African swine fever virus A238L inhibitor of NF-κB and of calcineurin phosphatase is imported actively into the nucleus and exported by a CRM1-mediated pathway

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This study examined nuclear and cytoplasmic shuttling of the African swine fever virus (ASFV) A238L protein, which is an inhibitor of NF-κB and of calcineurin phosphatase. The results showed that the protein was present in both the nucleus and the cytoplasm in ASFV-infected cells and that the higher molecular mass 32 kDa form of the A238L protein was the predominant nuclear form, which accumulated later in infection. In contrast, both the 28 and 32 kDa forms of the A238L protein were present in the cytoplasm. The A238L protein was actively imported into the nucleus and exported by a CRM1-mediated pathway, although a pool of the protein remained in the cytoplasm and did not enter the nucleus. By using a recombinant ASFV from which the A238L gene had been deleted, it was shown that expression of A238L did not inhibit nuclear import of the NF-κB p50 or p65 subunit and did not inhibit nuclear export of p65 by a CRM1-mediated pathway. The results were consistent with a model in which A238L functions within both the nucleus and the cytoplasm.

INTRODUCTION

African swine fever virus (ASFV) is a large cytoplasmic DNA virus that replicates primarily in macrophages in vivo and in macrophages and endothelial cells in vitro. ASFV causes an acute haemorrhagic fever in domestic pigs but inapparent infections in its natural hosts — warthogs, bushpigs and ticks of the species Ornithodoros moubata and Ornithodoros erraticus. In these hosts, and in domestic pigs, which recover from infection with less-virulent isolates, the virus can persist over long periods. The virus genome varies from about 170 to 192 kbp and encodes between 160 and 175 proteins. ASFV shares similarities in replication strategy with poxviruses but is classified in a separate family, the Asfarviridae (Dixon et al., 2005). Estimates indicate that about 90 poxvirus proteins are required for virus replication in cells, suggesting that ASFV may require a similar number (Upton et al., 2003). Other virus-encoded proteins include several with roles in evading host defence systems (Dixon et al., 2004).

One of these, the A238L protein, functions by several different mechanisms to interfere with signalling pathways in infected macrophages. The first functions demonstrated for A238L were inhibition of NF-κB activation and inhibition of calcineurin phosphatase activity. As a consequence, calcineurin-activated pathways, such as activation of the NFAT (nuclear factor of activated T cells) transcription factor are inhibited. More recently A238L has been shown to inhibit TNF-α expression through a CREB-binding protein (CBP)/p300 transcriptional co-activators pathway (Granja et al., 2004b; Miskin et al., 1998, 2000; Powell et al., 1996; Revilla et al., 1998). By interfering with the activation of several different host factors, A238L is predicted to inhibit transcriptional activation of a broad spectrum of host response genes. So far, A238L expression has been shown to inhibit transcription of the COX-2 gene and production of prostaglandins and to inhibit transcriptional activation of the TNF-α gene. This could reduce the inflammatory response to ASFV and help prevent virus elimination (Granja et al., 2006). Interestingly, deletion of the A238L gene from the genome of a virulent ASFV isolate did not reduce the virulence of the virus for domestic pigs (Neilan et al., 1997), suggesting either that the pathways inhibited by A238L do not play a critical role in virus pathogenesis or that the virus encodes other complementary genes which may compensate for the loss of A238L.

Mapping of the domain in the A238L protein required to bind to calcineurin has identified a 14 aa peptide located between residues 200 and 213, which binds with high affinity to calcineurin but does not inhibit its phosphatase activity. A
critical motif in this peptide, PxxITxCS/S, is similar to calcineurin-docking motifs in the NFAT substrate and other cellular inhibitors of calcineurin (Aubareda et al., 2006; Martinez-Martinez & Redondo, 2004). A purified recombinant peptide containing the C-terminal 82 aa of A238L alone was sufficient to act as a potent inhibitor of calcineurin (C. C. Abrams, R. Silk, D. Chapman & L. K. Dixon et al., unpublished results; Miskin et al., 2000).

