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Apoptosis Induced in an Early Step of African Swine Fever Virus Entry into Vero Cells Does Not Require Virus Replication

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Permissive Vero cells develop apoptosis, as characterized by DNA fragmentation, caspases activation, cytosolic release of mitochondrial cytochrome *c*, and flow cytometric analysis of DNA content, upon infection with African swine fever virus (ASFV). To determine the step in virus replication that triggers apoptosis, we used UV-inactivated virus, inhibitors of protein and nucleic acid synthesis, and lysosomotropic drugs that block virus uncoating. ASFV-induced apoptosis was accompanied by caspase-3 activation, which was detected even in the presence of either cytosine arabinoside or cycloheximide, indicating that viral DNA replication and protein synthesis were not required to activate the apoptotic process. The activation of caspase-3 was released from chloroquine inhibition 2 h after virus absorption, while the infection with UV-inactivated ASFV did not induce the activation of the caspase cascade. We conclude that ASFV induces apoptosis in the infected cell by an intracellular pathway probably triggered during the process of virus uncoating.

Key Words: African swine fever virus; apoptosis; caspases.

INTRODUCTION

Apoptosis is a tightly controlled cell suicide process, critical for the formation (embryonic development) and maintenance (tissue homeostasis) of multicellular organisms, which is activated by a variety of extracellular and intracellular signals (Vaux and Strasser, 1996). Programmed cell death also plays a protective role eliminating cells damaged by irradiation, chemical injuries, or virus infections (O'Brien, 1998). The elimination by apoptosis of virus-infected cells can be mediated by cytotoxic T lymphocytes recognition (reviewed by Oldstone, 1997), or by the expression of viral components, which can modify the intracellular dynamic balance between family members promoting and inhibiting apoptosis (White, 1996). On the other hand, viruses have evolved strategies to regulate apoptosis, either by blocking it to prevent premature death of the host cell and thus maximizing virus production (or facilitating persistent infections), or by actively promoting programmed cell death to spread virus progeny to neighboring cells while limiting host inflammatory and other immune responses (O'Brien, 1998). Although virus replication in the host cell is necessary for induction of apoptosis by many types of viruses, the induction of cell death in the absence of viral replication has been reported in cells infected by type 3 reovirus (Tyler *et al.,* 1995), avian leukosis virus (Brojatsch *et al.,* 1996), human herpes simplex 1 (Aubert *et al.,* 1999), bovine herpesvirus 1 (Hanon *et al.,* 1997), vaccinia virus (Ramsey-Ewing and Moss, 1998), and Sindbis virus (Jan and Griffin, 1999).

African swine fever, a highly contagious disease of domestic pigs, is caused by a large double-stranded DNA virus that infects a variety of cells of the mononuclear phagocytic system (Wilkinson, 1989; Malmquist and Hay, 1960; Plowright *et al.,* 1968; Casal *et al.,* 1984). Apoptosis has been described as a process associated with African swine fever virus (ASFV) replication in acute *in vivo* (Gomez-Villamandos *et al.,* 1995; Ramiro-Ibanez *et al.,* 1996; Oura *et al.,* 1998) and *in vitro* infections (Nogal *et al.,* 2001). The analysis of the complete nucleotide sequence of the genome of the BA71V strain of ASFV (Yáñez *et al.,* 1995) has revealed the existence of several genes potentially capable of modulating the virus–host interaction. One of these genes, A179L, which encodes a viral Bcl-2 homolog, has been shown to inhibit apoptotic cell death by several groups (Afonso *et al.,* 1996; Brun *et al.,* 1996, 1998; Revilla *et al.,* 1997). We have described another ASFV gene, A224L, with similarity to IAP genes, which encodes a late structural polypeptide (Chacón *et al.,* 1995) able to inhibit caspase activation and to promote cell survival in mammalian cells (Nogal *et al.,* 2001). Other virus genes may also be important in interfering with the host immune response to the viral infection, including A238L, EP402R, and EP153R, which encode an IkappaB-like protein (Powell *et al.,* 1996; Revilla *et al.,* 1998), a CD2 homolog (Rodríguez *et al.,* 1993), and a polypeptide with a C-type animal lectin-like domain (Galindo *et al.,* 2000), respectively.

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While a number of reports have been concerned with anti-apoptotic ASFV genes, little is known regarding how the virus induces apoptosis in the infected cell. To determine the step in the ASFV life cycle that triggers the signal for cell death, we have examined the requirements for induction of caspase-3 activation by ASFV in Vero cells, using UV-inactivated virus, inhibitors of protein and nucleic acid synthesis, and lysosomotropic drugs to block the virus uncoating. It is proposed that apoptosis is initiated in a postbinding step, during or after virus uncoating, and that ASFV gene expression and replication is not required to induce the apoptotic process.

RESULTS

Characterization of the ASFV-induced apoptosis in permissive cells

Incubation of Vero cells with the adapted ASFV isolate (BA71V) results in a lytic cycle during productive infection. To establish if apoptosis is the cause of cell death, we employed two different methods to assess the state of the DNA in the infected cells. As shown in Fig. 1A, flow cytometric measure of the DNA content indicates an increase of fragmented DNA in the hypoploid sub-G_0/G_1 peak (gate I in the figure) of the cell cycle from 16 h postinfection (hpi; 16% of apoptotic cells) until 24 hpi (54% of apoptotic cells).

To corroborate the DNA fragmentation during ASFV infection, Vero cells were infected with BA71V and the DNA analyzed at the indicated times after infection (Fig. 1B). Samples from mock-infected or infected cells, containing approximately 1500 ng of DNA, were electrophoresed in 1.5% agarose gels. Intense internucleosomal fragmentation, a pattern highly specific of apoptosis, was observed in ASFV-infected Vero cells at 16 and 18 hpi. No DNA laddering was observed in mock-infected Vero cells or in cells analyzed at 7 and 12 hpi. These results provide evidence that *in vitro* infection of Vero cells with ASFV BA71V causes DNA fragmentation from 16 hpi.

To obtain further insight into the mechanism of ASFV-induced apoptosis, we investigated the presence of cytochrome *c* (cyt *c*) in the cytosol of Vero cells at different times after ASFV infection. As shown in Fig. 1C, cyt *c* could be detected in the cytosol of BA71V-infected Vero cells as early as 8 hpi, although the specific band corresponding to cyt *c* was more markedly observed in the cytosolic fraction at 16 and 24 hpi.

