

# Virus uncoating is required for apoptosis induction in cultured mammalian cells infected with African horse sickness virus

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Infection of cultured mammalian cells with African horse sickness virus (AHSV) is known to induce cell death. To date, the trigger(s) of this response, the apoptotic pathways activated during AHSV infection and the functional consequences of apoptosis on the virus replication cycle have yet to be characterized. This study demonstrated that extracellular treatment of BHK-21 cells with both of the AHSV4 outer capsid proteins, VP2 and VP5, was sufficient to trigger apoptosis. Whether steps in AHSV4 replication subsequent to viral attachment were required for AHSV4-induced apoptosis was also investigated. Apoptosis was induced in BHK-21 cells infected with UV-inactivated AHSV4 and in ribavirin-treated cells infected with AHSV4. However, both AHSV4- and VP2/VP5-stimulated apoptotic responses were inhibited in the presence of the endosomal acidification inhibitors ammonium chloride and chloroquine. These results indicated that uncoating of AHSV4 virions, but not viral transcription or subsequent steps in viral replication, was required for AHSV4 to induce apoptosis in BHK-21 cells. Furthermore, this study showed that both the extrinsic (caspase-8) and intrinsic (caspase-9) apoptotic pathways were induced following AHSV4 infection. The inhibition of caspase activity in AHSV4-infected cells did not diminish AHSV4 replication, but reduced the release and dissemination of progeny viral particles. Taken together, the data indicated that uncoating of AHSV virions was required for apoptosis induction, and that apoptosis enhanced virus spread and release.

## INTRODUCTION

*African horse sickness virus* (AHSV), an arbovirus (genus *Orbivirus*; family *Reoviridae*), is the causative agent of African horse sickness, a highly infectious disease of equids of which the mortality rate in susceptible horse populations may exceed 90 % (Mellor & Hamblin, 2004). The disease is characterized by clinical signs that develop as a consequence of damage to the circulatory and respiratory systems, thereby giving rise to serious effusion and haemorrhage in various organs and tissues (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004). Microscopic examination of endothelial cells of the heart, lung, liver and spleen of AHSV-infected animals showed ultrastructural modifications that included the loss of intercellular junction integrity, the presence of cytoplasmic projections and condensation of the nucleus, thus suggesting that apoptosis may contribute to the pathogenesis of AHSV in the mammalian host (Laegreid *et al.*, 1992; Gómez-Villamandos *et al.*, 1999). Indeed, for various members of the family *Reoviridae*, including *Bluetongue virus* (BTV), *Epidemic haemorrhagic disease virus* (EHDV) and others, apoptosis appears to be integral to the cellular pathogenesis induced by these viruses (DeMaula *et al.*, 2001; Kominsky

*et al.*, 2002; Mortola *et al.*, 2004; Clarke *et al.*, 2005; O'Donnell *et al.*, 2005; Danthi *et al.*, 2008; Stewart & Roy, 2010; Shai *et al.*, 2013). In the case of BTV, the prototype member of the genus *Orbivirus*, the outer capsid proteins VP2 and VP5 were shown to be sufficient for the induction of apoptosis in cultured mammalian cells (Mortola *et al.*, 2004).

Apoptosis is the programmed destruction of cells and is characterized by distinctive changes in cellular morphology. These include plasma membrane blebbing, cell shrinkage, nuclear condensation and, subsequently, degradation of genomic DNA (Kerr *et al.*, 1972; Wyllie, 1997). The apoptotic pathways leading to cell death can be divided into two signalling cascades involving death receptors (extrinsic signalling) or mitochondria (intrinsic signalling) (Xu & Shi, 2007; Salvesen & Riedl, 2008). The extrinsic pathway is triggered by the binding of external (death) ligands to their cognate (death) receptors on cell membranes, causing oligomerization of the receptor. This induces formation of the death-inducing signalling complex (DISC), leading to the activation of the initiator caspase, caspase-8. Activated caspase-8 can, in turn, activate downstream effector caspases including caspase-3

(Ashkenazi & Dixit, 1998). Apoptosis via the intrinsic pathway involves signals that allow the release of several proapoptotic proteins, including cytochrome *c* and the second mitochondria-derived activator of caspase (Smac/DIABLO), from the mitochondrial inner membrane space into the cytosol (Susin *et al.*, 1999; Du *et al.*, 2000; Parone *et al.*, 2003). Cytosolic cytochrome *c*, Apaf-1 and procaspase-9 form an apoptosome complex, leading to the activation of caspase-9. Activated caspase-9, like activated caspase-8, can activate the downstream effector caspase, caspase-3 (Ahmad *et al.*, 1997; Zou *et al.*, 1999).

AHSV is known to induce apoptosis in infected cultured mammalian cells. The proapoptotic activity of AHSV appears to be a general feature of these viruses, as it is not specific for a particular virus serotype or to the infection of a specific type of cell (Osawa & Hazrati, 1965; Stassen *et al.*, 2012; Venter, 2014). However, the initiators of the apoptotic response, the nature of the caspase cascades activated and the functional consequences of their activation on the virus replication cycle are unknown. This study reports that uncoating of AHSV, but not viral gene expression, is required for the induction of apoptosis. The results furthermore demonstrate that AHSV-induced apoptosis in BHK-21 mammalian cells results in the activation of both the extrinsic and intrinsic apoptotic pathways, and that activation of these caspase-dependent pathways enhances virus spread.

