Available online at www.sciencedirect.com
SCIENCE DIRECT
Virology 326 (2004) 160–170
VIROLOGY
www.elsevier.com/locate/yviro

The C-type lectin homologue gene (EP153R) of African swine fever virus inhibits apoptosis both in virus infection and in heterologous expression

Carolina Hurtado,[a] Aitor G. Granja,[a] María J. Bustos,[a] María L. Nogal,[a] Gonzalo González de Buitrago,[b] Virginia G. de Yébenes,[a] María L. Salas,[a] Yolanda Revilla,[a] and Angel L. Carrascosa[a,*]

[a] *Centro de Biología Molecular "Severo Ochoa" (C.S.I.C.-U.A.M.), Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain*
[b] *Centro Nacional de Biotecnología (C.S.I.C.), Universidad Autónoma de Madrid, 28049 Madrid, Spain*

Received 29 March 2004; accepted 24 May 2004

Abstract

The open reading frame EP153R of African swine fever virus (ASFV) encodes a nonessential protein that has been involved in the hemadsorption process induced in virus-infected cells. By the use of a virus deletion mutant lacking the EP153R gene, we have detected, in several virus-sensitive cells, increased levels of caspase-3 and cell death as compared with those obtained after infection with the parental BA71V strain. Both transient and stable expression of the EP153R gene in Vero or COS cells resulted in a partial protection of the transfected lines from the apoptosis induced in response to virus infection or external stimuli. The presence of gene EP153R resulted in a reduction of the transactivating activity of the cellular protein p53 in Vero cell cultures in which apoptosis was induced by virus infection or staurosporine treatment. This is to our knowledge the first description of a viral C-type lectin with anti-apoptotic properties.
© 2004 Elsevier Inc. All rights reserved.

Keywords: ASFV; C-type lectin; EP153R gene; Caspase-3; p53; Apoptosis

Introduction

C-type animal lectins are proteins found in serum, extracellular matrix, and cellular membranes, which exhibit a Ca^{2+}-dependent carbohydrate-binding activity enabling the recognition of both endogenous and exogenous specific glycoconjugates in animals. Lectins are generally considered as non-enzymatic proteins acting as receptors for carbohydrate ligands (Gabius, 1997). It is known, on the other hand, that several cells of the immune system (macrophages, B, T, and natural killer cells) display receptors with sequence similarity to C-type lectins, thus involving the mammalian lectins both in cell-to-cell interactions and in pathogen recognition by their ability to mediate discrimination between self and nonself (Weis et al., 1998). Several proteins with C-type lectin-like domains have been reported in animal viruses, most of them in the poxviridae family (fowlpox (Afonso et al., 2000; Tomley et al., 1988), vaccinia (Blasco et al., 1993; Goebel et al., 1990; Wilcock et al., 1999), cowpox (Shchelkunov et al., 1998), myxoma (Cameron et al., 1999), and molluscum contagiosum (Senkevich et al., 1996)), although a lectin homologue has also been found in herpesviridae (cytomegalovirus (Voigt et al., 2001)) and in asfarviridae (African swine fever virus (ASFV) (Galindo et al., 2000; Neilan et al., 1999; Yáñez et al., 1995)). These proteins have proven or putative roles in virus infectivity, cell to cell spreading, inhibition of cell-mediated cytotoxicity, or hemadsorption. However, the actual function of many of the viral lectin homologues in immune evasion or in the virus infective cycle remains to be determined.

In addition to the immune response developed by the host to combat virus infections, the infected cell itself can activate a suicide program (O'Brien, 1998), which may have an adverse effect on virus replication. In turn, viruses have evolved different strategies to regulate this process,

* Corresponding author. Fax: +34-914974799.
E-mail address: acarrascosa@cbm.uam.es (A.L. Carrascosa).

0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2004.05.019

either by preventing premature cell death to allow infectious virus production or by promoting apoptosis for rapid cell-to-cell virus dissemination (Everett and McFadden, 1999).

African swine fever virus (ASFV), a large enveloped deoxyvirus, is the causative agent of a highly contagious disease of domestic pigs and infects a variety of cells of the mononuclear phagocytic system (reviewed in (Costa, 1990; Hess, 1981; Vinuela, 1985). ASFV replication can elicit the apoptotic response both in acute in vivo (Gomez-Villamandos et al., 1995; Oura et al., 1998; Ramiro-Ibanez et al., 1996) and in vitro infections (Nogal et al., 2001), in a process triggered in the absence of virus replication, after virus internalization, and before early virus protein synthesis, most probably during the virus uncoating (Carrascosa et al., 2002). Several ASFV genes have been reported to modulate the programmed cell death or to interfere with the host cell response; among them, A179L, which encodes a viral Bcl-2 homologue (Afonso et al., 1996; Revilla et al., 1997), and A224L, with similarity to IAP genes (Chacon et al., 1995; Nogal et al., 2001), have been shown to inhibit caspase activation and promote cell survival in mammalian cells. A224L is also able to activate the transcription factor NF-κB (Rodriguez et al., 2002), while another ASFV gene, A238L, which encodes an IkappaB-like protein (Powell et al., 1996; Revilla et al., 1998), behaves as an effective inhibitor of NF-κB activity (Revilla et al., 1998). As mentioned above, the ASFV genome also contains a gene (EP153R) with a C-type animal lectin-like domain (Yáñez et al., 1995), which has been involved in the hemadsorption process observed in ASFV-infected cells (Galindo et al., 2000). The EP153R gene is transcribed both during the early and late phases of the infectious cycle, the expression of the protein pEP153R being detected from early times (6 hpi) after infection. Besides the C-type lectin domain, the EP153R sequence reveals a cell attachment (RGD) sequence and significant homology with the N-terminal region of CD44 molecules involved in cellular adhesion and T-cell activation, respectively. We show in this report that the ASFV protein pEP153R has an inhibitory effect on the caspase-3 activation and the apoptosis induced both in ASFV-infected cells and in cell lines transfected, either stably or transiently, with the EP153R gene and treated with different pro-apoptotic stimuli. To our knowledge, this is the first description of a viral C-type lectin with anti-apoptotic properties.

Results

EP153R inhibits caspase-3 activity and cell death during ASFV infection

We have previously shown that permissive Vero cells develop apoptosis, as characterized by DNA fragmentation, caspase activation, cytosolic release of mitochondrial cytochrome *c*, and flow cytometric analysis of DNA content, upon infection with ASFV (Carrascosa et al., 2002). Proteolysis of caspase-3 from its 32-kDa precursor to the active form (17 kDa) was used in this study to quantify the apoptosis induced in Vero cells infected with either the parental strain BA71V or the EP153R deletion mutant (ΔEP153R). As shown in Figs. 1A and B and as previously reported, caspase-3 activation was first detected in virus-infected cells at 12 hpi, increasing until 20–25 hpi. It can also be noticed that cultures infected with ΔEP153R exhibited higher amounts of the 17-kDa band, indicating that the processing of procaspase-3 to its active form was enhanced in the absence of EP153R gene expression. As can be seen in Fig. 1C, the results of caspase-3 cleavage correlated perfectly well with the analysis of enzymatic activity. To confirm that the increase in caspase-3 activity was associated with programmed cell death, the cell cycle of virus-infected cultures was analyzed by flow cytometry after PI staining (Fig. 1D). As expected, the percentages of cell death were always higher from 12 hpi in cells infected with ΔEP153R than in BA71V-infected cultures. To provide a more statistical value of the increase in caspase-3 associated to the absence of EP153R, we have calculated in 28 independent determinations (including assays of biological activity and quantification of the17-kDa protein by several methods) the ratio between the level of caspase-3 in ΔEP153R-infected and BA71V-infected cultures, analyzed at late times of infection (15–30 hpi), resulting in a factor of 2.11 ± 0.58. From these results, we conclude that the absence of gene EP153R in the ASFV infection increases about 2-fold the activity of caspase-3.