Since caspases have been shown to play a central role in programmed cell death, we have analyzed whether ASFV infection induces caspase activity in Vero cells. The caspases exist as inactive procaspases that require proteolytic activation. To investigate the mechanism of caspase activation during ASFV infection and whether a correlation exists between induction of caspase-3 activity and the processing of proenzyme to the proteolytic fragment, we have studied in parallel samples from BA71V-infected Vero cells both the cleavage of pp32 precursor and the caspase-3 activity (Fig. 2). Cellular extracts obtained at the indicated times after infection were standardized for protein concentration (30 μg) and analyzed by Western blot using a specific antiserum anti-caspase-3. Figure 2A shows that infection with BA71V induces the processing of caspase-3 from its 32-kDa precursor (which is also recognized by the anti-caspase-3 antibody) into an active fragment of 17 kDa from 13 hpi and clearly increases at 19 and 24 hpi. No processing of caspase-3 could be found in mock-infected cells. Figure 2B also shows the temporal correlation between the cleavage of caspase-3 and its enzymatic activity: both curves fit reasonably well, supporting the use of immunoblotting to analyze caspase-3 activity in further experiments.

Although apoptosis clearly requires the participation of caspases, the particular enzymes involved vary according to cell type and the stimulus triggering cell death. Caspase activity in cell extracts prepared at different times after infection with BA71V was tested by *in vitro* hydrolysis of the specific substrates of caspase-3, 8, and 9. Figure 2C shows the caspase activities evaluated in mock- or ASFV-infected Vero cells incubated in different experimental conditions. Both caspases-3 and -9 were induced by ASFV infection, while caspase-8 showed only a slight increase. The induction of caspases by ASFV infection occurred also in the presence of cytosine arabinoside (CAR) or cycloheximide (CHX), a first indication that neither DNA nor protein synthesis was required to trigger the ASFV-induced apoptotic process. Moreover, Vero cells infected with UV-inactivated BA71V did not present caspase activities over the values of control samples.

Induction of apoptosis by ASFV does not require virus replication or protein synthesis

Metabolic inhibitors, such as CHX, may behave as apoptotic inducers when used in high concentrations or in addition to other stress signals. Although the induction of caspase-3 induced by ASFV was observed in the absence or presence of CHX, it cannot be completely ruled out that, in the latter case, it could be produced by the simultaneous action of the drug and the virus infection. To examine this, we analyzed extracts of Vero cells infected with BA71V (previously irradiated or not with UV light) in the presence of different concentrations of CHX. As shown in Fig. 3A, CHX did not induce the proteolytic activation of pp32 to the 17-kDa fragment in either mock-infected or in UV-inactivated ASFV-infected cultures, indicating that the induction of caspase-3 in Vero cells, even in the presence of 10 μg/ml of CHX, was produced only by the infection with ASFV. The quantification of the 17-kDa band in several experiments revealed in ASFV-