## RESULTS

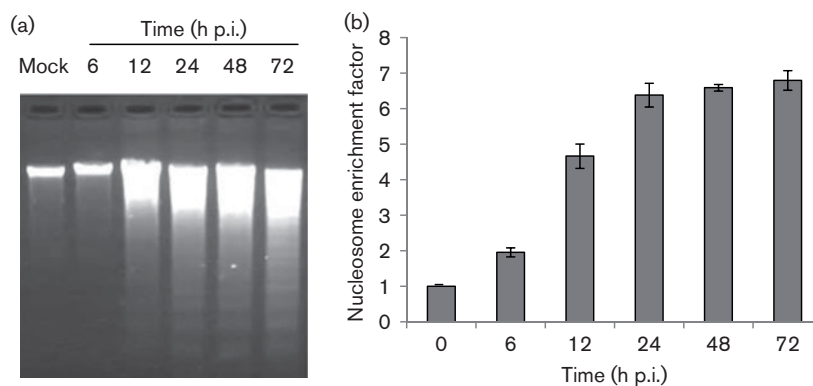
### Time frame of AHSV4-induced apoptosis in BHK-21 cells

Prior to undertaking studies regarding AHSV4-induced apoptosis in mammalian cells, the time frame in which

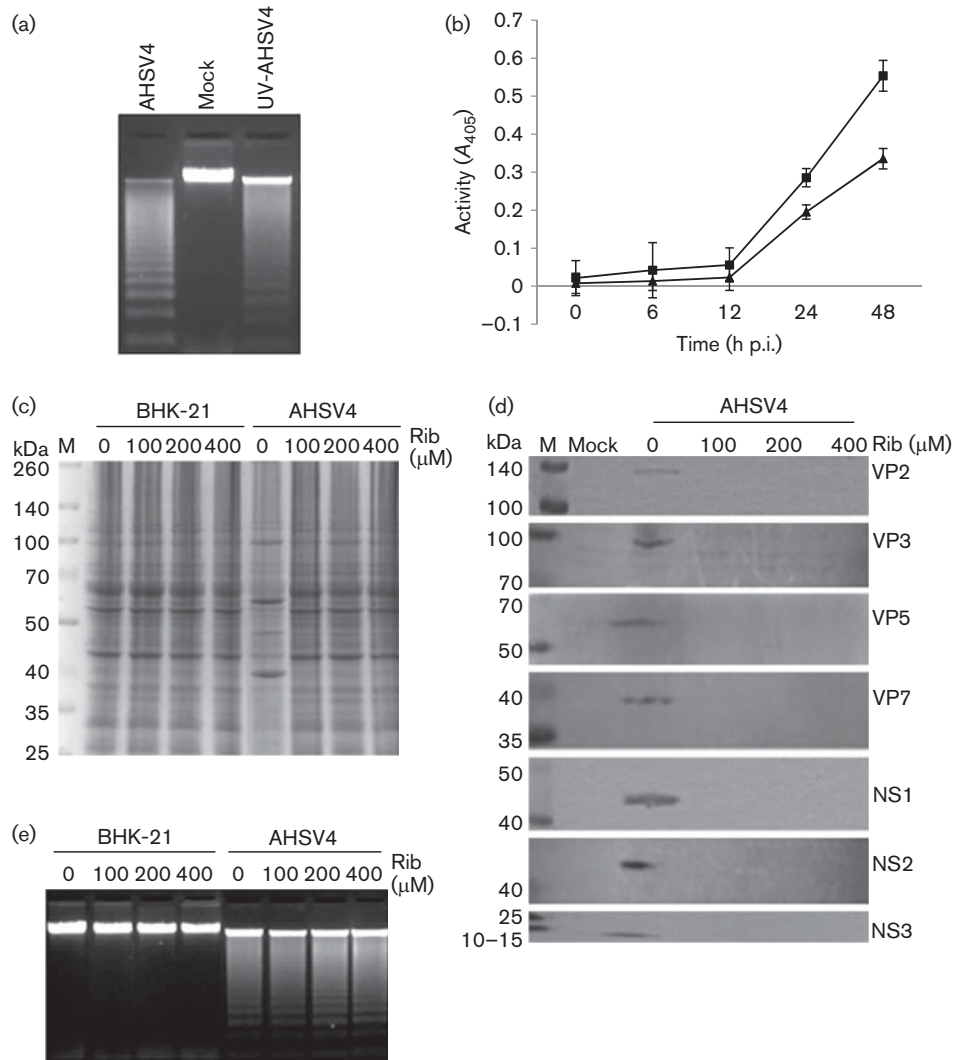
this process occurs was first established. BHK-21 cells were infected with AHSV4 at m.o.i. 1 p.f.u. per cell and, at different time points post-infection, infected cells were processed for DNA fragmentation assays. In contrast to mock-infected cells, a fragmented genomic DNA ladder typical of apoptosis was detected in AHSV4-infected cells from 12 to 72 h post-infection (p.i.) (Fig. 1a). Subsequent quantification of the nucleosomes released into the cytoplasm of virus-infected cells indicated that there was an increase in the release of nucleosomes with the progression of infection, peaking at 24 h p.i. (Fig. 1b). The data indicate that infection of BHK-21 cells with AHSV4 resulted in apoptosis at 12 h p.i. and culminated at 24 h p.i., thus establishing a time frame for the study of AHSV4-induced apoptosis in BHK-21 cells.

### AHSV4 gene expression is not required to induce apoptosis in BHK-21 cells

To determine whether viral gene expression was required for apoptosis induction, the effect of UV treatment on the capacity of AHSV4 to induce apoptosis was first investigated. BHK-21 cells were infected with UV-irradiated AHSV4 at m.o.i. 2 p.f.u. per cell. Complete inactivation of AHSV4 by UV treatment was confirmed by a plaque assay prior to its use in these experiments. The results indicated that apoptosis was triggered in response to treatment of the BHK-21 cells with UV-irradiated virus, as evidenced by the induction of DNA fragmentation (Fig. 2a) and activation of caspase-3, a key agent of apoptosis (Fig. 2b). For both the UV-irradiated and non-irradiated AHSV4, the results indicated that caspase-3 activity in the infected BHK-21 cells increased gradually from 0 to 12 h p.i., followed by a steep increase in caspase-3 activity until 48 h p.i.