Because other caspases have been shown to play a key role in apoptosis, we have studied the effect of EP153R expression on the enzymatic activity of caspases 6 and 8 in Vero cells infected with either BA71V or ΔEP153R virus isolates (Fig. 2A). The activity of caspases 6 and 8 was only slightly increased in BA71V-infected Vero cells, and this increase was not affected by the absence of the EP153R gene in cells infected with the mutant ΔEP153R virus. These findings also indicate the predominant role for caspase-3 (increased up to 7-fold) in the apoptosis induced by ASFV, as previously described (Nogal et al., 2001).

To rule out the possibility that the expression of the *LacZ* gene, inserted under the control of ASFV promoter p72 into the EP153R locus for the construction of ΔEP153R deletion mutant (Galindo et al., 2000), could be responsible for the enhanced caspase-3 activation observed in ΔEP153R-infected cells, we performed the analysis presented in Fig. 2B: Vero cells were infected with ASFV isolates containing either a β-galactosidase (ΔEP153R and vΔCD2) or a β-glucuronidase (v72GUS)

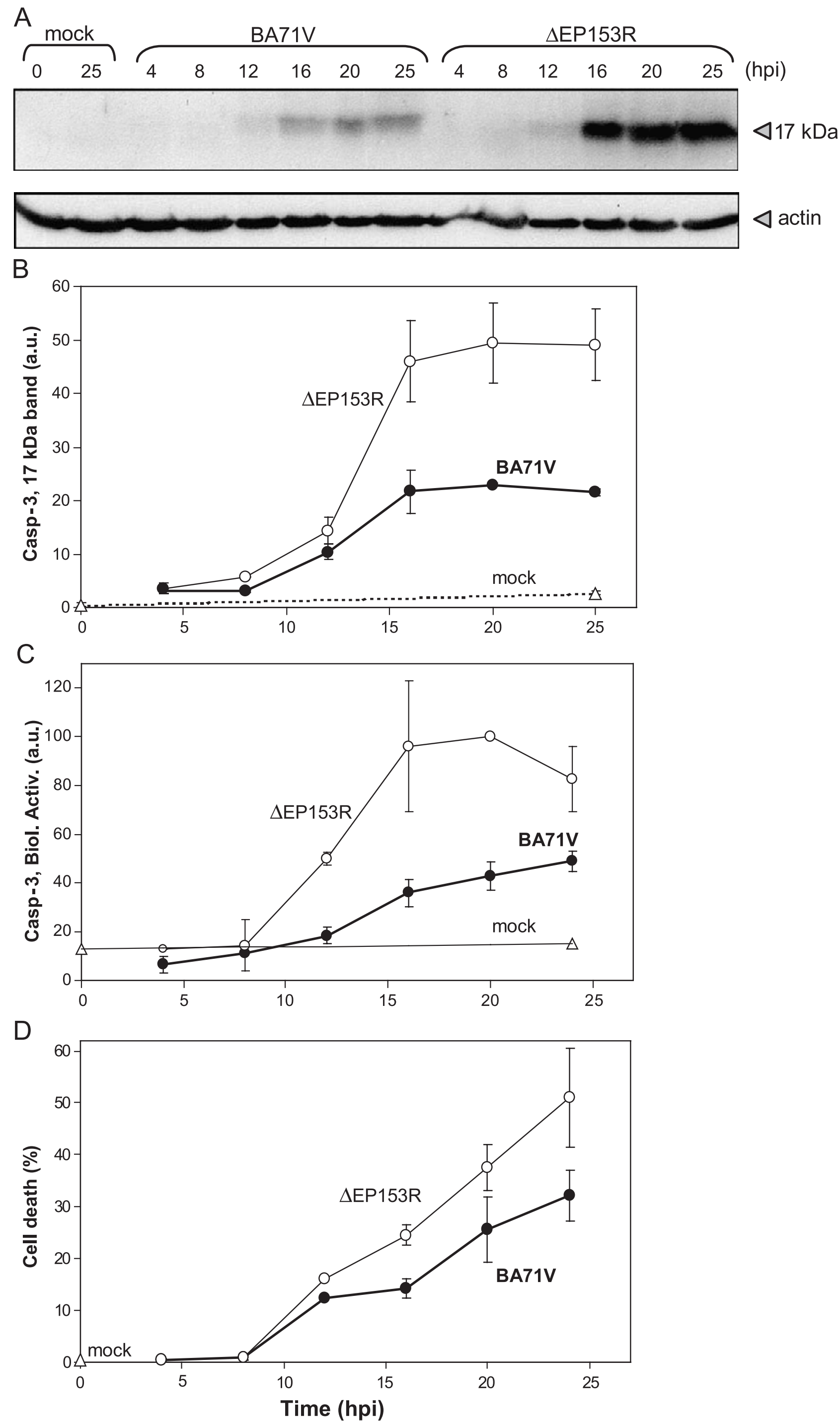

Fig. 1. Analysis of apoptosis in ASFV-infected Vero cells. Cultures were mock-infected or infected with BA71V or ΔEP153R at a moi of 3 pfu/cell and collected at the times indicated to determine the activation of caspase-3 by detection of the 17-kDa fragment in Western blots (A and B) or biological activity (C), and the percentage of cell death by flow cytometry after PI staining (D). The control of protein loading was performed with anti-actin antibodies and is shown in panel A. The quantification (mean ± SD) of the 17-kDa fragment from two separate experiments is shown in panel B. The values were corrected taking into account the actin content in each sample. Data on caspase-3 biological activity (panel C) and cell death (panel D) are also the average (±SD) of duplicate samples.

gene, and analyzed for caspase-3 cleavage by immunoblotting, as described above. Proteolytic cleavage was only enhanced in cells infected with ΔEP153R, indicating that the observed effect is due to the absence of gene EP153R, and not to the insertion of a foreign gene (*lac*Z or gusA) into the ASFV genome.