The A238L protein contains ankyrin repeats in the centre of the protein, which most closely resemble those in the IxBz protein, which acts as an inhibitor of NF-κB activation. This data suggests that, like IxB proteins, A238L may bind directly to NF-κB, inhibiting its activity. Further support for this model comes from experiments showing that expression of A238L inhibits NF-κB-dependent gene transcription and that A238L is co-precipitated from cell extracts with the p65 subunit of NF-κB (Revilla et al., 1998). Moreover, addition of A238L recombinant protein to nuclear extracts from activated cells showed that A238L inhibited binding of p65/p50 NF-κB heterodimers to κB-binding sequences and displaced preformed complexes from these sequences. Together, these data suggest that A238L can act as a homologue of IxBz. However, a direct interaction between A238L and p65 has not been demonstrated and it remains possible that A238L acts by a different mechanism to inhibit NF-κB. It has also been shown that A238L is recruited into a complex with p65 only after signal-induced degradation of IxB, suggesting that A238L does not displace IxB from a complex with NF-κB but instead is incorporated into a complex after degradation of IxB (Tait et al., 2000). Two different forms of the A238L protein have been detected with molecular masses of 28 and 32 kDa; these two forms are thought to result from post-translational modification, but the nature of the modification has not been defined. The 32 kDa form of A238L has been shown to co-precipitate with p65 (Tait et al., 2000).

The IxB family of proteins consists of at least seven members (IxBz, IxBβ, IxBc, p105, p100, IxBζ and Bcl-3), which contain a central domain with five to seven ankyrin repeats. These act by several mechanisms to inhibit the activity of NF-κB (Hayden & Ghosh, 2004; Israel, 2000). The crystal structures of IxBz and IxBz bound to NF-κB p65/p50 heterodimers show that the ankyrin repeats of IxB make multiple contacts with NF-κB to block both the DNA-binding site and the nuclear localization signal (NLS) of p65 (Huxford et al., 1998; Jacobs & Harrison, 1998; Malek et al., 2001, 2003). However, the exposed NLS on the p50 subunit coupled with a nuclear export signal (NES) present on both IxBz and p65 result in constant nuclear–cytoplasmic shuttling of NF-κB/IxB complexes, although the steady-state localization is in the cytoplasm (Huang et al., 2000; Johnson et al., 1999). Signal-induced degradation of IxB alters the dynamics of this shuttling, leading to nuclear accumulation of NF-κB and activation of NF-κB-dependent gene transcription. Transcription of IxB genes is activated by NF-κB and this provides a negative feedback loop to regulate NF-κB activation. Newly synthesized IxB enters the nucleus forming a complex with NF-κB, which leads to its displacement from target DNA sequences and export from the nucleus (Huang & Miyamoto, 2001; Israel, 2000). Although previous data have suggested that A238L may function within the nucleus, one study showed that, at 8 h post-infection (p.i.), A238L was found exclusively in the cytoplasm in a complex with NF-κB. This suggested that A238L may inhibit NF-κB activity by retaining the complex in the cytoplasm (Tait et al., 2000). However, detailed studies of the nuclear–cytoplasmic shuttling of A238L throughout a time course of infection have not been carried out. In this study, we showed that A238L is actively transported into the nucleus, with larger amounts present at late times p.i. We also demonstrated that a proportion of A238L is exported from the nucleus by a CRM1-mediated pathway. In addition, we showed that A238L does not inhibit nuclear import or export of p65.

**METHODS**

**Viruses and cells.** African green monkey (Cercopithecus aethiops) kidney (Vero) cells were maintained in Dulbecco’s modified Eagle’s medium containing 25 mM HEPEs and supplemented with 2 mM glutamine, 50 U penicillin ml⁻¹, 50 µg streptomycin ml⁻¹ and 5 % bovine fetal calf serum (FCS; PAA Laboratories GmbH).

ASFV strains were the BA71V tissue-culture-adapted ASFV isolate and recombinants of this isolate, which either had the simian virus 5 (SV5) Pk epitope tag (V5 tag) fused to the 5’ end of the A238L gene (SV5Gal) or had the coding region of A238L deleted (vIKGal). These viruses contained the β-galactosidase gene under the control of the ASFV p72 promoter inserted at the site of recombinant (Miskin et al., 1998).

**Virus infection.** Cells were washed and virus was added at an m.o.i. of 5 in serum-free medium. After incubation for 1.5 h at 37 °C, virus was removed. Cells were then washed twice and cultured in medium with FCS for various lengths of time as indicated.