**Fig. 1.** Time course of AHSV4-induced apoptosis in BHK-21 cells. (a) DNA fragmentation analysis of mock-infected BHK-21 cells at 72 h p.i. and of AHSV4-infected BHK-21 cells at the indicated time points. (b) Cytoplasmic extracts were prepared from mock-infected and AHSV4-infected BHK-21 cells, and the release of free nucleosomes into the cell cytoplasm was determined by ELISA. The data were used to calculate the nucleosome enrichment factor in AHSV4-infected cells and represent mean  $\pm$  SD of three independent experiments.



**Fig. 2.** Effect of UV treatment and ribavirin on the induction of apoptosis by AHSV4 in BHK-21 cells. (a) Agarose gel from a DNA laddering experiment performed 24 h after mock-infection of BHK-21 cells or infection with AHSV4 or UV-inactivated AHSV4 (UV-AHSV4). (b) Activation of caspase-3 in BHK-21 cells infected with AHSV4 (■) and UV-inactivated AHSV4 (▲). Cytoplasmic extracts were prepared from mock-infected and virus-infected BHK-21 cells at the indicated time points, and the caspase-3 activity determined using ELISA. The data are presented as an increase in caspase-3 activity in AHSV4-infected BHK-21 cells and represent mean  $\pm$  SD of three independent experiments. (c–e) Mock-infected and AHSV4-infected BHK-21 cell monolayers were incubated in the presence of ribavirin (Rib) at the concentrations shown. At 24 h p.i., cytoplasmic extracts were prepared and analysed by (c) SDS-PAGE and (d) immunoblot analysis with polyclonal antibodies raised against different AHSV4 structural and non-structural proteins, as shown on the right of the figure. The sizes of the Spectra Multicolor Broad Range Protein Ladder (Fermentas) are indicated to the left of the figure (M). (e) Genomic DNA was extracted from mock-infected and AHSV4-infected BHK-21 cells at 24 h p.i., and analysed by agarose gel electrophoresis.

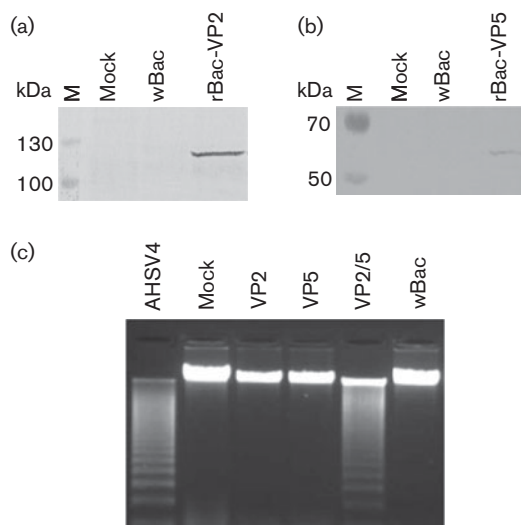
These results suggested that intracellular viral gene expression was dispensable for apoptosis induction in AHSV4-infected cells. To confirm this suggestion, the capacity of AHSV4 to induce apoptosis in the presence of ribavirin was subsequently investigated. Ribavirin is a guanosine nucleoside analogue that inhibits viral ssRNA and dsRNA production without affecting expression of cellular genes (Rankin *et al.*, 1989; Graci & Cameron, 2006).

BHK-21 cells were infected with AHSV4 and incubated for 24 h in the absence or presence of various concentrations of ribavirin. The presence of ribavirin in the culture medium of uninfected cells at concentrations up to 400  $\mu$ M did not affect their protein synthesis ability (Fig. 2c) and did not promote DNA fragmentation (Fig. 2e). In experiments with AHSV4-infected cells, ribavirin inhibited viral protein synthesis, as demonstrated by

immunoblot analyses of intracellular levels of different structural and non-structural proteins (Fig. 2d). However, AHSV4 induced DNA fragmentation in the presence of each concentration of ribavirin tested (Fig. 2e). Taken together, the data thus supported the notion that viral transcription and subsequent steps in viral replication were not needed for apoptosis induction in AHSV4-infected cells.

### Outer capsid proteins VP2 and VP5 of AHSV4 are sufficient to trigger apoptosis in BHK-21 cells

Based on the supposition that binding of cell receptors might trigger apoptosis, the ability of the outer capsid proteins VP2 and VP5 of AHSV4 to trigger apoptosis was investigated. The role of each individual outer capsid protein and their possible synergistic effect in inducing apoptosis was examined by treating uninfected BHK-21 cells extracellularly with recombinant baculovirus-expressed VP2 and/or VP5 proteins. As it was not possible to purify the recombinant proteins to homogeneity, an identically prepared cytoplasmic protein extract of WT baculovirus-infected Sf9 cells was included in the analyses to determine whether host and/or baculovirus proteins were capable of inducing apoptosis when added to BHK-21



**Fig. 3.** Induction of apoptosis in BHK-21 cells by the AHSV4 outer capsid proteins. (a, b) Immunoblot analyses of cell extracts prepared from Sf9 cells infected with the rBac-VP2 or rBac-VP5 recombinant baculoviruses. The immunoblots were incubated with polyclonal anti-VP2 and anti-VP5 antibodies to confirm expression of the AHSV4 (a) VP2 and (b) VP5 proteins, respectively. The sizes of the PageRuler Plus Prestained Protein Ladder (Fermentas) are indicated to the left of the figure (M). (c) BHK-21 cells were treated extracellularly with recombinant baculovirus-expressed AHSV4 VP2 and/or VP5, as indicated, and with a protein extract prepared from WT baculovirus-infected cells (wBac). At 24 h post-treatment, genomic DNA was extracted and analysed by agarose gel electrophoresis.

cells. Analysis of the genomic DNA at 24 h post-treatment indicated no signs of DNA fragmentation when the cells were treated with either VP2 or VP5 independently. In contrast, when both the VP2 and VP5 proteins were added together to the cells, DNA laddering typical of AHSV4-induced apoptosis was observed (Fig. 3). BHK-21 cells treated with a cytoplasmic protein extract derived from WT baculovirus-infected insect cells did not induce apoptosis in the BHK-21 cells, thus demonstrating that the recombinant VP2 and VP5 proteins were required to trigger apoptosis.

