Mechanism of Inactivation of NF-κB by a Viral Homologue of IκBα

SIGNAL-INDUCED RELEASE OF IκBα RESULTS IN BINDING OF THE VIRAL HOMOLOGUE TO NF-κB*

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Activation of the nuclear factor κB plays a key role in viral pathogenesis, resulting in inflammation and modulation of the immune response. We have previously shown that A238L, an open reading frame from African swine fever virus (ASFV), encoding a protein with 40% homology to porcine IκBα exerts a potent anti-inflammatory effect in host macrophages, where it down-regulates NF-κB-dependent gene transcription and proinflammatory cytokine production. This paper reveals the mechanism of suppression of NF-κB activity by A238Lp. A238Lp is synthesized throughout infection as two molecular mass forms of 28 and 32 kDa, and vaccinia-mediated expression of A238L demonstrated that both proteins are produced from a single gene. Significantly, the higher 32-kDa form of A238Lp, but not the 28-kDa form, interacts directly with RelA, the 65-kDa subunit of NF-κB, indicating that the binding is dependent on a post-translational modification. Immunoprecipitation analysis shows the NF-κB p65-A238L p32 heterodimer is a separate complex from NF-κB-IκBα, and it resides in the cytoplasm. Moreover, we show that ASFV infection stimulates the NFκB signal transduction pathway, which results in the rapid degradation of endogenous IκBα, although both forms of A238Lp are resistant to stimulun-induced degradation. Using the proteasome inhibitor MG132, we show that when degradation of IκBα is inhibited, A238Lp binding to NF-κB p65 is reduced. The results suggest that the virus exploits its activation of the NF-κB pathway to enable its own IκB homologue to bind to NF-κB p65. Last, we show that synthesis of IκBα is increased during ASFV infection, indicating RelA-independent transcription of the IκBα gene.

The NF-κB family of transcription factors is involved in regulation of the expression of numerous cellular genes involved in the immune response, inflammation and apoptosis (reviewed in Ref. 1). The NF-κB/Rel family is composed of homodimeric and heterodimeric complexes of the Rel family of proteins, which include RelA (p65), NF-κB1 (p50), NF-κB2 (p52), c-Rel, and RelB (2). The heterodimer of p50 and p65 is the most common form of NF-κB dimer. In resting cells, it is present in an inactive form bound to one of a family of IκB proteins, ensuring that proinflammatory gene expression is turned off. The family of IκBs, characterized by structural ankyrin repeat motifs that bind NFκB, includes IκBα, IκBβ, IκBε, the C-terminal ends of NF-κB precursors p100/p105, the Bcl3 protein, and the Drosophila protein Cactus (2–4). Upon cell stimulation, IκBα is first phosphorylated by IκB kinase, a large multisubunit complex (5–7); it is then ubiquitinated and degraded by the 26 S proteasome, allowing NFκB to translocate to the nucleus and bind target κB sites (reviewed in Ref. 8). Activated NFκB is then responsible for the rapid induction of cellular anti-viral activity.

Viruses exploit diverse and complex strategies to counteract the host response to infection. Large DNA viruses encode proteins that are nonessential for viral replication but that can inhibit a range of cellular functions, including blocking of cytokines and their receptors (reviewed in Ref. 9), the cytotoxic immune defense (10), or proteins that regulate cellular signaling (11). A novel strategy has been elucidated for the porcine virus, African swine fever virus (ASFV).1 ASFV is a large double-stranded DNA virus that infects macrophages, and virulent isolates cause a fatal hemorrhagic disease of pigs (12, 13). In addition to hemorrhage, the pathology is characterized by lymphoid tissue destruction due to apoptosis (14). We found that secretion and transcription of proinflammatory cytokines were inhibited in ASFV-infected macrophages (15). The ASFV genome of 170–180 kilobase pairs was sequenced (16) and revealed at least 150 open reading frames, one of which, designated A238L in the BA71V isolate, has ankyrin repeat motifs that bind NFκB precursors p100/p105, the Bcl3 protein, and the Drosophila protein Cactus (16). Interestingly, we found that expression of the A238L gene alone inhibited NFκB-dependent gene transcription and prevented NFκB binding to its cognate κB target sequence (15).

In this study, we investigated the mechanism of inhibition of NFκB by A238Lp. We show here that there are two forms of A238Lp synthesized throughout infection. One is the 28-kDa protein predicted from the ORF; the second is a higher molecular mass 32-kDa form produced by post-translational modification. Significantly, the higher molecular mass 32-kDa form and not the 28-kDa form bound NFκB p65, thus demonstrating that the post-translational modification was required to inhibit the NFκB pathway. We also show that, in common with many viruses (reviewed in Ref. 17), infection with ASFV acti-

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1 The abbreviations used are: ASFV, African swine fever virus; ORF, open reading frame; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; PAGE, polyacrylamide gel electrophoresis; RANTES, regulated on activation normal T cell expressed; IL, interleukin; hpi, hours postinfection; TNFα, tumor necrosis factor α; SUMO, small ubiquitin-related modifier.
vated the NF-κB signal transduction pathway. Remarkably, ASFV exploits the resulting signal-induced degradation of IκBα to enable its own IκBα homologue to bind NF-κB p65. The result is a A238Lp-NF-κB heterodimer that forms an inactive complex in the cytoplasm that is not subject to signal-induced degradation. Furthermore, ASFV infection was found to increase IκBα synthesis, although NF-κB p65 activity was inhibited, thus supporting previous studies showing RelA-independent control of IκBα gene transcription (18–20).