**Preparation of cell extracts.** Cells were washed in PBS, harvested by scraping and pelleted by centrifugation. To prepare nuclear and cytoplasmic fractions, cells were resuspended in 200 µl pre-chilled fractionation buffer [150 mM Tris/HCl (pH 8.8), 10 mM KCl, 1 mM EDTA, 0.2 % NP-40, 10 % glycerol, 1 mM PMSE, 1 µg small protease inhibitors (leupeptin, pepstatin, antipain and chymostatin) ml⁻¹] and incubated on ice for 10 min. The suspension was then centrifuged for 5 min at 13,200 r.p.m. in a benchtop centrifuge to pellet the nuclei. The supernatant was removed and added to 200 µl SDS-PAGE sample buffer [100 mM Tris/HCl (pH 6.8), 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol, 100 mM dithiothreitol]. The nuclear pellet was then harvested in 50 µl SDS-PAGE sample buffer and the DNA sheared by repeated passage through a 21-gauge needle. To prepare total extracts, cell pellets were resuspended in SDS-PAGE sample buffer and the DNA sheared as described above. To confirm that fractions were not cross-contaminated, parallel blots were probed with antibody against histone H1 as a nuclear marker or against α-tubulin as a cytoplasmic marker.

**Western blotting.** Proteins were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in blocking solution [3 % milk powder in PBS with 0.1 % Tween 20] at 4 °C overnight or at room temperature for 3 h. Membranes were incubated with antibodies in blocking solution for 1 h at room temperature and then washed in PBS plus 0.05 %
Tween 20, before incubation with HRP-conjugated protein A (ab7456-1, diluted 1:2000; Abcam). Bound antibodies were detected by enhanced chemiluminescence (ECL). Digital images of gels were collected using a Bio-Rad GS-710 densitometer.

Indirect immunofluorescence. Cells were grown on glass coverslips to approximately 90% confluence and then transfected for 4 h using Lipofectamine 2000 (Invitrogen). Following transfection, cells were washed in serum-free medium and then, where appropriate, infected with ASFV following the above protocol. At the appropriate times p.i., the coverslips were washed with PBS, fixed in 4% paraformaldehyde for 30 min and permeabilized for internal staining with PBS containing 0.2% Triton X-100 (Sigma) for 15 min. Cells were blocked in PBS containing 3% BSA (Sigma) for 1 h and then incubated with primary antibody diluted in PBS/3% BSA. V5-tagged proteins were detected with mouse anti-Pk tag (diluted 1:100; Serotec) and p65 was detected using anti-p65 (sc-372, diluted 1:200; Santa-Cruz). After 30 min, the cells were washed three times in PBS plus 0.2% Tween 20 (Sigma) and then incubated with secondary antibodies diluted 1:300 in PBS/3% BSA for 30 min. Primary mouse antibodies were detected using Alexa 488-conjugated goat anti-mouse IgG and rabbit polyclonal serum was detected using Alexa 568-conjugated goat anti-rabbit IgG secondary antibody (Molecular Probes). Cells were washed twice in PBS/0.2% Tween and three times in PBS. The coverslips were mounted onto slides with Vectashield containing DAPI (Vector Laboratories). The cells were visualized using a Leica TCS SP2 confocal laser scanning microscope. Data were collected sequentially to prevent cross-talk between detection channels and were analyzed using LCS (Leica Confocal Software). The images shown are representative of three independent experiments.

RESULTS

A238L accumulates in the nucleus at late times p.i.

The subcellular localization of the 28 and 32 kDa forms of the A238L protein at various times p.i. was investigated. To do this, Vero cells were infected with a recombinant ASFV (SV5Gal) in which the wild-type A238L gene had been replaced with an A238L gene containing the Pk tag from SV5 fused to the 5’ end. This fusion has previously been shown not to affect either function of A238L (Miskin et al., 1998).

At various times p.i., cell extracts were separated into nuclear and cytoplasmic fractions. These fractions were separated by SDS-PAGE, blotted onto nitrocellulose membranes and probed with an antibody against the Pk tag to detect the A238L protein. As shown in Fig. 1(a), both the 28 and 32 kDa forms of the A238L protein were detected early in infection (3 or 4 h p.i.). At early times, most of the A238L protein was present in the cytoplasm, although overexposure of the gels showed that small amounts of A238L could be detected in the nucleus from 3 h p.i. (Fig. 1b). Increasing amounts of A238L could be detected in the nuclear fraction from 16 h p.i., reaching a maximum at 16–24 h p.i. Slightly more of the 28 kDa form of A238L was detected at early times p.i. compared with the 32 kDa form, but from 16 h p.i. onwards, the 32 kDa form of the protein was the main form detected, particularly in the nuclear fraction. The protein detected in the nucleus at late times p.i. ran slightly more slowly than the form detected in the cytoplasm, raising the possibility that it had been modified further. Parallel infected cultures were incubated for 16 h in the presence of cytosine arabinoside, an inhibitor of virus DNA replication and late virus gene expression. Similar amounts of both forms of the A238L protein were detected in the nuclear and cytoplasmic fractions compared with cultures incubated without cytosine arabinoside (Fig. 1c).