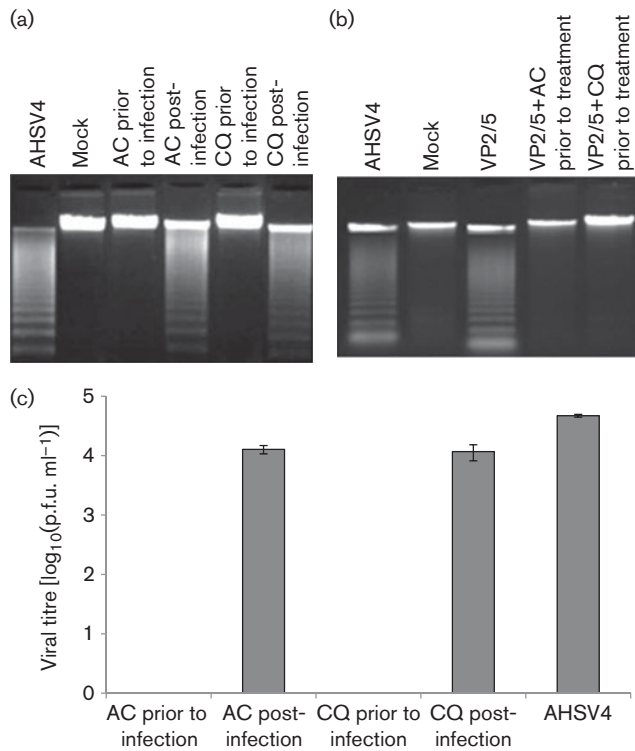
### Induction of apoptosis by AHSV4 in BHK-21 cells requires virus uncoating

Following receptor-mediated endocytosis, conversion of the orbivirus virions to core particles that lack the VP2 and VP5 proteins occurs in early endosomes and requires acidic pH (Forzan *et al.*, 2007; Zhang *et al.*, 2010). To investigate whether acid-dependent virus uncoating was required for AHSV4 to trigger apoptosis, the capacity of AHSV4 to elicit apoptosis in the presence of two inhibitors of endosomal acidification, i.e. ammonium chloride and chloroquine, was assessed. BHK-21 cell monolayers were exposed to 10 mM ammonium chloride or 100  $\mu$ M chloroquine before or after infection with AHSV4. DNA fragmentation analysis of cells harvested 24 h p.i. revealed that the respective inhibitors protected the BHK-21 cells from apoptosis induced by AHSV4 only when added 2 h prior to virus infection (Fig. 4a). Similar results were obtained when BHK-21 cells were exposed to ammonium chloride or chloroquine prior to treatment with a combination of the recombinant baculovirus-expressed VP2 and VP5 proteins (Fig. 4b). Moreover, AHSV4 replication in cells that had been pre-treated with ammonium chloride or chloroquine was blocked compared with that in untreated virus-infected cells (Fig. 4c). The results indicated that conversion of AHSV4 virions to core particles was necessary for induction of apoptosis following AHSV4 infection.

### Extrinsic and intrinsic apoptotic pathways are activated in AHSV4-infected BHK-21 cells

It was demonstrated previously that the executioner caspase, caspase-3, is activated during AHSV4 infection of BHK-21 cells (Fig. 2b). However, caspase-3 is an effector caspase common to both the extrinsic (caspase-8) and intrinsic (caspase-9) apoptotic pathways. To determine whether these respective apoptotic pathways were activated during AHSV4 infection, BHK-21 cells were thus infected with AHSV4, and the activity of caspase-8 and caspase-9 was determined over the time course of infection.

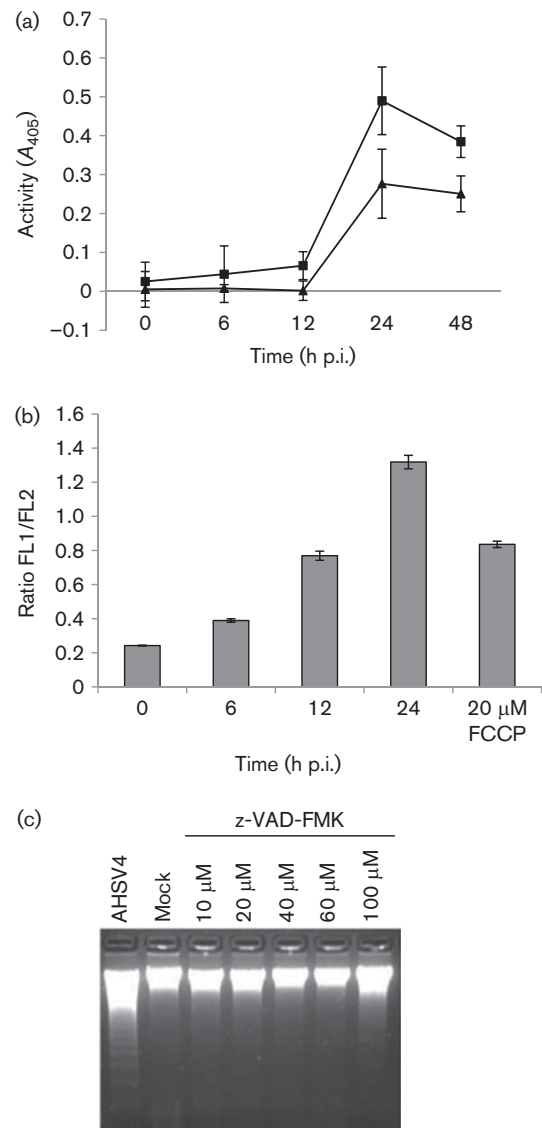
In virus-infected BHK-21 cells, caspase-8 activity increased gradually from 0 to 12 h p.i. Between 12 and 24 h p.i., there was a steep increase in caspase-8 activity and, after 24 h p.i.,



