast, A224L has no effect on caspase-6 and -8. Interestingly, we have also shown that ASFV IAP interacts with the proteolytic fragment of processed caspase-3, suggesting that A224L can not only inhibit the cleavage of the pro enzyme but also control the activity of the caspase-3. The modulation of caspase-3 activity by these two mechanisms has been previously described for human IAP (14). Those authors showed that, besides inhibiting the proteolytic activity of caspase-3, XIAP also bound directly to this protease in vitro.

In contrast with the results presented here, Neilan et al. (33) found no significant differences in the apoptosis induced in swine macrophages by the virulent ASFV Malawi Lil-20/1 strain and the corresponding IAP deletion mutant. Although the IAP genes of both strains are highly conserved, the differ-

ent system used might explain the discrepancy in the apoptotic effect of the viral IAP during infection. It should also be taken into account that, since the quantitative determination of apoptotic cell death presented by these authors was limited to 16 or 24 hpi (using a different method in each case), a more detailed kinetic study of apoptotic induction in Malawi strain-infected macrophages would be needed to compare the results obtained in this system with those presented in our work.

IAP family proteins are conserved throughout evolution and can suppress apoptosis when expressed in cells from multiple species. The extensive similarity shared by IAP family proteins across evolution (particularly in the BIR domains) and the ability of IAPs to block cell death across species suggests that these proteins target a common component in the apoptosis pathways (30, 35, 39). The ability of baculovirus OpIAP protein to protect against cell death in mammalian cells is an example of conservation of apoptotic mechanisms between distantly related species, and demonstrates that components of the mammalian apoptotic machinery exist that can interact with viral IAP proteins (11, 21, 40). In agreement with this, we have demonstrated that the ASFV A224L gene can control apoptosis and caspase-3 activity induced by staurosporine or TNF-CHX when transfected in Vero cells. Furthermore, the stable expression of A224L in these cells was shown to rescue the proteolytic processing of caspase-3 induced by the infection with the IAP knockout virus.

Given the homology in the BIR sequence and the functional relationship between ASFV IAP and cellular IAPs, it is possible that the ASFV IAP gene derives from a cellular gene. Genes that modulate apoptosis might be incorporated into viral genomes to sustain host cell viability (15, 25), in a similar way as viral genomes have incorporated genes that control cell proliferation to enhance viral replication. Our results suggest that viral acquisition of an IAP-related gene can promote cell survival in vitro. On the other hand, the high degree of conservation of this viral gene among ASFV isolates (33) indicates that A224L has a significant function in the virus-host interaction and is consistent with our data.

Although virus production does not seem to be affected by the absence of the A224L gene, it should be taken into account that, given the percentage of apoptosis (approximately 50%) observed at 24 hpi in cells infected with the wild-type virus, the increase in apoptosis with the deletion mutant may not be sufficient to allow the detection of possible differences in virus production by plaque assay. On the other hand, as previously mentioned, other ASFV genes, such as the antiapoptotic Bcl-2 homologue, migth contribute to support the production of the virus infected cell. Multiple deletion mutants in the ASFV genes controlling cell survival would be useful in assessing this point.

In conclusion, our results demonstrate that the ASFV survival gene IAP promotes cell survival in mammalian cells either when expressed after transfection or during ASFV infection and reveal the viral gene as a functional homologue of the highly conserved IAP family.

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