This showed that most of the A238L protein was synthesized early in infection and that late virus gene expression was not required for nuclear accumulation of A238L or for the increase in the relative amount of the 32 kDa form at late times p.i.

Fig. 1. Localization of the A238L protein in different subcellular fractions during ASFV infection. (a) Vero cells were infected with SV5Gal. At various times after infection, cells were harvested and separated into nuclear and cytoplasmic fractions. Proteins from each fraction were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-Pk tag antibodies followed by HRP-conjugated anti-mouse secondary antibodies. Bound antibodies were detected by ECL. The time (h p.i.) at which extracts were harvested is indicated. (b) Overexposure of extracts harvested 3 h p.i. (c) Extracts harvested from cells at 16 h p.i. in the presence (+) or absence (−) of cytosine arabinoside. N, Nuclear fraction; C, cytoplasmic fraction.
A238L is exported from the nucleus by a CRM1-mediated pathway

To investigate whether A238L was exported from or retained in the nucleus, the subcellular localization of A238L was examined in the presence of leptomycin B (LMB), a specific inhibitor of the CRM1-dependent nuclear export pathway (Huang et al., 2000). Levels of A238L detected in infected cells by immunofluorescence were low and difficult to quantify. Therefore, we transfected Vero cells with a plasmid containing a Pk-tagged A238L gene under the control of the A238L promoter. Cells were then infected with ASFV isolate BA71V. Three hours prior to fixation, cells were treated or not with LMB in the presence or absence of PMA and LPS. Cells transfected with a plasmid containing Pk-tagged IxBz were used as a positive control for LMB: IxBz contains a CRM1-dependent NES and has previously been shown to accumulate exclusively in the nucleus in the presence of LMB (Huang et al., 2000). These cells were treated with PMA/LPS to activate NF-κB and enhance IxB nuclear import. At 8 and 16 h p.i., cell cultures were fixed, permeabilized and stained with an antibody specific to the Pk tag, followed by a fluorescently labelled secondary antibody. Cells were then examined using confocal microscopy and the subcellular localization of A238L was determined.

As shown in Fig. 2(a), in cells stimulated with PMA/LPS in the presence of LMB, all of the IxB was present in the nucleus as expected. In the absence of LMB, although most IxB was in the nucleus, a detectable amount was present in the cytoplasm. This is consistent with the nuclear export of IxB via the CRM1-mediated pathway and showed that the LMB was functionally active.

In ASFV-infected cells expressing A238L under the control of its own promoter, A238L exhibited a distribution that could be characterized into three distinct patterns of subcellular localization: preferentially nuclear, a uniform distribution throughout the cytoplasm and nucleus, or preferentially cytoplasmic [see Fig. 2b(i)]. One hundred cells were scored for these three cellular distributions under each experimental condition and in three independent experiments. In the presence of LMB, at both 8 and 16 h p.i., there was a significant increase in the number of cells exhibiting a predominantly nuclear localization compared with in the absence of LMB (~50 % compared with <10 %) [Fig. 2b(ii)]. This demonstrated that at least a proportion of the nuclear A238L was exported to the cytoplasm using the CRM1-dependent nuclear export pathway. In cells treated with LMB, A238L was predominantly present in the nucleus in more cells; nevertheless, in approximately half of the cells