**Fig. 4.** Effect of endosomal acidification inhibitors on the induction of apoptosis by AHSV4 infection of BHK-21 cells. BHK-21 cells were treated with 10 mM ammonium chloride (AC) or 100  $\mu$ M chloroquine (CQ), as indicated. (a, b) Genomic DNA was extracted from BHK-21 cells at 24 h after AHSV4 infection (a) or at 24 h after extracellular treatment with a mixture of recombinant VP2 and VP5 proteins (b), and analysed by agarose gel electrophoresis. (c) Growth of AHSV4 in BHK-21 cells treated with either AC or CQ pre- or post-infection, or left untreated. Viral titres were determined at 24 h after infection by a plaque assay. Data represent mean  $\pm$  SD of three independent experiments performed in duplicate.

the caspase-8 activity showed a steady decrease (Fig. 5a). Caspase-9 activity was first detected from 12 h p.i. and reached a maximum at 24 h p.i., followed by a decrease in activity (Fig. 5a). To further characterize activation of the intrinsic apoptotic pathway, we next determined whether AHSV4 infection caused mitochondrial damage. For this purpose, the mitochondrial transmembrane potential of AHSV4-infected BHK-21 cells was assessed using the lipophilic cation DePsipher. As shown in Fig. 5(b), flow cytometric analysis of AHSV4-infected BHK-21 cells stained with the DePsipher reagent indicated a progressive loss of mitochondrial potential from 6 h p.i.

To substantiate the data obtained in the caspase activity assays, AHSV4-infected BHK-21 cells were incubated in the presence or absence of the pan-caspase inhibitor z-VAD-FMK. At 24 h post-treatment, no DNA



**Fig. 5.** AHSV4 infection of BHK-21 cells induces activation of the extrinsic and intrinsic apoptotic signalling pathways. (a) The activity of caspase-8 (■) and caspase-9 (▲) was determined using caspase-specific ELISAs by making use of cytoplasmic extracts prepared from mock-infected and AHSV4-infected cells at the indicated time points. The data represent mean  $\pm$  SD of three independent experiments and are presented as an increase in the activity of the respective caspases in AHSV4-infected BHK-21 cells. (b) AHSV4-infected cells were treated with DePsipher at the indicated times points and analysed by flow cytometry. BHK-21 cells treated with FCCP were included as a positive control in the analysis. An increase in the ratio of green DePsipher monomer (FL1)/red DePsipher aggregate (FL2) fluorescence is indicative of mitochondrial membrane depolarization. The data represent mean  $\pm$  SD of three independent experiments. (c) BHK-21 cells were infected with AHSV4 in the absence or presence of different concentrations of the pan-caspase inhibitor z-VAD-FMK. The genomic DNA was extracted at 24 h p.i. and analysed by agarose gel electrophoresis.

fragmentation was observed in virus-infected cells incubated in the presence of the inhibitor (Fig. 5c), indicating that AHSV4-induced apoptosis in BHK-21 cells was indeed caspase-dependent.

### Effect of caspase inhibition on AHSV4 replication in BHK-21 cells

As the pan-caspase inhibitor z-VAD-FMK was shown to block apoptosis in AHSV4-infected cells, it was of interest to examine the role of apoptosis in AHSV4 replication. BHK-21 cell monolayers were infected and either left untreated or treated with 100  $\mu$ M z-VAD-FMK, and the production of total virus (intracellular and cell culture medium) and released virus (cell culture medium) was determined at 24 h p.i. by plaque assays. Although z-VAD-FMK did not significantly change the production of infectious virions, there was a slight but significant ( $P < 0.05$ ) reduction in virus release when the cells were incubated in the presence of the inhibitor (Fig. 6a). These results suggested that apoptosis may thus not be required for the production of infectious progeny in cultured cells, but rather may enhance dissemination of progeny virions from infected cells. This notion was supported by the

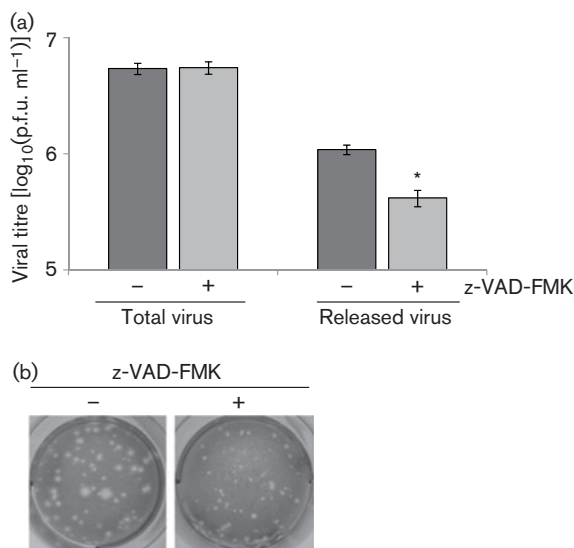
observation that the presence of z-VAD-FMK during plaque assay of the AHSV4 stock caused a reduction in the size of the viral plaques (Fig. 6b), suggesting that the inhibitor reduced cell-to-cell virus spread over the different rounds of replication, release and reinfection that occurred during plaque formation.

## DISCUSSION

Although it has been reported that AHSV infection of cultured mammalian cells induces apoptosis and results in the activation of caspase-3, these studies have characterized apoptosis mainly in terms of morphological markers of apoptosis (Stassen *et al.*, 2012; Venter, 2014). During the course of the viral life cycle, there are several obligate interactions between viruses and cells capable of triggering apoptosis, including virus-receptor engagement, fusion with or penetration of cellular membranes, and disruption of host cell transcriptional and translational machineries (Roulston *et al.*, 1999; Danthi, 2011). Here, a series of experiments was undertaken to examine the induction of apoptosis by AHSV4 infection of BHK-21 mammalian cells and the functional consequences of apoptosis on the viral replication cycle.