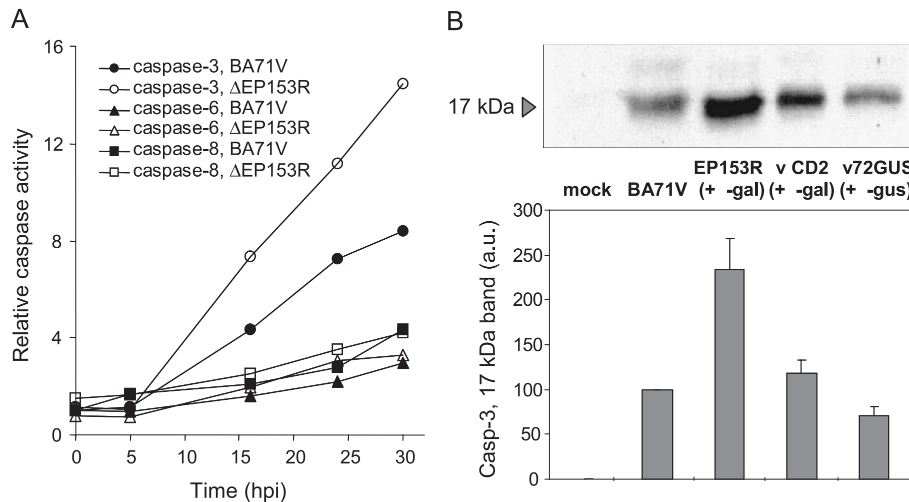

Fig. 2. Caspase activation in ASFV-infected Vero cells. (A) Cultures were infected with BA71V or ΔEP153R at a moi of 3 pfu/cell and collected at the indicated times to determine the protease activity of caspases on specific substrates (Ac-DVED-AMC, Ac-VEID-AMC, and Ac-IETD-AMC for caspase -3, -6, and -8, respectively) after normalizing the cell extracts for total protein content (1 mg/ml). Data represent caspase activities relative to the values at zero time of infection with BA71V for each protease. (B) Vero cells were mock-infected or infected with ASFV isolates containing or not either a β-galactosidase (ΔEP153R and vΔCD2) or a β-glucuronidase (v72GUS) gene, and analyzed for caspase-3 cleavage by immunoblotting, as indicated in the legend to Fig. 1. The quantification (mean ± SD) of the 17-kDa fragment from triplicate samples is shown below.

EP153R protects different virus-sensitive cells from ASFV-induced apoptosis

To further analyze the role of gene EP153R, we studied the effect of its absence in infections performed in three ASFV-sensitive cell lines. Cultures of Vero, COS, and BHK cells were infected with either the parental strain BA71V or ΔEP153R and analyzed for their DNA content by flow cytometry at 20 hpi. As shown in Fig. 3A, the percentage of cell death was always higher in each particular cell line when infected with the EP153R deletion mutant. To determine more precisely the apoptotic response in swine macrophages, the natural target cell for ASFV, we analyzed the biological activity of caspase-3 and the percentage of cell death along the infective cycle in cultures infected either with BA71V or ΔEP153R (Figs. 3B and C). From these results, we could establish that (i) the onset of apoptosis occurred earlier in infected swine macrophages than in infected Vero cells, (ii) in the interval of 8–15 hpi, macrophages infected with ΔEP153R displayed higher levels of apoptotic response than those of BA71V-infected cultures, and (iii) at later times of infection (from 20 hpi), the values obtained from both infections tend to equalize.

Taken together, these results supported the conclusion that the absence of EP153R expression resulted in an enhancement of the apoptotic response developed in virus-sensitive infected cells.

Transient or stable transfection of EP153R gene results in protection from drug or virus-induced apoptosis

To confirm the role of EP153R in the control of apoptosis, we performed a series of experiments to analyze the effect of EP153R expression in heterologous systems. After subcloning the ASFV EP153R gene into the pcDNA3.1 mammalian expression vector, the plasmids were transfected for transient or stable expression into Vero and COS cells as described under Materials and methods. Stably transfected cell lines displayed growing kinetics and saturation densities similar to their original cell types, indicating that the transfection process had no deleterious effect on them. The analysis of EP153R mRNA expression by RT-PCR (Fig. 4A) revealed the presence of EP153R-specific transcripts in cell cultures transfected for transient or stable expression with the EP153R-containing vector and their absence in the corresponding controls transfected with the plasmid alone. To analyze the effect of EP153R gene in heterologous expression, transiently transfected Vero cell cultures were treated, 24 h after transfection, with different apoptotic drugs (2 μg/ml actinomycin D, 1 μM staurosporine, or 4 μM camptothecin) and further incubated for 24 h. Cell extracts were prepared and assayed for biological activity of caspase-3. As shown in Fig. 4B, all of the drugs were able to induce an approximately 5-fold activation of caspase-3 in control pcDNA-transfected Vero cell cultures, while little or no induction was detected in EP153R-transfected cells, revealing a considerable protection from apoptosis in Vero cells transiently expressing the EP153R gene. As we had previously determined that one of the best apoptosis inducers in virus-sensitive cells was precisely the ASFV infection, we used the deletion mutant ΔEP153R EP153R virus to provoke the apoptotic process and then analyzed the possible protective effect of EP153R expression in COS cells stably transfected with control or EP153R-containing pcDNA (Fig. 4C), as described above. As expected, ASFV infection was able to induce a level of

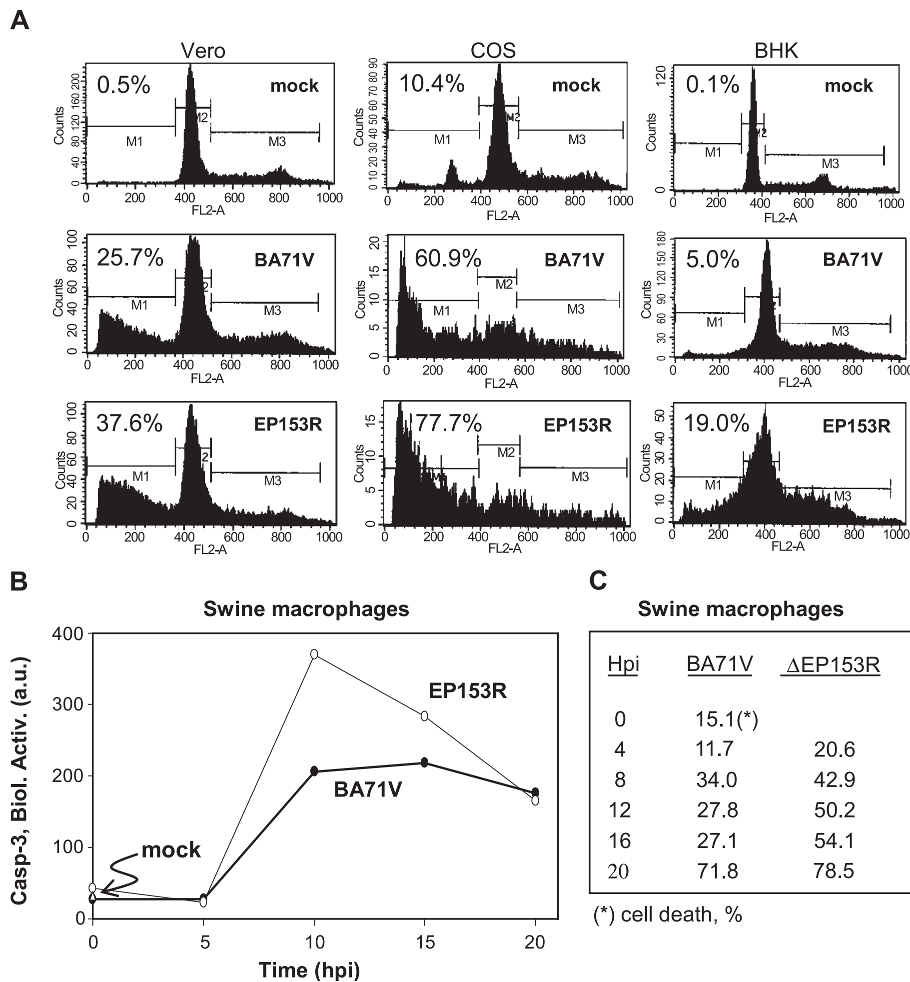

Fig. 3. Analysis of cell cycle and caspase-3 activity in ASFV-sensitive cell lines infected with BA71V or ΔEP153R isolates. Cultures of Vero, COS, and BHK cells were mock-infected or infected with BA71V or ΔEP153R at a moi of 3 pfu/cell and subjected at 20 hpi to cell cycle analysis by flow cytometry after PI staining (panel A). The percentage of total cell death (M1 value) is indicated at the left side of each graph. Cultures of swine macrophages were infected in parallel and processed at the indicated times to determine the biological activity of caspase-3 (panel B) or the percentage of cell death by flow cytometry (panel C).