![Fig. 2. A238L is exported from the nucleus by a CRM1-mediated pathway. (a) Vero cells were transfected for 16 h with a plasmid expressing Pk-tagged IxBz. Three hours prior to fixation, cells were treated with LPS (10 μg ml⁻¹) and PMA (100 nM) in the presence or absence of LMB (40 nM). (b) BA71V-infected Vero cells expressing Pk-tagged A238L under the control of its own promoter. Three hours prior to fixation, cells were treated with or without LPS and PMA in the presence or absence of LMB, as above. At 8 or 16 h p.i., cells were fixed and stained. In both (a) and (b), Pk-tagged proteins were detected with anti-Pk antibody, followed by Alexa 488-conjugated goat anti-mouse IgG secondary antibody, and imaged using confocal microscopy. The subcellular localization of Pk-tagged A238L was characterized as preferentially nuclear (N), both nuclear and cytoplasmic (N/C) or preferentially cytoplasmic (C). Representative images are shown (i). For each condition tested, 100 cells were counted and the percentages of cells exhibiting N, N/C and C distributions were determined. Cells were examined in the absence (ii) or presence (iii) of PMA/LPS, with or without LMB as indicated. Data presented is the mean (±SD) of three independent experiments. Bars, 10 μm.](image-url)
treated with LMB, A238L exhibited an even distribution of A238L throughout the nucleus and cytoplasm. It is possible that a proportion of A238L was either not imported into the nucleus and remained in the cytoplasm, or, if it was imported into the nucleus and then exported, a CRM1-independent nuclear export pathway may have been involved.

Infection of cells is likely to activate a number of signalling pathways and this probably explains why no difference was observed in the distribution of A238L in PMA/LPS-treated cells compared with untreated cells. The Western blot results shown in Fig. 1 indicated that at 9 h p.i. most A238L was in the cytoplasm. In contrast, the results from the immunofluorescence experiments shown in Fig. 2 suggested that similar amounts of A238L were present in the nucleus at 8 compared with 16 h p.i. One possible explanation is that protein expression was higher from the plasmid transfected into infected cells in the immunofluorescence experiments. The complex interactions of A238L with components of the NF-kB and calcineurin signalling pathways and with nuclear import and export machinery mean that altering expression levels of A238L may alter its distribution. Nevertheless, our key conclusion regarding the nuclear export of A238L remained valid.

Nuclear import of A238L occurs by an energy-dependent process

Both forms of A238L are less than 60 kDa, which is the diffusion exclusion limit of the nuclear pore complex. To determine whether A238L is imported into the nucleus by an active process, cells were treated with cycloheximide to block protein synthesis and then incubated at 37 or 4 °C. At 4 °C, all active transport processes are blocked, whereas diffusion is unaffected. In the majority of cells at 4 °C, A238L was found to be evenly distributed between the nucleus and cytoplasm, both in the presence and absence of LMB. However, when cells were incubated at 37 °C, the results were dramatically different and A238L exhibited a predominantly nuclear localization in almost 50% of the cells examined (Fig. 3). This suggested that, although passive diffusion may play a role in the nuclear import of A238L, an additional active nuclear transport process is involved.

This comparison also showed that there was no significant difference in the distribution of A238L in cells treated with cycloheximide compared with untreated cells (see Fig. 3).

As inhibition of protein synthesis did not prevent the nuclear accumulation of A238L, we concluded that a pool of pre-existing cytoplasmic A238L was imported into the nucleus.

Expression of A238L does not inhibit nuclear translocation of NF-κB

To investigate whether expression of A238L inhibited nuclear translocation of NF-κB, we compared nuclear translocation of the p65 and p50 subunits of NF-κB in cells infected with ASFV BA71V isolate expressing the Plk-tagged A238L gene (SV5Gal) or a recombinant BA71V isolate from which the A238L gene had been deleted (vIKGal). Deletion of the A238L gene has been shown previously not to affect virus replication in cells and, as demonstrated in Fig. 4(b), the patterns of virus protein synthesis were not significantly different during a time course of infection with these viruses. To determine whether the total cellular pool of the p65 and p50 proteins was altered following infection with SV5Gal or vIKGal virus, total cell extracts were separated by SDS-PAGE, blotted and probed with antibody against p65 or p50. No change was detected in the total cellular pool of p50 during infection with either virus (Fig. 4a). In contrast, an increase in the amount of p65 was detected at 3 h p.i. Total amounts of p65 decreased at 6 and 9 h p.i. but increased again at 12 and 18 h p.i. These results suggested that regulation of the total cellular pool of p65 may contribute to regulation of NF-κB activity during ASFV infection. However, similar changes in amounts of p65 were observed in cells infected with both wild-type BA71V and the deletion mutant lacking A238L, suggesting that A238L expression does not regulate the amount of p65.
At various times after infection, cells were fractionated into nuclear and cytoplasmic extracts and these were separated by SDS-PAGE, blotted onto nitrocellulose membranes and probed with antibodies that recognized either the p50 or the p65 NF-κB subunit. Fig. 5(a) shows that in cells infected with SV5Gal, which expressed A238L, the p65 subunit of NF-κB was detected in both nuclear and cytoplasmic fractions throughout infection. Larger amounts were detected in the nucleus at late times (16–24 h p.i.). At early times, the p50 subunit was detected almost exclusively in the cytoplasm but was detected in both nuclear and cytoplasmic fractions at late times (16–24 h p.i.). Fig. 5(b) shows the distribution of the p65 and p50 subunits following infection with the vIKGal ASFV A238L deletion mutant. There was no significant difference in the distribution of p65 and p50 between the nucleus and cytoplasm when results were compared with those from cells infected with virus expressing A238L. At 16 and 24 h p.i., an additional 55 kDa form of p65 was detected in the nucleus. The appearance of this band is consistent with cleavage of p65 late in infection by caspase-3 (Kang et al., 2001), which has been shown to be induced in ASFV-infected cells (Nogal et al., 2001).