One of the goals of the present study was to assess the stage of the AHSV life cycle at which apoptosis is induced. The finding that apoptosis is induced by infection of BHK-21 cells with UV-inactivated AHSV4 suggested that viral gene expression is dispensable for apoptosis induction. In agreement with this finding, the capacity of AHSV4 to induce apoptosis was not diminished by the presence of the viral RNA synthesis inhibitor ribavirin, notwithstanding that this inhibitor was very effective in inhibiting the intracellular production of viral proteins. Although these experiments do not exclude a contribution of viral non-structural proteins to enhancing the magnitude of AHSV4-induced apoptosis, these findings do, however, strongly suggest that viral transcription and subsequent steps in virus replication are dispensable for apoptosis induction in AHSV4-infected cells. A similar non-dependence of apoptosis on viral gene expression has also been reported for cells infected with other members of the family *Reoviridae*, including BTV (Mortola *et al.*, 2004), mammalian and avian reoviruses (Connolly & Dermody, 2002; Labrada *et al.*, 2002), as well as various other viruses such as *Human herpesvirus 1* (*Herpes simplex virus*) (Koyama & Adachi, 1997), *Sindbis virus* (Jan & Griffin, 1999) and *African swine fever virus* (Carrascosa *et al.*, 2002).

The apparent non-dependence of apoptosis on viral gene expression indicates that apoptosis is induced by parental virus particles at a stage prior to virus transcription. Following virus attachment and receptor-mediated endocytosis, the virions are uncoated to form core particles. This process is dependent on an acidic pH (Forzan *et al.*, 2007), and is characterized by removal of VP2 and exposure of VP5, which is subsequently responsible for



**Fig. 6.** Effect of z-VAD-FMK on AHSV4 replication and dissemination. (a) BHK-21 cell monolayers were infected with AHSV4, and the production of both total virus (intracellular and culture medium) and released virus (culture medium) was determined at 24 h p.i. by plaque assays on BHK-21 cells. Data represent mean  $\pm$  SD of three independent experiments performed in duplicate. \*Statistically significant differences ( $P < 0.05$  determined by a paired, two-tailed Student's *t*-test). (b) Representative images of plaques obtained by titrating an AHSV4 stock on BHK-21 monolayers in the absence or presence of 100  $\mu$ M z-VAD-FMK. The pictures are of plaque assays that correspond to a  $10^{-4}$  dilution of the same virus stock.

permeabilization of the endosomal membrane to allow the delivery of the transcriptionally active core across the membrane into the cell cytoplasm (Hassan *et al.*, 2001; Zhang *et al.*, 2010). Parental viral particles could therefore trigger apoptosis during receptor engagement, during cell entry or upon virus uncoating.

Prior treatment of BHK-21 cells with either ammonium chloride or chloroquine, both of which inhibit endosomal acidification, was shown to abolish the ability of AHSV4 to induce apoptosis. Furthermore, extracellular treatment of the cells with the cellular receptor binding protein VP2 (Hassan & Roy, 1999) or with the membrane permeabilization protein VP5 (Hassan *et al.*, 2001; Stassen *et al.*, 2011) did not result in an apoptotic response. An apoptotic response was, however, elicited in cells treated with VP2 and VP5 together. As this apoptotic response was also inhibited by prior treatment of the cells with ammonium chloride or chloroquine, we hypothesize that a complex of VP2 and VP5 is internalized in cells treated exogenously with a mixture of the recombinant proteins. Based on the results, it appears unlikely that receptor binding is sufficient to elicit an apoptotic response but rather that downstream events dependent on acidification of the endosome, such as viral uncoating, are required for induction of apoptosis following AHSV4 infection. Taking previously published reports into account, it appears that virus uncoating or disassembly may be a general requirement for apoptosis induction by members of the genera *Orbivirus* and *Orthoreovirus* (Connolly & Dermody, 2002; Labrada *et al.*, 2002; Mortola *et al.*, 2004; Danthi *et al.*, 2006). The findings reported here furthermore suggest that activation of VP5 by acidification of endosomal vesicles and subsequent alteration of the integrity of the endosomal membrane may be critical to the induction of apoptosis. In this regard, it is interesting to note that cytosol acidification and excessive leakage of endosomal and lysosomal enzymes to the cytosol have been suggested to be associated with apoptosis and to promote cytochrome *c*-mediated activation of caspases (Matsuyama *et al.*, 2000; Turk *et al.*, 2000).

The results of the present study demonstrate that both the extrinsic and intrinsic apoptotic pathways are activated in BHK-21 cells infected with AHSV4. Activation of apoptosis is one of the first protective defences initiated by the host cell to restrict viral amplification and spread. When triggered early in infection, apoptosis could limit viral replication, thus explaining why several viruses encode anti-apoptotic factors aimed at blocking the induction and/or execution of the apoptotic program (Best, 2008; Galluzzi *et al.*, 2010). However, other viruses induce apoptosis late in infection to facilitate virus progeny release and spread to neighbouring cells whilst evading host inflammatory or immune responses (O'Brien, 1998; Hay & Kannourakis, 2002). The results of this study suggest that apoptosis is induced during an early stage of the AHSV replication cycle. This is supported by the finding that endosomal acidification inhibitors block virus-induced apoptosis

when present from the onset of infection, but lose their inhibitory capacity when added 1 h p.i. to the cells. Moreover, post-uncoating events are also dispensable for apoptosis induction as was evidenced by the ability of AHSV4 to induce apoptosis in ribavirin-treated cells. Yet, the results shown in Fig. 6 would suggest that such early apoptosis triggering does not have adverse effects on the production of infectious AHSV progeny. This apparently conflicting result can be reconciled considering that orbiviruses have a short replication time, with progeny viruses observed as early as 12 h p.i. in infected BHK-21 cells (Lecatsas & Erasmus, 1967; Lecatsas, 1968). Thus, it is conceivable that the short replication time would allow the virus to advance its life cycle successfully until completion and to reach satisfactory intracellular production of progeny viral particles before the infected cell dies or is severely damaged by the execution of apoptosis. Similar results have recently been reported for avian reovirus (Rodríguez-Grille *et al.*, 2014), indicating that in the case of both these viruses they activate rather than suppress caspases, and that this activation is necessary for efficient virus release and spread.