caspase-3 activity much higher than pro-apoptotic drugs (compare ordinates in panels B and C of Fig. 4), and a partial protection (up to approximately 45%) from virus-induced caspase-3 activation was observed in EP153R-transfected COS cells at 24 hpi, indicating again a protective role for EP153R gene in ASFV-induced apoptosis. These results were confirmed in stably transfected Vero cells, which were analyzed by determination of the percentage of cells undergoing early apoptosis (stained by Annexin V) (Fig. 4D), after mock-infection or infection with ΔEP153R virus. The Annexin V apoptotic indicator corroborated that the expression of gene EP153R in Vero cells conferred resistance to the ASFV-induced programmed cell death.

Effect of EP153R gene on p53 activity

In an effort to elucidate the mechanism by which the viral EP153R gene inhibits the apoptotic process, we searched for possible differences in the expression or intracellular distribution of protein p53, a cellular component involved both in the apoptotic cascade and in cell cycle control, in Vero cells infected with BA71V or ΔEP153R. Previous experiments from our laboratory had shown a clear induction of p53 in ASFV-infected cultures (Granja et al., in press). The examination of Vero cell cultures infected with BA71V or ΔEP153R by confocal microscopy, using a specific anti-p53 antibody (Fig. 5A), showed a nuclear localization for the ASFV-induced p53, which accumulated in this compartment throughout the virus cycle until 20 hpi. However, no relevant differences could be detected in the distribution or level of expression of p53 associated to the presence (BA71V) or absence (ΔEP153R) of gene EP153R. To analyze whether the action of EP153R could interfere with the biological activity of p53, we studied the p53-transactivating activity induced in pcDNA- or EP153R-stably transfected Vero cells at 15 h after staurosporine treatment. A slight (about 20%), but consistent, reduction of p53-transactivating activity was obtained in cells transfected

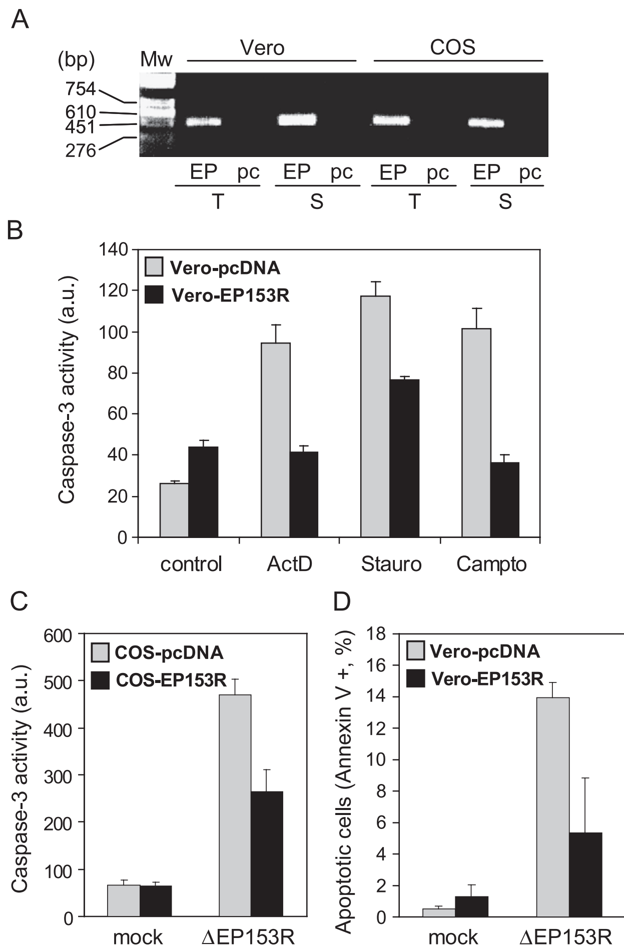

Fig. 4. EP153R mRNA expression, caspase-3 activity, and Annexin V staining in transiently or stably EP153R-transfected Vero and COS cells. (A) Cultures of Vero or COS cells, transiently (T) or stably (S) transfected with pcDNA3.1 (pc) or pcDNA3.1-EP153R (EP) plasmids, were collected for RT-PCR analysis. Total RNA was reverse-transcribed to cDNA and PCR-amplified with primers specific for EP153R viral gene before being analyzed by agarose gel electrophoresis. (B) Vero cell cultures were transfected with 2 μg of pcDNA3.1 or pcDNA3.1-EP153R plasmids and, 24 h later, the cultures were treated with pro-apoptotic drugs (2 μg/ml actinomycin D, 1 μM staurosporine, or 4 μM camptothecin). The biological activity of caspase-3 was determined in triplicate samples collected 24 h after drug treatment. (C) Cultures of stably transfected COS cells were infected with ΔEP153R at a moi of 3 pfu/cell and collected at 20 hpi to analyze the biological activity of caspase-3 in triplicate samples. (D) Cultures of stably transfected Vero cells were mock-infected or infected with ΔEP153R at a moi of 3 pfu/cell and collected at 20 hpi to analyze the percentage of apoptotic cells (Annexin V positives) in triplicate samples.

with EP153R (Fig. 5B), suggesting that the presence of EP153R might reduce to some extent the response of cells to external injuries. To confirm this effect, we made the same analysis in stably transfected Vero cells after 18 h of infection with BA71V as genotoxic inducer: a reduction of approximately 75% in p53 activity was observed in cells transfected with the viral gene (Fig. 5C). From these results, it should be inferred that a possible role of EP153R would be the inhibition of p53 function in ASFV-infected cells; accordingly, when p53 activity was assayed in Vero cells infected either with BA71V or ΔEP153R virus isolates, a higher activity was observed in cultures infected with the EP153R-defective virus (Fig. 5D). The fact that EP153R was less efficient in inhibiting the p53 activity induced by staurosporine (Fig. 5B) than that induced by ASFV infection (Figs. 5C and D) indicates that the mode of triggering the p53 response is important to determine its chances to be inhibited.