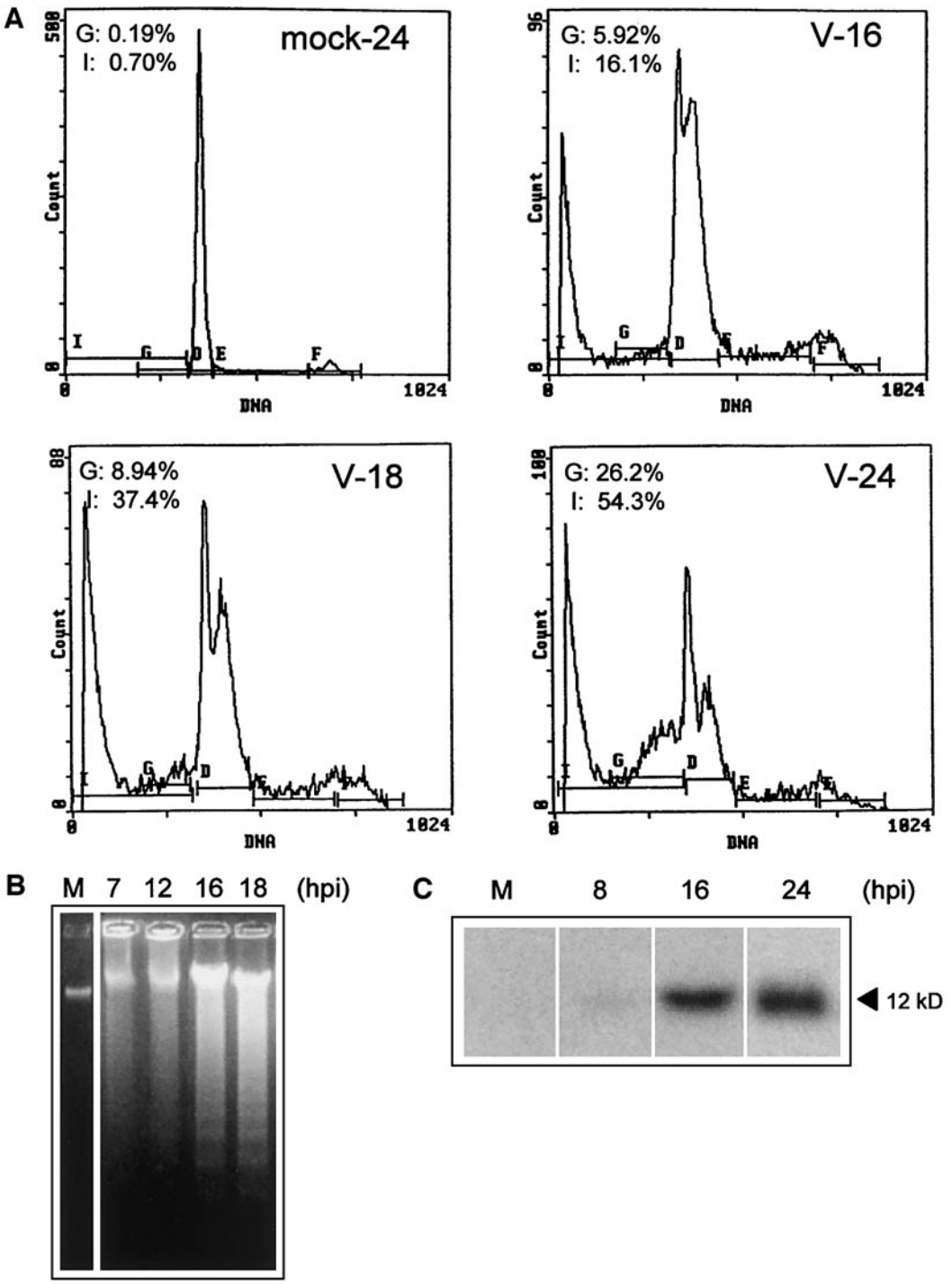

FIG. 1. Apoptosis induced by ASFV in Vero cells. (A) Flow cytometric histograms of mock- or ASFV-infected cells. 1×10^6 Vero cells were mock-infected or infected with 10 PFU/cell of BA71V. After 16, 18, and 24 hpi, cells were washed twice with PBS and the DNA content was determined by flow cytometry after propidium iodide staining. Gates D and I represent populations with euploid and hypoploid DNA content, respectively, while gate G should include cells in early apoptosis. (B) Agarose gel electrophoresis of the internucleosomal DNA laddering. At the indicated times after infection, DNA from mock- (M) or BA71V-infected Vero cells was obtained and subjected to electrophoresis on a 1.5% agarose gel. (C) Western blot of cytochrome *c* in the cytosol of infected cells. Cytosolic extracts (30 μg) from mock- or BA71V-infected Vero cells were subjected to SDS–12% polyacrylamide gel electrophoresis. Immunoblot analysis with specific anti-cytochrome *c* antibody was used for detection of the protein using an ECL-based method.

infected cultures levels of caspase-3 induction ranging from five- to eightfold, as compared with the mock-infected cell culture values. Although the presence of CHX in ASFV-infected cultures resulted in variable levels of caspase-3 activation as compared to those obtained in the absence of CHX (Figs. 2C and 3A), statistical analy-

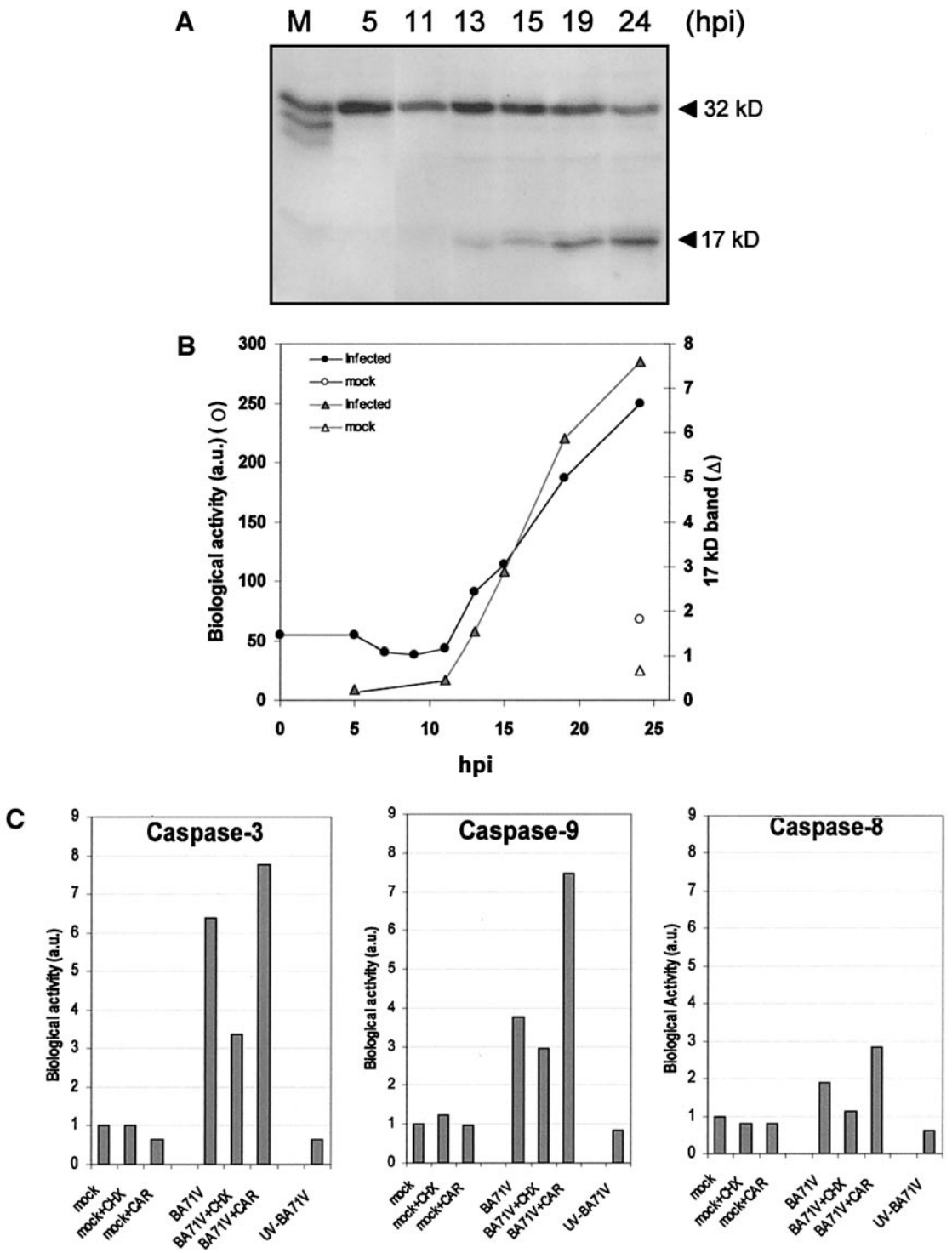

FIG. 2. Caspases activation induced by ASFV infection. (A) Cleavage of caspase-3 induced by ASFV infection. Cell lysates (30 μg) from mock-infected or BA71V-infected Vero cells obtained at the indicated times after infection were subjected to SDS–12% polyacrylamide gel electrophoresis. Immunoblot analysis with anti-caspase-3 antibody was used for detection using an ECL-based method. (B) Correlation of protease activity (determined in fluorogenic assays, ○●) and proteolytic cleavage of caspase-3 (evaluated by quantification of 17-kDa band, △▲) during ASFV infection. Samples were the same as described in A. (C) Biological activity of caspases-3, -8, and -9 in cytosolic extracts from ASFV-infected Vero cells. Cell lysates (30 μg) from mock- or BA71V-infected Vero cells, incubated in the presence or absence of CHX or CAR, or infected with UV-inactivated ASFV, were obtained at 20 hpi and analyzed for protease activity in fluorogenic assays with specific substrates. Data were normalized to the corresponding value of each caspase in lysates from mock-infected Vero cells.