Fig. 6 shows cells that were infected with ASFV and stained with antibodies against an early ASFV protein, p30 (green), and the p65 subunit of NF-κB (red); nuclei are shown in blue. Translocation of p65 to the nucleus was observed in cells that were stained with the anti-p30 antibody. There was no difference in the amount of p65 in the nucleus of uninfected cells that did not show positive staining with the anti-p30 antibody, demonstrating that nuclear translocation of p65 was not inhibited in cells infected with ASFV. To determine whether expression of A238L inhibited nuclear export of p65, we compared localization of p65 in cells infected with BA71V or vIKGal ASFV in the presence and absence of LMB. The results showed that, as expected, treatment of cells with LMB resulted in an increase in the percentage of cells showing a predominantly nuclear localization of p65. In the absence of LMB, an increase in the amount of cytoplasmic p65 was observed, showing that p65 is exported from the nucleus to the cytoplasm by a
CRM1-mediated pathway. No difference was observed in the cells infected with the two viruses, demonstrating that A238L does not inhibit nuclear export of p65.

**DISCUSSION**

The A238L protein of ASFV inhibits activation of the NF-κB transcription factor and the phosphatase activity of calcineurin. In addition, A238L has been shown to inhibit transcription from the TNF-α promoter by a mechanism involving the CBP/p300 transcriptional co-activators. The A238L protein thus has the potential to act as a potent immunosuppressant by inhibiting transcriptional activation of the many immune response genes regulated by these factors (Miskin et al., 1998, 2000; Powell et al., 1996; Revilla et al., 1998). Evidence to date has shown that, in a T-cell line, A238L inhibition of cyclo-oxygenase 2 (COX2) gene transcription is dependent on an NFAT element within the gene promoter and involves inhibition of the p300 transcriptional co-activator (Granja et al., 2004a). Similarly, inhibition of TNF-α expression has been shown to involve inhibition of CBP/p300 function (Granja et al., 2006). The NF-κB and NFAT pathways are targeted by other viruses (Bowie et al., 2004; Camus-Bouclainville et al., 2004; Li et al., 2004; Scott et al., 2001), although A238L is the only protein known to combine both of these functions in a single protein.

Little is known of the detailed mechanism by which A238L functions. Previous work has suggested that the protein may function in both the cytoplasm and the nucleus (Granja et al., 2006; Revilla et al., 1998; Tait et al., 2000), but a detailed study of the location and cytoplasmic nuclear shuttling of A238L during ASFV infection has not previously been carried out.

The similarity between the ankyrin repeats of A238L and those of members of the IκB family suggests that A238L may make direct contact via these ankyrin repeats with NF-κB subunits. The observation that A238L can be co-precipitated with the NF-κB p65 subunit supports this hypothesis, although direct contact between A238L and p65 has not been demonstrated. IκB proteins contain between five and seven ankyrin repeats, and crystallographic structures of IκBa and IκBb in complex with p65/p50 NF-κB heterodimers show that the IκB ankyrin repeats make multiple contacts with NF-κB, blocking both the NLS and DNA-binding domain on the p65 subunit (Huxford et al., 1998; Jacobs & Harrison, 1998; Malek et al., 1998, 2003). In contrast, A238L contains only four ankyrin repeats, and protein structure modelling (D. Chapman & L. K. Dixon, unpublished data), based on the structure of IκB in complex with NF-κB, suggests that A238L may block the DNA-binding site but not the nuclear localization signal on p65, and vice versa.