Discussion

The recognition of a broad repertoire of carbohydrate specificities in cell surfaces by lectins has important implications in many biological processes. The binding of mitogenic lectins to glycoproteins expressed on the plasma membrane of T-cells results in the stimulation of lymphocytes as well as other cells (Ashraf and Khan, 2003), although the induction of apoptosis by Concanavalin A in cultured murine macrophages has also been reported (Suen et al., 2000). In the case of C-type animal lectins, their main roles are derived from its activity as membrane-bound receptors that mediate recognition or endocytosis of glycoproteins (Drickamer and Taylor, 1993). The involvement of C-type lectins in the modulation of apoptosis has been recently proposed in the case of human HIP/PAP C-type lectin encoding gene, which stimulates liver regeneration and protects hepatocytes against TNF-alpha plus actinomycin-D-induced apoptosis (Simon et al., 2003), and for CD94/NKG2 murine NK cell receptors, whose expression is also correlated with a lower level of apoptosis (Gunturi et al., 2003), but has never been described in C-type lectin genes encoded by animal viruses. Members of the poxviridae (Afonso et al., 2000; Blasco et al., 1993; Cameron et al., 1999; Goebel et al., 1990; Senkevich et al., 1996; Shchelkunov et al., 1998; Tomley et al., 1988; Wilcock et al., 1999), herpesviridae (Voigt et al., 2001), and asfarviridae (Galindo et al., 2000; Neilan et al., 1999; Yáñez et al., 1995) families have been reported to encode proteins with C-type lectin-like domains, with activities modulating virus infectivity, cell to cell spreading, or hemadsorption.

In the case of the ASFV gene EP153R, which has been involved in the hemadsorption process observed in ASFV-infected cells (Galindo et al., 2000), the sequence of the BA71V strain revealed, besides the C-type lectin domain and the homology with the N-terminal region of CD44 molecules, the presence of a cell attachment (RGD) sequence (Yáñez et al., 1995) that could be involved in the modulation of apoptosis by direct caspase-3 activation, as reported for RGD peptides (Buckley et al., 1999). Consequently, we have analyzed in this work the effect of gene EP153R on the apoptosis induced in ASFV-sensitive Vero cells infected either with the parental virus BA71V or with the deletion mutant virus ΔEP153R that lacks the EP153R gene. Our results clearly show an enhanced activation of caspase-3 (determined either by processing of procaspase-3

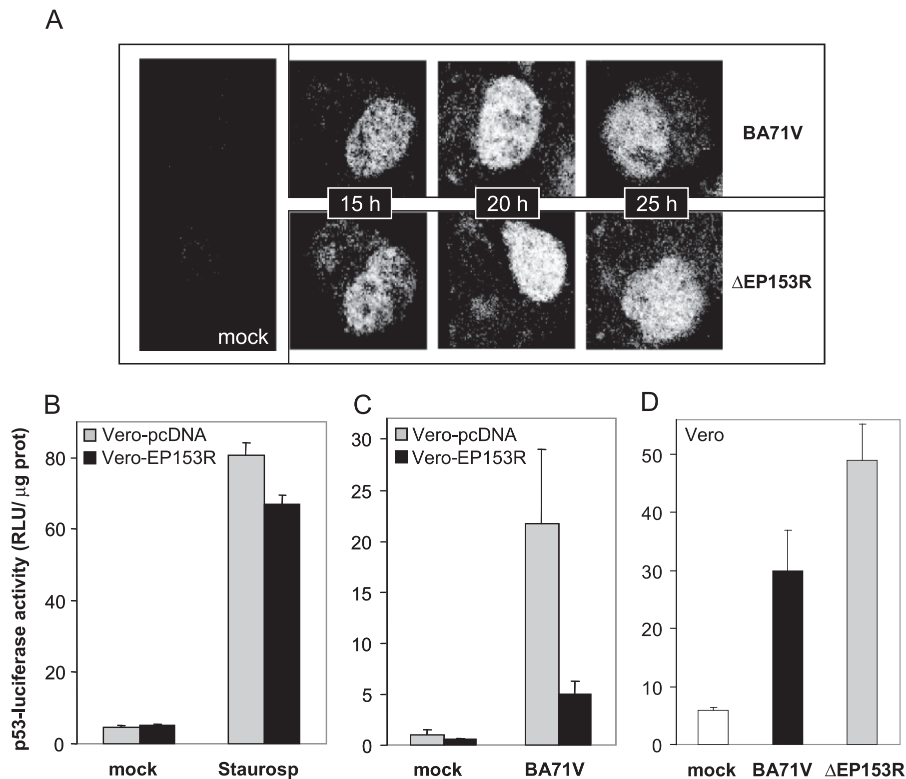

Fig. 5. (A) Subcellular localization of ASFV-induced p53. Vero cell cultures were mock-infected or infected with BA71V or ΔEP153R isolates at a moi of 3 pfu/cell. At the times indicated, cultures were labeled with anti-p53 antibody and then examined by confocal microscopy. (B and C) p53 transactivating activity in pcDNA or EP153R stably transfected Vero cells. Cultures of stably transfected Vero cells were transfected with p53RE-luc plasmid (100 ng per 10^6 cells) and incubated for 24 h before the addition of 1 μM staurosporine (panel B) or infection with BA71V at a moi of 3 pfu/cell (panel C). Cell extracts prepared after 15 h of staurosporine treatment or after 18 h of virus infection were analyzed for p53-transactivating activity. Data in panel B are the mean ± SD of duplicate samples and correspond to one of three independent experiments. Panel C shows the average values of luciferase activity and standard deviations from five independent experiments (with, at least, triplicate samples). (D) p53 transactivating activity in ASFV-infected Vero cells. Cultures were transfected with p53RE-luc plasmid (as in B and C) 24 h before infection with BA71V or ΔEP153R virus isolates at a moi of 3 pfu/cell. At 22 hpi, p53 activity was assayed in triplicate samples. Data correspond to the average values of luciferase activity and standard deviations from two independent experiments.

to its active form of 17 kDa or by biological activity), which resulted in an increase in the percentage of cell death, in Vero cell cultures infected with ΔEP153R as compared to those infected with the parental BA71V isolate. This was the opposite effect to that expected if the RGD sequence present into gene EP153R would activate caspase-3 (as reported for soluble RGD peptides), which should inhibit the apoptosis in cells infected with the deletion mutant ΔEP153R. Thus, the anti-apoptotic action of gene EP153R must not be related to the RGD sequence, but to another part of the molecule. Interestingly, the analysis of caspase-3 activation in swine macrophages at different times after infection with BA71V or ΔEP153R revealed that ASFV gene EP153R was also able to modulate the apoptotic process in the natural target cell for ASFV in a similar manner to that observed in other ASFV-sensitive cell lines (Vero, COS, and BHK).

To further confirm the modulation of apoptosis assigned to the viral protein, we have tested the effect of the expression of EP153R in a heterologous system by stable or transient transfection of the ASFV gene into Vero or COS cells. Using this protocol, we were also able to associate the presence of EP153R with a protective effect from the apoptosis triggered in cell cultures by chemical inducers (actinomycin D, staurosporine, or camptothecin) or by ASFV infection. Taken together, these results demonstrate a role in the control of apoptosis for ASFV gene EP153R.