Although the findings reported here establish a foundation to define the signalling events that culminate in AHSV-induced apoptosis, further studies are needed to understand the molecular and cellular mechanisms that govern AHSV-mediated induction of apoptosis. Increased understanding of the signalling pathways used by AHSV to induce apoptosis may contribute important new information regarding mechanisms by which AHSV produces cell death and disease. However, it will be imperative to investigate caspase activation and the role of apoptosis in AHS disease in susceptible animal hosts.

## METHODS

**Cells and virus.** Baby hamster kidney-21 cells (BHK-21; ATCC CL-10) were cultured at 37°C and 5 % CO<sub>2</sub> in Eagle's minimal essential medium (EMEM) supplemented with Earle's Balanced salt solution, 2 mM L-glutamine, 1 % (v/v) non-essential amino acids, 5 % (v/v) FBS, and antibiotics (penicillin, streptomycin and amphotericin B) (HyClone). *Spodoptera frugiperda* clone 9 insect cells (Sf9; ATCC CRL-1711) were cultured at 27°C in Grace's insect medium supplemented with 10 % (v/v) FBS and an antibiotics/antimycotic solution (streptomycin, penicillin and fungizone) (Lonza).

AHSV serotype 4 (AHSV4) was used for cell infection. BHK-21 cell monolayers were rinsed twice with incomplete EMEM (lacking FBS and antibiotics) and then infected with AHSV4 at m.o.i. 1 p.f.u. per cell. Virus adsorptions were performed at 37 °C for 1 h, followed by incubation of the cell monolayers in complete EMEM.

**DNA fragmentation analyses.** Genomic DNA from BHK-21 cells under the different experimental conditions was extracted with an Apoptotic DNA-Ladder kit (Roche Applied Science) according to the manufacturer's instructions and separated by electrophoresis in 1 % (w/v) agarose gels containing ethidium bromide. These assays were performed at least three independent times and representative results are shown. A Cell Death Detection ELISA<sup>PLUS</sup> kit (Roche Applied Science) was used according to the manufacturer's instructions for the *in vitro* quantification of nucleosomes released into the cytoplasm of cells that died from apoptosis.

**UV inactivation of virus.** AHSV4 was exposed to UV light (312 nm) for 30 min using a UV transilluminator (UVP model M-15). Virus inactivation was verified by a plaque assay on BHK-21 cells (Oellermann, 1970). Confluent BHK-21 cell monolayers were infected with the UV-inactivated virus at m.o.i. 2 p.f.u. per cell, as described above for non-irradiated AHSV4.

**Generation of recombinant baculoviruses.** Recombinant pCR-XL-Topo vectors, harbouring cloned cDNA copies of the VP2 and VP5 genome segments of AHSV4, were kindly provided by Dr W. Fick (Department of Genetics, University of Pretoria). The coding sequence of VP2 was PCR amplified with primers VP2F (GCCGAATTCATGGCGTCCGAGTTTGG) and VP2R (GGCGGTACCTATTCTGTTTTGCGAG). The 3.201 kb amplicon was digested with *EcoRI* and *KpnI* (as underlined), and cloned into the identical sites of the bacmid donor plasmid pFastBac1 (Invitrogen) to generate pFB-VP2. A similar strategy was used to construct the bacmid donor plasmid pFB-VP5, except that primers VP5F (CCC GCGGCGCATGGGAAAGTTCACATC) and VP5R (CCCCTGCAGTCAAGCTATTTTCACACC) were used, and the 1.538 kb amplicon was digested with *NotI* and *PstI* (as underlined) prior to cloning. The nucleotide sequence and orientation of cloned insert DNA was verified by automated sequencing procedures. Recombinant baculoviruses were obtained by means of the Bac-To-Bac baculovirus expression system (Invitrogen) following the manufacturer's instructions. In brief, *Escherichia coli* DH10Bac was transformed with the recombinant pFastBac1 vectors, after which recombinant bacmids harbouring the genes of interest were extracted and then transfected into Sf9 cells with CellFectin reagent (Invitrogen). High-titre stocks of the recombinant baculoviruses were prepared from plaque-purified viruses. Expression of the full-length VP2 and VP5 proteins by the recombinant baculoviruses rBac-VP2 and rBac-VP5, respectively, was confirmed by immunoblot analysis.

**Treatment of BHK-21 cells with AHSV4 outer capsid proteins.** For purification of recombinant VP2 and VP5, suspension cultures of Sf9 cells ( $1.5 \times 10^8$  cells ml<sup>-1</sup>) were infected at m.o.i. 5 p.f.u. per cell with the recombinant baculoviruses. At 72 h p.i. the cells were harvested by low-speed centrifugation, lysed by three successive freeze/thaw cycles and fractionated by centrifugation at 6600 r.p.m. for 30 min at 4°C. The soluble VP2 and VP5 proteins were quantified on Coomassie blue-stained SDS-polyacrylamide gels by comparison to known concentrations of BSA with EZQuant-Gel software version 2.1 (EZQuant).