In this study, we have shown that A238L is imported into the nucleus by an active process in virus-infected cells. Western blotting of nuclear and cytoplasmic cell extracts suggested that the 32 kDa higher molecular mass form of A238L predominantly accumulated in the nucleus at later times post-infection. Previous data has shown that this form of the protein is co-precipitated with the NF-κB p65 subunit, suggesting that, particularly at late times p.i., A238L functions within the nucleus to inhibit NF-κB. The difference between the two forms of A238L has been suggested to be due to post-translational modification of the

![Fig. 6. p65 nuclear import and export is not inhibited in ASFV-infected cells.](http://vir.sgmjournals.org)
protein, although the nature of the modification has not been defined (Tait et al., 2000). Possible explanations for accumulation of the 32 kDa form of A238L in the nucleus are that the modification to the protein takes place in the nucleus or that this modification is required for nuclear translocation of the protein. NLSs on A238L have not been defined to date. However, sequence analysis of A238L using PSORT (Horton et al., 2006), which predicts possible subcellular localization sites, has identified two putative NLSs. These consist of single, short stretches of amino acids highly enriched in basic residues. One of these is located at the N terminus towards the tail end of the first ankyrin repeat domain and the second is located at the C terminus within the PxIxTxxC/S calcineurin-binding domain. A proportion of the A238L protein is also present in the cytoplasm. We showed a greater nuclear accumulation of A238L in cells when LMB was added to inhibit CRM1-mediated nuclear export. Thus, we concluded that A238L is exported actively from the nucleus by a CRM1-mediated pathway. No NESs were identified and it is not known whether A238L interacts directly with the nuclear export pathways or translocates in association with another protein that contains a NES.

Our experiments indicated that the cytoplasmic A238L protein comprises both molecules that do not enter the nucleus and molecules that are actively exported from the nucleus. If the higher molecular mass 32 kDa form of A238L shuttles between the nucleus and cytoplasm, this could explain why both forms are present in the cytoplasm. The 28 kDa form of A238L is not co-precipitated with p65, suggesting that this may be the form of the protein that binds to and inhibits calcineurin (Tait et al., 2000). If the predicted NLS within the calcineurin-docking motif in the A238L protein is functional, then this would be masked when A238L binds to calcineurin. Hence, this A238L–calcineurin complex would be predicted to remain in the cytoplasm. Calcineurin is not thought to contain specific NLSs and instead is thought to shuttle into the nucleus in a complex with other proteins such as NFAT (Crabtree & Olson, 2002).

Our data also showed that expression of A238L did not inhibit nuclear translocation of the NF-κB p50 and p65 subunits. This is consistent with a model whereby A238L may act within the nuclei to inhibit binding of NF-κB to DNA. Previous experiments have shown that recombinant A238L, when added to nuclear extracts from stimulated cells, inhibits binding of NF-κB to κB enhancer sequences and can also displace preformed NF-κB transcription complexes from DNA (Revilla et al., 1998).

p65 can be exported from the nucleus either using NESs contained in the p65 protein or as part of a complex with IκB utilizing export signals on IκB (Huang et al., 2000). Our data showed that A238L did not inhibit nuclear export of p65, although we did not define the form in which p65 is exported in ASFV-infected cells.

Accumulating evidence suggests that IκB proteins play critical roles within the nucleus in regulating NF-κB-dependent gene transcription. IκBζ acts within the nucleus and can either inhibit or activate NF-κB transcriptional activity. IκBζ binds to the p50 subunit of NF-κB in both p50/p50 and p50/p65 NF-κB dimers. Transcription of the IκBζ gene has been shown to be activated in response to IL-1 and signalling through Toll-like receptors. Evidence from IκBζ-deficient cells shows that IκBζ is required for transcriptional activation of IL-6 and a number of other immune response genes (Yamamoto et al., 2004; Yamazaki et al., 2001). Evidence to date suggests that A238L acts as an inhibitor of NF-κB-dependent gene transcription. However, we cannot exclude the possibility that A238L may also have some role in activating gene transcription. This may be revealed by global transcription profiling.

As both NF-κB and calcineurin are important targets for immunomodulatory drugs, there is considerable interest in defining how a single protein can mediate inhibition of both of these pathways. Understanding how the two functions of A238L are regulated is also important for understanding its role during ASFV infection.

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