Regarding the mechanism by which EP153R accomplishes the inhibition of apoptosis, we investigated for possible differences in the expression of several components involved in the apoptotic induction pathway, upstream the activation of effector caspase-3, in cells infected with BA71V or ΔEP153R. No relevant differences were observed by immunoblotting studies revealed with antibodies specific against p53, p21, Mdm2, Bcl-2, and Bax (data not shown). However, we could determine that the presence of gene EP153R in stably transfected Vero cells resulted in a reduced ability to induce p53 transactivating activity after staurosporine treatment or ASFV infection. The analysis by confocal microscopy of the expression and distribution of p53 in Vero cells infected with BA71V or ΔEP153R revealed that p53 was equally induced and accumulated into the nucleus in both infections. However, the trans-

activating activity of p53 was enhanced at late times in cells infected with the deletion mutant virus ΔEP153R, suggesting that EP153R might be involved in the control of p53 activity rather than in its expression or distribution. It must be noted that the activation of p53 can be modulated at three levels: increase of p53 expression, transformation of the protein from a latent to an active conformation through phosphorylation or acetylation, and translocation of p53 to the nucleus, where it acts as a transcriptional factor (Prives and Hall, 1999; Qian et al., 2002). In the case of ASFV infection, it seems that translocation of p53 to the nucleus occurs at early times of infection (Granja et al., in press) and that activation of p53 as a transcriptional factor is partially inhibited by the presence of EP153R. The possible connection between the inhibition of p53 activity and the protection from apoptosis by EP153R in ASFV-infected cells is still speculative. Further experiments are needed to determine the effect of gene EP153R in p53 posttranslational modifications and in successive steps downstream of p53 activation to elucidate the involvement of the viral gene in the control of cell cycle and death. The presence of a double lysine motif (preserved in many ASFV isolates, Neilan et al., 1999) within the cytoplasmic (C-terminal) tail of pEP153R, a characteristic shared with several proteins from human, rat, hamster, and viral (human adenovirus) origin that reside in the endoplasmic reticulum (Jackson et al., 1990), may target pEP153R to this subcellular compartment, which might exclude the viral protein from direct interaction with caspase-3, but should allow it to produce some disturbance in interactions between resident elements or in the Ca^{+} metabolism in the reticulum, resulting in the alteration by inhibition of the apoptotic process.

In conclusion, we report here that the ASFV gene EP153R behaves as an inhibitor of the apoptosis induced by the virus in infected cells. It is interesting to note that there are at least two other ASFV genes involved in the inhibition of apoptosis: A179L, which encodes a viral Bcl-2 homologue (Afonso et al., 1996; Revilla et al., 1997) and A224L, with similarity to IAP genes (Chacon et al., 1995; Nogal et al., 2001), which inhibit caspase activation and promote cell survival in mammalian cells. The reason why ASFV carries such an anti-apoptotic battery of genes is unknown, but it is tempting to speculate that, because the programmed cell death is a process triggered at early times and developed at intermediate and late times after ASFV infection (Carrascosa et al., 2002), it must be controlled from the virus uncoating process and throughout the infective cycle to prolong cell survival and maximize the production of viral progeny. This could account for the presence of anti-apoptotic genes transcribed either late (A224L) after virus infection or at both early and late (A179L and EP153R) times of infection and for the presence of anti-apoptotic proteins in the viral particle (A224L), which might confer protection from apoptosis induced during virus internalization, transcription, replication, or in any other step in the infectious cycle. Besides the different times of expression, the existence of several anti-apoptotic genes should confer other advantages to the virus, such as the increase of the response against cell suicide by the sum of activities, the development of resistance to the apoptosis induced by the cytolytic action of natural killer cells and cytolytic T lymphocytes, or the interference with different routes of apoptosis induction: in the case of ASFV, genes A179L (Bcl-2), A224L (IAP), and EP153R (C-type lectin) might interfere with the induction of the mitochondrial pathway, the efficiency of effector caspase-3, and the activation of p53, respectively. In any case, it becomes evident that ASFV is especially concerned about the control of apoptosis in the infected host cell. Additionally, it must be considered that ASFV encodes proteins with the capacity to regulate the transcription machinery of the host cell, like A224L, reported to activate the NF-κB activity (Rodriguez et al., 2002), and A238L (Powell et al., 1996; Revilla et al., 1998), which encodes an IkappaB-like molecule that prevents the activation of both NF-κB and NFAT transcription factors (Miskin et al., 1998; Miskin et al., 2000; Revilla et al., 1998; Tait et al., 2000), extending even more the capacity of ASFV to interfere with the expression of cellular genes involved in the activation of immune response, inflammation, and lastly in the control of cell viability.

Materials and methods

Cells and viruses

The established cell lines used in this report were seeded from working stocks kept in store in our laboratory and can be obtained from the American Type Culture Collection. Swine alveolar macrophages were prepared by bronchoalveolar lavage as previously described (Carrascosa et al., 1982). Cells were cultured at 37 °C in Dulbecco's modified Eagle (DME) medium supplemented with either 5% newborn calf serum (Vero), 5% heat-inactivated fetal calf serum (COS and BHK), or 10% homologous swine serum (swine macrophages).

The Vero-adapted ASFV strain BA71V was propagated and titrated by plaque assay on Vero cells as described (Carrascosa et al., 1982; Enjuanes et al., 1976). The construction and evaluation of the ASFV deletion mutant lacking gene EP153R (ΔEP153R) have been previously reported (Galindo et al., 2000). The ASFV recombinants vΔCD2, lacking the virus CD2 homologue, and v72GUS, with the gusA gene under the control of the viral p72.4 promoter inserted into the thymidine kinase locus, have been described before (Garcia et al., 1995; Rodriguez et al., 1993).

Transfections

The viral EP153R gene was subcloned into the pcDNA3.1 mammalian expression vector (Invitrogen). For transient expression, Vero or COS cells were plated at 1×10^6 per 6 cm dish 24 h before being transfected with 2 μg of

pcDNA3.1 or pcDNA3.1-EP153R using 12 μl of Lipofectamine (2 mg/ml, Invitrogen) per dish. To generate stably expressing lines, cells were transfected as described above and incubated for 2 days before being trypsinized, and plated at 1:10 dilution in 10-cm dishes. Next day, antibiotic selection was applied (2 mg/ml G418, Sigma) and cells were re-fed with medium with fresh antibiotic every 3 days until colonies were apparent (2–3 weeks).

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 ×100) supplemented immediately before use with protease inhibitor cocktail tablets (Roche). Protein concentration was determined by the bicinchoninic acid (BCA) method using the BCA Protein Assay Reagent from Pierce. Proteins (30 μg) were subjected to sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis, and then electroblotted onto a nitrocellulose (Protran, Schleicher and Schuell) membrane. After reacting with specific primary antibodies (anti-caspase-3 or anti-actin), membranes were exposed to horseradish peroxidase-conjugated secondary anti-species (rabbit or mouse) antibody (Amersham), followed by chemiluminescence (ECL, Amersham) detection by autoradiography.

Analysis of apoptosis

(1) Detection of the 17-kDa fragment of caspase-3. The cleavage of procaspase-3 (32 kDa) to the activated form of 17-kDa was detected in cell extracts, obtained as indicated above, by Western blot analysis revealed by incubation with a 1/400th dilution of polyclonal antibody specific for recombinant human caspase-3 (anti-caspase-3, Ab-4, NeoMarkers). Quantification of protein bands was performed by densitometry (Image Quant and TINA) in films developed after ECL exposure.
(2) Biological activity of caspases. Cell extracts (aliquots containing about 1 mg of protein per ml) 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 ovoalbumin) and incubated at 37 °C for 2 h with a 10 mM concentration of fluorescent substrate for specific (3, 6, or 8) 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.
(3) Flow cytometry: (I) Annexin V-FITC staining can identify apoptosis at earlier stages than assays based on DNA fragmentation by recognition of cells undergoing translocation of phosphatidylserine from the inner to the outer side of the plasma membrane. We used the Annexin V-FITC apoptosis detection kit I (BD Pharmingen), which labels cells in conjunction with the vital dye propidium iodide (PI), to distinguish between early apoptosis (annexin+) and total death (double-labeled cells). Cultures were stained according to the manufacturer's staining protocol, except that cells were labeled in monolayer, then detached by trypsinization and examined using a BD Biosciences FACSCalibur flow cytometer. (II) DNA content measurement was performed in cells suspended by trypsinization, permeabilized with 70% ethanol, and stained for 30 min at room temperature with PI (50 μg/ml in 0.3% Nonidet P40, 0.1% sodium citrate, and 20 μg/ml RNase) before being analyzed by flow cytometry. The percentage of total cell death was assigned from the data observed in the hypodiploid range (M1 value) of the cell cycle, which corresponded to apoptotic + necrotic cell population.