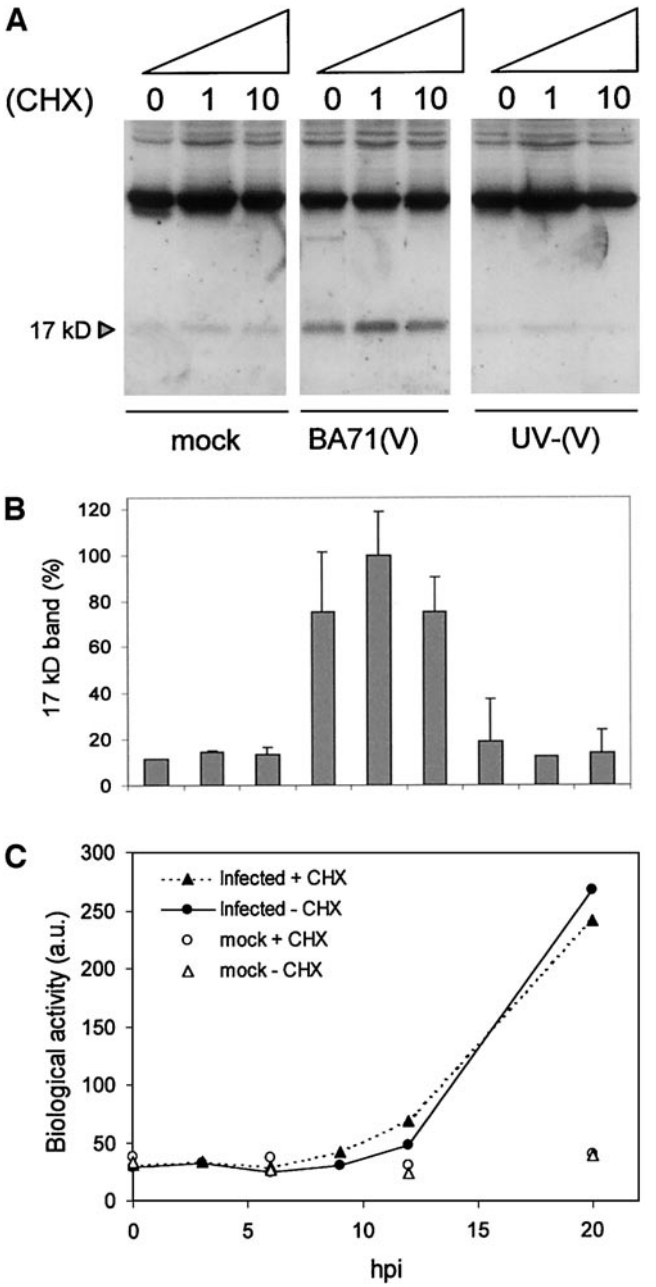

FIG. 3. Induction of caspase-3 by ASFV in the presence of CHX. (A) Cleavage of caspase-3. Cell lysates obtained at 20 hpi from mock-, BA71V-, or UV-inactivated BA71V-infected Vero cells (20 μg), incubated in the presence of different concentrations (0, 1, and 10 μg/ml) of CHX, were subjected to SDS–12% polyacrylamide gel electrophoresis and analyzed by Western blot with anti-caspase-3 antibody, as indicated above. (B) Quantification of the 17-kDa band of samples as described in A. Data represent the means ± SE of values from two independent experiments. (C) Time course of caspase-3 induction. Cell lysates from mock-infected or BA71V-infected Vero cells, obtained at the indicated times after infection in the presence or absence of CHX (10 μg/ml), were analyzed for protease activity in fluorogenic assays with caspase-3-specific substrate.

ses established that the induction in the presence of CHX was 96.6 ± 32.1% of that obtained in its absence (100%) in 13 independent determinations, indicating that the level of caspase-3 induction in ASFV-infected Vero cell cultures was equivalent in the presence or absence of an inhibitor of protein synthesis.

To determine whether the presence of CHX could affect the magnitude or kinetics of ASFV-induced apoptosis, we infected Vero cells with BA71V in the presence or absence of CHX and evaluated the caspase-3 activity induced at different times after infection. As shown in Fig. 3C, no differences could be detected in the onset, slope, or level of caspase-3 induction, supporting the conclusion that ASFV protein synthesis had no role in the induction of apoptosis in Vero cells.

On the other hand, the fact that the induction of caspase-3 was never observed with UV-inactivated ASFV (Figs. 2C, 3A, and 3B) indicates that apoptosis was not induced with these inactivated virus particles.

Apoptosis is induced after ASFV internalization and before early viral protein synthesis

Although it may be deduced from the inhibition mechanism expected for each inhibitor (CHX or CAR), we were interested to define in our system the step that was blocked by them in the ASFV infective cycle. To this end, we have studied the successive steps of the virus cycle in Vero cells infected with BA71V in the presence of CHX or CAR, or infected with UV-inactivated virus. As expected, low amounts of infective virus were produced in cells infected by ASFV in the presence of CHX or CAR (Fig. 4E), and cytoplasmic viral DNA synthesis or late ASFV proteins were only detected in cultures infected with the active virus in the absence of inhibitors (Figs. 4C and 4D). The induction of the early ASFV protein p32 was observed in BA71V-infected cultures, even in the presence of CAR (Fig. 4B), as expected for an inhibitor of DNA replication. There was no synthesis of ASFV proteins in cultures infected either by UV-inactivated BA71V or by the infective virus in the presence of CHX (Figs. 4B and 4D). Regarding the first steps of the virus cycle, we could not detect any significant difference in the binding and internalization steps (Fig. 4A), indicating that the process of virus entry progressed normally despite the UV inactivation or the presence of CHX.