For treatment of BHK-21 cells with the recombinant VP2 and VP5 proteins, confluent BHK-21 cell monolayers in six-well tissue culture plates ( $1 \times 10^6$  cells per well) were rinsed twice with incomplete EMEM. As VP5 is toxic for cells in high concentrations (Martínez-Torrecuadrada *et al.*, 1994; Stassen *et al.*, 2011) and considering the lesser abundance of VP5 relative to VP2 in intact virions, a lower concentration of VP5 compared with VP2 was used in the assays. Thus, 10 µg VP2 and/or 5 µg VP5 were added to the cells. Protein adsorptions were performed for 1 h, after which the protein preparations were aspirated and replaced with complete EMEM. As a control, a soluble protein extract prepared from WT baculovirus-infected Sf9 cells was used to treat BHK-21 cells.

**Apoptosis inhibitor treatments.** Confluent BHK-21 cell monolayers, grown in six-well tissue culture plates ( $1 \times 10^6$  cells per well), were infected with AHSV4 and the cells were incubated in the presence of 100–400 µM ribavirin (Sigma-Aldrich) for 24 h. Cells were then processed for SDS-PAGE, immunoblot and DNA fragmentation analyses.

The endosomal acidification inhibitors ammonium chloride and chloroquine were both purchased from Sigma-Aldrich. Confluent BHK-21 cell monolayers, grown in six-well tissue culture plates ( $1 \times 10^6$  cells per well), were exposed to 10 mM ammonium chloride or 100 µM chloroquine in EMEM for 2 h prior to infection or 1 h

following AHSV4 infection. After incubation for 24 h, the cell lysates were processed for viral replication by plaque assay and detection of apoptosis by DNA fragmentation analysis. BHK-21 cells were also treated with a VP2/VP5 protein mixture, as described above, in the presence of ammonium chloride and chloroquine at the same concentrations. The cell lysates were then processed for the detection of apoptosis by DNA fragmentation analysis.

The pan-caspase inhibitor z-VAD-FMK [carbobenzoxy-valyl-ananyl-aspartyl-(*O*-methyl)-fluoromethylketone] was purchased from Promega. Confluent BHK-21 cell monolayers in six-well tissue culture plates ( $1 \times 10^6$  cells per well) were rinsed twice in EMEM and then incubated with 10–100 µM z-VAD-FMK for 1 h prior to infection with AHSV4. At 24 h p.i., the cells were harvested and processed for DNA fragmentation analysis. Confluent BHK-21 cell monolayers were also incubated in the presence of 100 µM z-VAD-FMK prior to infection with AHSV4, as described above. At 24 h p.i., the total and extracellular virus titres were determined by plaque assays (Rodríguez-Grille *et al.*, 2014).

**Caspase activity assays.** The activity of caspase-3, -8 and -9 was determined with the appropriate ApoTarget Caspase Colorimetric Protease Assay kit (BioSource International) according to the manufacturer's protocol. Mock-infected and AHSV4-infected BHK-21 cells ( $2.5 \times 10^6$  cells per time point) were harvested, lysed and clarified by centrifugation. The protein concentration of each cytoplasmic extract was determined with a Quick Start Bradford Protein Assay kit (Bio-Rad) and BSA as the standard. Each sample (100 µg protein per sample) was mixed with reaction buffer and 4 mM appropriate substrate (DEVD-pNA for caspase-3, IETD-pNA for caspase-8 and LEHD-pNA for caspase-9), and incubated at 37 °C for 2 h. The liberated pNA was quantified at 405 nm with a Multiscan Ascent version 1.24 ELISA plate reader and the increases in caspase activities calculated.

**Detection of mitochondrial membrane depolarization.** Mitochondrial membrane depolarization was assessed using DePsipher (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; Trevigen) according to the manufacturer's instructions. DePsipher has the property of aggregating upon mitochondrial membrane polarization, forming a red fluorescent compound. If the membrane potential is disrupted, the dye cannot access the mitochondrial transmembrane space and remains in its green fluorescent monomeric form. BHK-21 cells, seeded in six-well tissue culture plates ( $1 \times 10^6$  cells per well), were mock-infected or infected with AHSV4. The cells were harvested at different time points post-infection, suspended in 1 ml DePsipher solution (5 µg ml<sup>-1</sup>) and incubated in the dark for 20 min at 37 °C. The cells were washed twice in  $1 \times$  PBS and resuspended in 1 ml PBS buffer. Finally, cells were analysed with a BD FACSAria flow cytometer (BD Biosciences) and the data analysed with FACSDiva version 6.1.1 software (BD Biosciences). In these assays, BHK-21 cells treated with 20 µM FCCP [carbonyl cyanide *p*-(trifluoro-methoxy) phenylhydrazone] served as a positive control for cells with depolarized mitochondrial membranes (Dispersyn *et al.*, 1999). The green DePsipher monomer was detected using the fluorescein channel (FITC-A; FL1) and the red DePsipher aggregates were detected using the propidium iodide channel (PE-A; FL2). The results are presented as a green/red fluorescence ratio (geomean FL1/FL2), the increase of which indicates mitochondrial membrane depolarization (Markovic *et al.*, 2007; Isakovic *et al.*, 2008).

**Immunoblot analysis of viral proteins.** Proteins were resolved by 10 % SDS-PAGE and transferred to Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech AB) with a semi-dry electroblotter (Hoefer) using standard protocols (Sambrook & Russell, 2001). For detection of expressed AHSV4 outer capsid proteins, polyclonal anti-VP2 and anti-VP5 antibodies (a gift from Dr W. Fick,



Department of Genetics, University of Pretoria) were used. Polyclonal antibodies specific to the NS1, NS2, NS3, VP3 and VP7 proteins of AHSV4 were generated by immunizing rabbits with the following peptides: NS1 aa 487–501 (CKNVLDGRDLTVLED), NS2 aa 202–115 (CLDEAGPSRTPRKLS), NS3 aa 77–91 (CEALRDPEPIRQIKK), VP3 aa 152–165 (ATKDYRGAEIADPEC) and VP7 aa 252–165 (CSLDRSLDTPVELAP). Peptide synthesis and antibody production were carried out by GenScript. Protein-A conjugated to HRP (Sigma-Aldrich) was used as the secondary antibody in all immunoblots.

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