RT-PCR analysis of EP153R mRNA expression

Total RNA from transfected Vero or COS cell cultures was isolated by using the Trizol reagent (Invitrogen) following the manufacturer's recommendations. RNA was reverse transcribed to single-stranded cDNA with the avian myeloblastosis virus reverse transcriptase and oligo(dT) primers from Promega. DNA was PCR-amplified with Amplitaq DNA polymerase (Roche) and the following primers: 5′-ATGTATTTTAAGAAAAAATACATCGG and 5′-TTATTTACCACAAATAAATAATAAATC and then analyzed by electrophoresis on 0.7% agarose gels containing ethidium bromide.

p53 transactivating activity

The p53RE-luc reporter plasmid contains 14 tandem repeats of the p53 consensus binding motif (Stratagene). Cell cultures (300 000 cells) were transfected with p53RE-luc plasmid (100 ng per 10^6 cells) and Lipofectamine (Invitrogen) as described above, and incubated for 24 h. After the indicated treatments, cell extracts in CCLR lysis buffer (Luciferase Assay System, Promega) were obtained and processed according to the manufacturer's recommendations. Light produced was measured and recorded in a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Results were expressed as luminescence units after normalization of protein concentration determined by the BCA method.

Confocal microscopy

Vero cells were grown on coverslips to about 2×10^5 cells/cm^2. Cultures were extensively rinsed (three times) with PBS and fixed with cold 99.8% methanol for 15 min at −20 °C before rehydrating twice with PBS and blocking

with 1% BSA in PBS (BSA/PBS) for 10 min at room temperature. Cells were then incubated with specific primary antibodies against p53 (sc-6243, Santa Cruz Biotechnologies), diluted 1/20 in BSA/PBS for 2 h, and rinsed extensively with PBS before the secondary antibody (Alexa 488 goat anti-rabbit, Molecular Probes) was added (diluted 1/500) for 1 h at room temperature in the dark. Finally, the cells were rinsed successively with PBS, distilled water and ethanol, and mounted with a drop of Mowiol on a precleaned microslide. Visualization of stained cultures was performed in a Confocal Microradiance (BioRad) coupled to a vertical Axioskop2 (Zeiss) microscope. Confocal images were digitized, processed, and organized with Lasersharp2000 v.4, Adobe Photoshop 7.0 and Microsoft PowerPoint SP-2 software.

Acknowledgments

This work was supported by grants from Ministerio de Ciencia y Tecnología (BMC2000-1485 and AGL2002-10220-E), the European Commission (QLRT-2000-02216), and by an institutional grant from the Fundación Ramón Areces. C. Hurtado was a fellow from Fundación Ramón Areces.

References

Afonso, C.L., Neilan, J.G., Kutish, G.F., Rock, D.L., 1996. An African swine fever virus Bc1-2 homolog, 5-HL, suppresses apoptotic cell death. J. Virol. 70 (7), 4858–4863.

Afonso, C.L., Tulman, E.R., Lu, Z., Zsak, L., Kutish, G.F., Rock, D.L., 2000. The genome of fowlpox virus. J. Virol. 74 (8), 3815–3831.

Ashraf, M.T., Khan, R.H., 2003. Mitogenic lectins. Med. Sci. Monit. 9 (11), RA265–RA269.

Blasco, R., Sisler, J.R., Moss, B., 1993. Dissociation of progeny vaccinia virus from the cell membrane is regulated by a viral envelope glycoprotein: effect of a point mutation in the lectin homology domain of the A34R gene. J. Virol. 67 (6), 3319–3325.

Buckley, C.D., Pilling, D., Henriquez, N.V., Parsonage, G., Threlfall, K., Scheel-Toellner, D., Simmons, D.L., Akbar, A.N., Lord, J.M., Salmon, M., 1999. RGD peptides induce apoptosis by direct caspase-3 activation [see comments]. Nature 397 (6719), 534–539.

Cameron, C., Hota-Mitchell, S., Chen, L., Barrett, J., Cao, J.X., Macaulay, C., Willer, D., Evans, D., McFadden, G., 1999. The complete DNA sequence of myxoma virus. Virology 264 (2), 298–318.

Carrascosa, A.L., Santaren, J.F., Vinuela, E., 1982. Production and titration of African swine fever virus in porcine alveolar macrophages. J. Virol. Methods 3 (6), 303–310.

Carrascosa, A.L., Bustos, M.J., Nogal, M.L., Gonzalez de Buitrago, G., Revilla, Y., 2002. Apoptosis induced in an early step of African swine fever virus entry into vero cells does not require virus replication. Virology 294 (2), 372–382.

Costa, J., 1990. Molecular biology of Iridoviruses. In: Darai, G. (Ed.), Kluwer Academic Publisher, Boston, pp. 247–270.

Chacon, M.R., Almazan, F., Nogal, M.L., Vinuela, E., Rodriguez, J.F., 1995. The African swine fever virus IAP homolog is a late structural polypeptide. Virology 214 (2), 670–674.

Drickamer, K., Taylor, M.E., 1993. Biology of animal lectins. Annu. Rev. Cell Biol. 9, 237–264.

Enjuanes, L., Carrascosa, A.L., Moreno, M.A., Vinuela, E., 1976. Titration of African swine fever (ASF) virus. J. Gen. Virol. 32 (3), 471–477.

Everett, H., McFadden, G., 1999. Apoptosis: an innate immune response to virus infection. Trends Microbiol. 7 (4), 160–165.

Gabius, H.J., 1997. Animal lectins. Eur. J. Biochem. 243 (3), 543–576.

Galindo, I., Almazán, F., Bustos, M.J., Viñuela, E., Carrascosa, A.L., 2000. African swine fever virus EP153R open reading frame encodes a glycoprotein involved in the hemadsorption of infected cells. Virology 266 (2), 340–351.

Garcia, R., Almazan, F., Rodriguez, J.M., Alonso, M., Vinuela, E., Rodriguez, J.F., 1995. Vectors for the genetic manipulation of African swine fever virus. J. Biotechnol. 40 (2), 121–131.

Goebel, S.J., Johnson, G.P., Perkus, M.E., Davis, S.W., Winslow, J.P., Paoletti, E., 1990, The complete DNA sequence of vaccinia virus, Virology 179 (1), 247–266, 517–563.

Gomez-Villamandos, J.C., Hervas, J., Mendez, A., Carrasco, L., Martin de las Mulas, J., Villeda, C.J., Wilkinson, P.J., Sierra, M.A., 1995. Experimental African swine fever: apoptosis of lymphocytes and virus replication in other cells. J. Gen. Virol. 76 (Pt 9), 2399–2405.