ASFV uncoating is necessary to induce apoptosis

To delimitate further the step after ASFV internalization needed to trigger the apoptotic process, we studied the effect of inhibitors of virus uncoating in the induction of apoptosis by BA71V in Vero cells. We have reported previously (Alcamí *et al.,* 1989b) that ASFV enters into the cell by a receptor-mediated endocytosis mechanism, concluding with the presence of viral cores in the cytosol from 60 min after a synchronic ASFV infection and the inhibition of virus uncoating by lysosomotropic drugs. Preliminary results indicated that both chloroquine and dansyl-cadaverine were able to inhibit the activation of

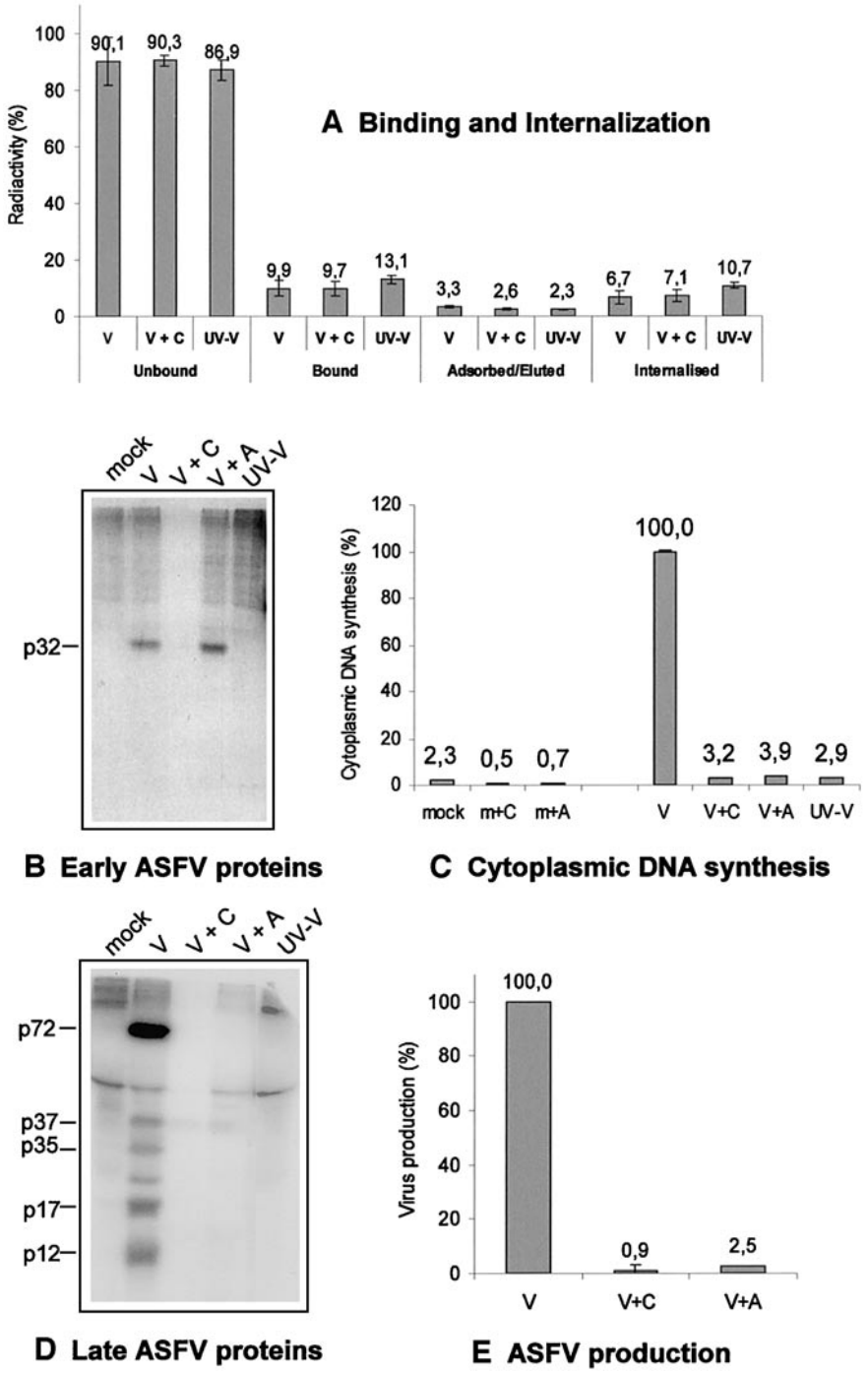

FIG. 4. ASFV cycle in Vero cells infected under different conditions. (A) ASFV binding and internalization. Vero cell cultures were infected with [^{3}H]ASFV in the absence (V) or presence (V+C) of CHX (10 μg/ml), or with UV-inactivated ^{3}H-ASFV (UV-V), and then assayed for ASFV binding and internalization as described under Materials and Methods. Mean values and standard errors for unbound, bound, adsorbed, and internalized radioactivity, from triplicate samples, are shown. (B) Early ASFV protein synthesis. Vero cells, infected with UV-inactivated (UV-V) or infective BA71V in the absence (V) or presence of CHX (V+C) or CAR (V+A), were labeled with [^{35}S]methionine and cysteine at 0–5 hpi and lysed before being subjected to immunoprecipitation with rabbit antiserum specific for the early ASFV protein p32. Radioactive protein bands were separated in SDS–14% polyacrylamide gel electrophoresis and analyzed after fluorographic amplification with Amplify. (C) Cytoplasmic DNA synthesis. Vero cells, infected (V) or not (m) with infective or UV-inactivated BA71V (UV-V) and labeled at 12–16 hpi with methyl-(^{3}H)-thymidine, in the absence or presence of CHX or CAR, were collected and resuspended in a solution of 0.6% Nonidet P-40 in PBS. After incubation at 4°C for 30 min, the nuclear fraction was removed by centrifugation and the acid-insoluble radioactivity was determined in the supernatant (cytoplasmic) fraction. Mean values and standard errors from duplicate samples are shown. Data are represented as percentages referred to the value obtained in cells infected with infective ASFV (V). (D) Late ASFV protein synthesis. Determined as indicated in B, in extracts of cells labeled at 17–22 hpi. (E) Infective ASFV production. Vero cells were infected with 5 PFU/cell of BA71V, washed away from virus inoculum after the adsorption period, and incubated until 24 hpi, when infective ASFV yields were titrated by plaque assay. Virus infections were made in duplicate samples and in the absence or presence of CHX or CAR, which were maintained throughout the infective cycle.

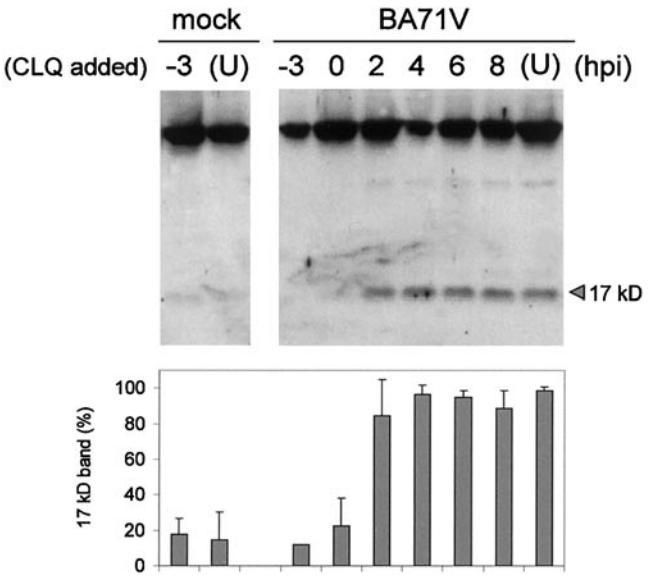

FIG. 5. Chloroquine inhibition of ASFV-induced caspase-3 activation. Vero cells were infected or not with BA71V and, at the indicated times of infection (from −3 to 8 hpi), 0.03 mM chloroquine was added to the culture and maintained throughout the rest of the virus cycle. Cell lysates were obtained at 20 hpi and subjected (15 μg) to SDS–12% polyacrylamide gel electrophoresis before being analyzed by Western blot with anti-caspase-3 antibody, as indicated above. Samples from cultures untreated (U) with chloroquine were processed in parallel. The quantification (mean ± SE) of the 17-kDa band from two independent experiments is shown as a histogram in the lower panel. Note that the virus adsorption period proceeded from −2 to 0 hpi.

caspase-3 in ASFV-infected cultures. To define more accurately the time at which the lysosomotropic drug performed its inhibitory action, caspase-3 activation was analyzed in extracts of ASFV-infected Vero cells which were incubated with chloroquine added at different times (−3 to 8 hpi) and collected at 20 hpi. As shown in Fig. 5, there was only a slight induction of the 17-kDa caspase-3 fragment in mock-infected or ASFV-infected cultures that were incubated in the presence of chloroquine from −3 to 0 hpi. The activation of caspase-3 was released from chloroquine inhibition at 2 hpi (or 4 h after virus inoculation, including the virus absorption period from −2 to 0 hpi), a time at which it can be expected that ASFV uncoating step was completed (Alcamí *et al.,* 1989b).

DISCUSSION

Apoptosis is the process whereby cells undergo systematic self-destruction in response to a wide variety of stimuli. In the case of virus-infected cells, the induction of early cell death would severely limit virus production and reduce or eliminate the spread of progeny virus in the host. Thus, many animal viruses have evolved strategies to evade or delay early apoptosis to allow production of high yields of progeny virus. However, apoptosis also represents a very efficient mechanism by which the virus can induce cell death and disseminate progeny while limiting induction of inflammatory and immune responses (Cuff and Ruby, 1996; Koyama *et al.,* 2000; Roulston *et al.,* 1999).

Because less information is available on the process of ASFV-induced apoptosis in terms of cellular pathways and specific proteins involved, we have studied in the present work the mechanism by which the virus induces apoptosis in the infected cell. Interestingly, the fragmentation of the cellular DNA could be only demonstrated after 16 hpi in ASFV-infected Vero cells, a time at which viral morphogenesis is well underway. Similar results were obtained when cells were monitored by flow cytometry, which showed apoptosis in 16.1% of the infected cells at 16 hpi, and in the analysis of caspase-3 activation (Nogal *et al.,* 2001; this report), which was induced from 13 h after ASFV infection. Therefore, the fragmentation of cellular DNA would not have great consequences in the production of progeny virus. However, a substantial portion (more than a half) of the infected cells displayed the apoptotic condition at later times (24 hpi) of infection. Thus, apoptosis in ASFV-infected cells must be a tightly regulated process in which the action of inductors is balanced by the expression of viral anti-apoptotic genes along the infectious cycle.

Cyt *c* has been identified as a factor released from mitochondria into the cytosol of cells undergoing apoptosis. Cyt *c* and Apaf-1, after leaking out of the mitochondria, act as cohorts in the activation of caspase-9. Cyt *c*, Apaf-1, and procaspase-9 come together to form a complex known as the apoptosome. Thus, cyt *c* release from the mitochondria could be considered a key signal that initiates the irreversible events in cell death (Kroemer *et al.,* 1997; Zamzami *et al.,* 1997). During ASFV infection, cyt *c* could be identified in the cytosolic fraction of infected cells after 16 hpi, suggesting that apoptosis induced by the virus involves the mitochondrial pathway. Activation of caspases is also an event associated with apoptosis. Caspases are activated either via the receptor-mediated or the mitochondrial pathways. The receptor-mediated pathway leads to the activation of caspase-8. In the mitochondrial pathway, however, pro-apoptotic members of the Bcl-2 family associate with mitochondria and direct the release of cyt *c* and other proteins which activate procaspase-9, leading to the activation of effector caspases such as caspase-3. We report here that ASFV infection activates caspase-3 and caspase-9, and to a lesser extent, caspase-8. These results, together with those obtained on the cyt *c* release, suggest that ASFV induces apoptosis through the mitochondrial rather than the receptor-mediated pathway. In a recent report (Nogal *et al.,* 2001), we have shown that infection of Vero cells by ASFV also induces the cleavage of PARP, a nuclear protein specifically cleaved by caspases-3 and -6, illustrating a next step in the route of activation of ASFV-induced apoptosis.

It is interesting to note that the activation of caspase-3

and caspase-9 was also observed in ASFV-infected Vero cells incubated in the presence of CAR, an inhibitor of the macromolecular synthesis. This last observation indicates that apoptosis induced by ASFV infection is independent of the synthesis of the late viral proteins. Although viral replication is required for the induction of apoptosis by many types of viruses, cell death in the absence of viral replication has been reported for several virus infections. Apoptosis was triggered in these cases in an early step of the infective cycle, and one structural virus protein was involved in the process: sigma-1 attachment protein in type 3 reovirus (Tyler *et al.,* 1995), Env protein interacting with TNF receptor component in avian leukosis virus (Brojast *et al.,* 1996), virion L1R protein in vaccinia virus (Ramsey-Ewing and Moss, 1998), or surface glycoproteins E2 and E1 in Sindbis virus (Jan and Griffin, 1999). In the case of ASFV, we have found that genome replication was not required for the induction of apoptosis since activation of caspase-3 was detected in Vero cells infected with ASFV in the presence of concentrations of CAR that were able to reduce both the cytoplasmic DNA synthesis to the level found in mock-infected cultures and the production of infective virus to less than 3%. The synthesis of early ASFV proteins was also not required to induce apoptosis in sensitive cells, as it was found in experiments carried out in the presence of CHX, in which the magnitude and kinetics of virus-induced activation of caspase-3 was not affected by this inhibitor. The possibility that CHX, either alone or in association with some event in the virus entry, could induce the activation of caspase-3 observed in cells infected with ASFV was excluded by the fact that the activation was not observed in mock-infected cells or in cells infected with UV-inactivated ASFV in the presence of the inhibitor. In contrast to the induction of apoptosis reported in nonpermissive CHO cells by UV-inactivated vaccinia virus (Ramsey-Ewing and Moss, 1998), ASFV was not able to trigger cell death in Vero cells after inactivation by UV light, a result similar to that obtained with UV-inactivated herpes simplex virus 1 in human HEp-2 cells (Aubert *et al.,* 1999).