Granja, A.G., Nogal, M.L., Hurtado, C., Salas, J., Salas, M.L., Carrascosa, A.L., Revilla, Y. Modulation of p53 cellular function and cell death by African swine fever virus. J Virol (in press).

Gunturi, A., Berg, R.E., Forman, J., 2003. Preferential survival of CD8 T and NK cells expressing high levels of CD94. J. Immunol. 170 (4), 1737–1745.

Hess, W.R., 1981. African swine fever: a reassessment. Adv. Vet. Sci. Comp. Med. 25, 39–69.

Jackson, M.R., Nilsson, T., Peterson, P.A., 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J. 9 (10), 3153–3162.

Miskin, J.E., Abrams, C.C., Goatley, L.C., Dixon, L.K., 1998. A viral mechanism for inhibition of the cellular phosphatase calcineurin. Science 281 (5376), 562–565.

Miskin, J.E., Abrams, C.C., Dixon, L.K., 2000. African swine fever virus protein A238L interacts with the cellular phosphatase calcineurin via a binding domain similar to that of NFAT. J. Virol. 74 (20), 9412–9420.

Neilan, J.G., Borca, M.V., Lu, Z., Kutish, G.F., Kleiboeker, S.B., Carrillo, C., Zsak, L., Rock, D.L., 1999. An African swine fever virus ORF with similarity to C-type lectins is non-essential for growth in swine macrophages in vitro and for virus virulence in domestic swine. J. Gen. Virol. 80 (Pt 10), 2693–2697.

Nogal, M.L., Gonzalez de Buitrago, G., Rodriguez, C., Cubelos, B., Carrascosa, A.L., Salas, M.L., Revilla, Y., 2001. African swine fever virus IAP homologue inhibits caspase activation and promotes cell survival in mammalian cells. J. Virol. 75 (6), 2535–2543.

O'Brien, V., 1998. Viruses and apoptosis. J. Gen. Virol. 79 (Pt 8), 1833–1845.

Oura, C.A., Powell, P.P., Parkhouse, R.M., 1998. African swine fever: a disease characterized by apoptosis. J. Gen. Virol. 79 (Pt 6), 1427–1438.

Powell, P.P., Dixon, L.K., Parkhouse, R.M., 1996. An IkappaB homolog encoded by African swine fever virus provides a novel mechanism for downregulation of proinflammatory cytokine responses in host macrophages. J. Virol. 70 (12), 8527–8533.

Prives, C., Hall, P.A., 1999. The p53 pathway. J. Pathol. 187 (1), 112–126.

Qian, H., Wang, T., Naumovski, L., Lopez, C.D., Brachmann, R.K., 2002. Groups of p53 target genes involved in specific p53 downstream effects cluster into different classes of DNA binding sites. Oncogene 21 (51), 7901–7911.

Ramiro-Ibanez, F., Ortega, A., Brun, A., Escribano, J.M., Alonso, C., 1996. Apoptosis: a mechanism of cell killing and lymphoid organ impairment during acute African swine fever virus infection. J. Gen. Virol. 77 (Pt 9), 2209–2219.

Revilla, Y., Cebrian, A., Baixeras, E., Martinez, C., Vinuela, E., Salas, M.L., 1997. Inhibition of apoptosis by the African swine fever virus Bcl-2 homologue: role of the BH1 domain. Virology 228 (2), 400–404.

Revilla, Y., Callejo, M., Rodriguez, J.M., Culebras, E., Nogal, M.L., Salas, M.L., Vinuela, E., Fresno, M., 1998. Inhibition of nuclear factor kappaB

activation by a virus-encoded IkappaB-like protein. J. Biol. Chem. 273 (9), 5405–5411.

Rodriguez, J.M., Yanez, R.J., Almazan, F., Vinuela, E., Rodriguez, J.F., 1993. African swine fever virus encodes a CD2 homolog responsible for the adhesion of erythrocytes to infected cells. J. Virol. 67 (9), 5312–5320.

Rodriguez, C.I., Nogal, M.L., Carrascosa, A.L., Salas, M.L., Fresno, M., Revilla, Y., 2002. African swine fever virus IAP-like protein induces the activation of nuclear factor kappa B. J. Virol. 76 (8), 3936–3942.

Senkevich, T.G., Bugert, J.J., Sisler, J.R., Koonin, E.V., Darai, G., Moss, B., 1996. Genome sequence of a human tumorigenic poxvirus: prediction of specific host response-evasion genes. Science 273 (5276), 813–816.

Shchelkunov, S.N., Safronov, P.F., Totmenin, A.V., Petrov, N.A., Ryazankina, O.I., Gutorov, V.V., Kotwal, G.J., 1998. The genomic sequence analysis of the left and right species-specific terminal region of a cowpox virus strain reveals unique sequences and a cluster of intact ORFs for immunomodulatory and host range proteins. Virology 243 (2), 432–460.

Simon, M.T., Pauloin, A., Normand, G., Lieu, H.T., Mouly, H., Pivert, G., Carnot, F., Tralhao, J.G., Brechot, C., Christa, L., 2003. HIP/PAP stimulates liver regeneration after partial hepatectomy and combines mitogenic and anti-apoptotic functions through the PKA signaling pathway. FASEB J. 17 (11), 1441–1450.

Suen, Y.K., Fung, K.P., Choy, Y.M., Lee, C.Y., Chan, C.W., Kong, S.K., 2000. Concanavalin A induced apoptosis in murine macrophage PU5-1.8 cells through clustering of mitochondria and release of cytochrome *c*. Apoptosis 5 (4), 369–377.

Tait, S.W., Reid, E.B., Greaves, D.R., Wileman, T.E., Powell, P.P., 2000. Mechanism of inactivation of NF-kappa B by a viral homologue of I kappa b alpha. Signal-induced release of i kappa b alpha results in binding of the viral homologue to NF-kappa B. J. Biol. Chem. 275 (44), 34656–34664.

Tomley, F., Binns, M., Campbell, J., Boursnell, M., 1988. Sequence analysis of an 11.2 kilobase, near-terminal, *Bam*HI fragment of fowlpox virus. J. Gen. Virol. 69 (Pt 5), 1025–1040.

Vinuela, E., 1985. African swine fever virus. Curr. Top. Microbiol. Immunol. 116, 151–170.

Voigt, S., Sandford, G.R., Ding, L., Burns, W.H., 2001. Identification and characterization of a spliced C-type lectin-like gene encoded by rat cytomegalovirus. J. Virol. 75 (2), 603–611.

Weis, W.I., Taylor, M.E., Drickamer, K., 1998. The C-type lectin superfamily in the immune system. Immunol. Rev. 163, 19–34.

Wilcock, D., Duncan, S.A., Traktman, P., Zhang, W.H., Smith, G.L., 1999. The vaccinia virus A4OR gene product is a nonstructural, type II membrane glycoprotein that is expressed at the cell surface. J. Gen. Virol. 80 (Pt 8), 2137–2148.

Yáñez, R.J., Rodríguez, J.M., Nogal, M.L., Yuste, L., Enríquez, C., Rodriguez, J.F., Viñuela, E., 1995. Analysis of the complete nucleotide sequence of African swine fever virus. Virology 208, 249–278.