As UV irradiation could have a deleterious effect also on viral proteins, resulting in a less efficient virus entry (Jan and Griffin, 1999), a study of the ASFV cycle was performed. Interestingly, the statistics of virus binding and internalization were similar in all the virus infections, indicating that the presence of CHX or the UV-inactivation of ASFV did not affect the process of virus entry. This result is of great relevance since it rules out the possibility of induction of apoptosis by interactions between viral and cellular components involved in the process of ASFV entry, which should proceed normally in Vero cells infected by UV-inactivated ASFV (in the absence of caspase-3 activation), and establishes a model for apoptosis induction different from those of HIV-1 or bovine herpesvirus-1, in which cell death was also induced early in the infection, but by the interaction of virus components with a cellular protein in the plasma membrane (Banda *et al.,* 1992; Hanon *et al.,* 1998). In the case of ASFV, the lack of involvement of the virus proteins p12, p54, or p32, which have been reported to play a role in the early steps of the virus infection (Carrascosa *et al.,* 1991; Gómez-Puertas *et al.,* 1998), in the process of induction of apoptosis in infected cells, was further supported by two types of experiments: (i) purified recombinant p12 and p32 did not induce caspase-3 activation when incubated on Vero cell cultures, and (ii) infective ASFV preincubated with anti-p12 or anti-p54 antiserum was able to induce the activation of caspase-3 at the same extent as the control virus (unpublished results). However, a possible role for p54, postulated on the basis of sequence homology in the regulation of ASFV-induced apoptosis, has been recently suggested (Alonso *et al.,* 2001).

Consequently, the next step to analyze in the ASFV cycle was the virus uncoating. We have previously shown that several lysosomotropic drugs inhibited ASFV infection of Vero cells (Alcamí *et al.,* 1989b) by interfering with the uncoating of virus particles, resulting in the accumulation of virions in cytoplasmic vacuoles and the absence of viral cores in the cytosol. In the present study, we have found that caspase-3 activation does not occur in ASFV-infected Vero cells incubated in the presence of dansyl-cadaverine or chloroquine, indicating that proper uncoating of ASFV particles was needed to induce the apoptotic signal in infected cells. Furthermore, the effect of time of addition of chloroquine was consistent with that expected for an inhibitor of virus uncoating: the activation of caspase-3 was released from chloroquine inhibition at 2 hpi (or 4 h after virus inoculation), a result similar to that obtained in the inhibition of Sindbis virus infection by monensin (Jan and Griffin, 1999). It is possible that a death signal could arise by interactions between viral components of the endocytosed virus particle and cellular molecules in the endosome membrane, and this is the main thrust of future investigations. Regarding the role of anti-apoptotic viral genes, it is also important to consider that the mechanisms of viral evasion of the host response may depend on the cell used in the study and that Vero cells have exhibited a different apoptotic response than that of human HEp-2 cells when infected by herpes simplex virus 1 (Aubert and Blaho, 2001).

From these results we conclude that the apoptotic signal in ASFV-infected Vero cells is triggered in the absence of virus replication, after virus internalization and before early ASFV protein synthesis, most probably during the virus uncoating. A possible requirement of early ASFV transcription, or another RNA metabolic event as it has been postulated for herpes simplex virus 1 (Aubert *et al.,* 1999), could not be absolutely excluded, since the addition (not shown) of a conventional inhibitor of transcription such as actinomycin-D, resulted in the

activation of caspase-3, even in mock-infected cells, when the cultures were incubated with the concentration of inhibitor (above 10 μg/ml) needed to prevent the early ASFV protein synthesis. However, we have been able to detect the induction of 17-kDa band in cells infected in the presence of coumermycin A1 (data not shown), an inhibitor of type II DNA topoisomerases, which strongly inhibits the RNA synthesis directed *in vitro* by ASFV particles (Salas *et al.,* 1983). In summary, we have studied the event(s) required to induce apoptosis in ASFV infection, a subject much less studied than the possible role of viral anti-apoptotic genes, concluding that ASFV induces apoptosis in the infected cell in the absence of protein synthesis and virus replication, by an intracellular pathway probably triggered during the process of virus uncoating.

MATERIALS AND METHODS

Cells and virus

Vero cells were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% newborn calf serum. The Vero-adapted ASFV strain BA71V was propagated and titrated by plaque assay on Vero cells, as previously described (Enjuanes *et al.,* 1976). Cell cultures were infected at a multiplicity of infection (m.o.i.) of 3 PFU per cell (unless specified) in a small volume of medium (supplemented with 2% serum) for 2 h at 37°C, washed away from virus inoculum, and further incubated in fresh culture medium.

UV inactivation of ASFV

BA71V was inactivated by exposing the virus to a germicidal lamp at a distance of 10 cm for 30 min at room temperature. Inactivation was confirmed by titration of samples before and after UV exposition, and by the absence of cytopathic effect after inoculation of Vero cell monolayers with the UV-irradiated virus samples.

Radiolabeling of ASFV-infected cells and purification of virions

To monitor the synthesis of ASFV DNA, Vero cells were infected with BA71V at an m.o.i. of 5 and labeled from 12 to 16 hpi with 20 μCi/mL of methyl-(^{3}H)-thymidine (Amersham). For virus binding and internalization assays, stocks of [^{3}H]ASFV were prepared from Vero cell cultures infected with BA71V at an m.o.i. of 0.2 and metabolically labeled with 20 μCi of methyl-(^{3}H)-thymidine per milliliter as above. Supernatant fluids were collected at 72 hpi and the extracellular virus was purified by Percoll sedimentation, as previously described (Carrascosa *et al.,* 1985). To detect the viral protein synthesis, Vero cell monolayers were infected with BA71V at an m.o.i. of 5 and pulse-labeled, either at 0–5 or 17–22 hpi, with 100 μCi/mL of [^{35}S]methionine and cysteine (Redivue PROMIX [^{35}S] Cell Labeling Mix, Amersham) in a medium containing reduced (one-tenth) methionine and cysteine concentrations.

Virus binding and internalization assays

Cells were grown on 24-well plates to about 2×10^5 cells per well and then incubated with 5×10^4cpm of purified [^{3}H]ASFV for 4 h at 37°C. Cultures were extensively washed with PBS to remove the unbound material before incubation with Pronase E (1 mg/ml in PBS) for 1 h at 4°C and 15 min at 37°C to release the adsorbed virus. Radioactivity retained after the final washing of cell pellets was considered "internalized," and the sum of internalized plus the "adsorbed/eluted" radioactivity represented the "bound" virus.

Drug treatments

All inhibitors were purchased from Sigma Chemical Co. and used at the indicated concentration unless specified. Vero cell monolayers were pretreated with CHX (10 μg/ml), CAR (40 μg/ml), or chloroquine (0.03 mM) for 1 h before exposure to ASFV. Inhibitors were also present at the indicated concentrations in the virus inoculum (during absorption of viral particles), in the radioactive medium for labeling, and in the culture medium along the infective cycle.

Western blot analysis

Cells were washed twice with PBS and lysed in TNT buffer (20 mM Tris–HCl pH 7.5, 0.2 M NaCl, 1% Triton X-100) supplemented immediately before use with protease inhibitor cocktail tablets (Roche). Protein concentration was determined by the bicinchoninic acid method with BCA Protein Assay Reagent from Pierce. Proteins (10–50 μg) were subjected to sodium dodecyl sulfate (SDS)–11 or 14% polyacrylamide gel electrophoresis, and then electroblotted onto a nitrocellulose (Protran, Schleicher & Schuell) or Immobilon (Amersham) membrane. After reacting with specific primary antibodies, membranes were exposed to a dilution 1/10,000 of horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody (Amersham), followed by chemiluminescence (ECL, Amersham) detection by autoradiography.

Assessment of viral protein synthesis and DNA replication

To monitor the synthesis of ASFV proteins, Vero cells, infected with infective or UV-inactivated BA71V and labeled with [^{35}S]methionine and cysteine at early (0–5 hpi) or late (17–22 hpi) times of infection, in the absence or presence of CHX (10 μg/ml) or CAR (40 μg/ml), as indicated above, were lysed in dissociation buffer (1% Nonidet P-40 and 0.3% SDS in PBS) supplemented immediately before use with protease inhibitor cocktail tablets

(Roche). Extracts were incubated with one-twentieth dilution of rabbit antiserum specific either for the early ASFV protein p32 (Prados *et al.,* 1993) or for ASFV-infected Vero cells (Alcamí *et al.,* 1989a), before immunoprecipitation with protein A-Sepharose, as described elsewhere (Harlow, 1988; Simón-Mateo *et al.,* 1997). Radioactive protein bands were separated by SDS–polyacrylamide gel electrophoresis in 14% polyacrylamide gels and analyzed after fluorographic amplification with Amplify (Amersham).

To determine the viral DNA synthesis in mock- or ASFV-infected cultures, we analyzed the synthesis of cytoplasmic DNA, which should correspond almost entirely (with the exception of mitochondrial DNA synthesis) to ASFV DNA replication. Vero cells, infected with infective or UV-inactivated BA71V and labeled at 12–16 hpi with 20 μCi/mL of methyl-(^{3}H)-thymidine (Amersham), in the absence or presence of CHX or CAR, were collected at the end of the labeling period, washed with PBS, and resuspended in a solution of Nonidet P-40 (0.6% in PBS). After incubation at 4°C for 30 min, the nuclear fraction was removed by centrifugation and the acid-insoluble radioactivity was determined in the supernatant (cytoplasmic) fraction.

Analysis of apoptosis

DNA fragmentation assay. Equal numbers of uninfected or BA71V-infected Vero cells (10^6) were harvested after 7, 12, 16, and 18 hpi by centrifugation, washed twice with ice-cold PBS, and lysed in TNT buffer. To remove high molecular weight DNA and cellular proteins, samples were adjusted to 1 M NaCl, incubated on ice for 3 h, and subjected to two rounds of centrifugation at 15,000 *g* for 10 min. The cleared lysates were then treated with 500 μg of proteinase K (Sigma) for 30 min at 37°C, extracted twice with phenol, once with chloroform, and precipitated with ethanol for 2 h at −20°C. Precipitates were collected by centrifugation at 15,000 *g* for 10 min, washed with 70% ethanol, resedimented, dried, and resuspended in TE (10 mM Tris–HCl, pH 7.4, 1 mM EDTA) containing 20 μg/ml RNase A (Boehringer Mannheim, IN). Samples were incubated at 37°C for 15 min and aliquots were analyzed by electrophoresis on a 1.5% agarose gel. DNA was visualized by illumination with ultraviolet light after soaking the gel in electrophoresis buffer supplemented with 0.5 μg/ml ethidium bromide.

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 μg/ml) containing 20 μg/ml RNase A. Cells were analyzed using a Becton–Dickinson FACScan to determine the percentage of apoptotic cells in the cellular cycle.

Cytosolic cyt c *assay.* The preparation of cytosolic extracts was 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 M Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride, aprotinin (10 μg/ml), and leupeptin (10 μg/ml). After 30 min of incubation on ice, the cell lysate was centrifuged at 20,000 *g* for 30 min, and the supernatant was used as cytosolic extract to determine the presence of cyt *c* by immunoblotting (with the purified IgG fraction of a rabbit anti-horse cyt *c* as primary antibody, kindly provided by Dr. González-Porqué, Hospital Ramón y Cajal, Madrid, Spain) as described above.

Fluorogenic assays. To analyze the biological activity of caspases, the cytosolic extracts (aliquots containing 1 mg/ml of protein) were 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 mM concentration of fluorescent substrate for specific (3, 8, or 9) caspases. The reaction was stopped by addition of HPLC buffer (H_2O-acetonitrile 75/25, 0.1% trifluoroacetic acid). The cleaved fluorescent substrate was determined by C_{18} reverse-phase high-performance liquid chromatography using fluorescent detection. Control experiments (not shown) confirmed that the release of substrate was linear with time and with protein concentration under the specified conditions.

Caspase-3 activation. The cleavage of procaspase 3 (32 kDa) to the activated form of 17 kDa was detected in cell extracts, obtained as above indicated, by Western blot analysis revealed with a polyclonal antibody (diluted 1/2000) specific for recombinant human caspase 3 (anti-CPP32, Pharmingen). Quantification of protein bands was performed by densitometry (Image Quant) in films developed after ECL exposure.

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