**EXPERIMENTAL PROCEDURES**

**Cells and Viruses—**Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Vero cells were infected with the tissue culture adapted strain of ASFV, BA71V, at a multiplicity of infection of 5:1 for 1 h and then incubated in complete medium for the indicated times. In some experiments, cells were stimulated with 10 μM phorbol 12-myristate 13-acetate (PMA) and 10 μg/ml lipopolysaccharide (LPS). In experiments where proteasome inhibitor was used, MG132 (Calbiochem) was added at 25 μM to the medium after the initial 1-h infection for the rest of the incubation period (7 h).

**Western Blot Analysis—**Cells and Viruses—Cells and Viruses—Cells and Viruses—Cells were infected for 1 h at 37°C with the MVA-T7 virus expressing T7 RNA polymerase was grown in baby hamster kidney cells (21).

**Antibodies and Plasmids—**Peptides corresponding to the N and C terminus of A238Lp (MEHMPFPEREIQVFVKWIKHRRGNLTLF and VFFRWFKPKKPIIITGCWNNYVEKLPEQNP, respectively) were conjugated to maleimide-activated KLH and OVA and used to raise antibodies in rabbits. Two antibodies raised to the N-terminal peptide were characterized as cross-reacting with A238Lp and termed N1 and N2. One antisera was raised to the C-terminal peptide cross-reacted with A238Lp and termed C1. Rabbit anti-NF-κB p65 antisum was against an N-terminal peptide (SC109) was used for Western blot analysis and, where indicated, for immunoprecipitation, and a goat antibody against a C-terminal peptide of NF-κB (SC-372-G) was used in separate immunoprecipitations (Santa Cruz, CA). IκBα was detected by Western blot with a monoclonal antibody (SC-1643) and by immunoprecipitation by rabbit polyclonal antibody (SC 203). Antibody recognizing ERα was raised in rabbits as described previously (22). Normal rabbit serum was from Sigma. Anti-ASFV vpo was from Dan Rock (USD, Plum Island Animal Disease Center). A238Lp in pcDNA3 was as described previously (15). NF-κB p50 and IκBα cDNAs in RCmV was from Heike Pahl (University of Freiberg).

**Vaccinia Virus/T7 RNA Polymerase-mediated Expression of A238L—**BSC40 cells were infected for 1 h at 37°C with the MVA-T7 strain of vaccinia (21) expressing bacteriophage T7 RNA polymerase. Cells were washed in serum-free medium and transfected with pA238L or pcDNA3 alone (Invitrogen) using SuperFect (Qiagen). Cells were lysed in immunoprecipitation buffer 24 h post-transfection.

**Metabolic Labeling and Immunoprecipitation—**Cells (2 × 10⁶) were preincubated with methionine- and cysteine-free Eagle’s medium for 30 min at 37°C. They were pulse-labeled for 60 min using [35S]methionine (Du Pont NEN) or [35S]cysteine (Du Pont NEN). The characteristics of the virus IκB homologue, A238Lp, were followed during infection by raising antibody to peptides representing the N and C termini of the protein encoded by the open reading frame. One antibody, termed N2, recognized denatured protein and was used in Western blotting experiments to follow the time course of A238Lp expression during infection (Fig. 1A). A238Lp was first detected 2 h after infection with levels rising over 16 h. Interestingly, the antibody recognized two forms of A238Lp throughout the infectious cycle. One form migrated at 28 kDa, the molecular mass anticipated from translation of the A238L reading frame. The second form migrated at 32 kDa, suggesting post-translational modification of the protein or alternative splicing of the gene. When the A238L gene was expressed transiently from a T7 promoter using a recombinant vaccinia virus expressing T7 RNA polymerase (MVA-T7), two forms of the protein were again identified at 32 and 28 kDa by Western blot analysis (Fig. 1C, lane 3). These bands were not seen in uninfected cells (lane 1) or in cells transfected with vector alone (lane 2). Since both forms of the protein were expressed from a single cDNA, we concluded that the 32-kDa form was a post-translational modification of the faster migrating 28-kDa form. Moreover, since the two forms occur in transfected cells in the absence of the rest of the ASFV genome, the modification is either the result of an endogenous host cell process or, more unlikely, caused by a vaccinia virus-encoded protein.

Further antibodies were identified that immunoprecipitated A238Lp from infected cell lysates. Specifically, one antibody (C1) raised against the C terminus of A238Lp immunoprecipitated only the 28-kDa form of the protein, while a second antibody (N1) raised against the N terminus recognized only the 32-kDa form (Fig. 1B). Both antibodies failed to recognize A238Lp by Western blotting, suggesting that they recognize conformational epitopes. Importantly for this study, the antibodies could be used to distinguish between the two forms of A238Lp.

**The 32-kDa Form of A238L Coprecipitates with the 65-kDa Subunit of NF-κB—**Given the homology between A238Lp and IκBα, the potential association of the A238L gene product with NF-κB was analyzed. Control cells or cells infected with ASFV for 8 h prior to the experiment were pulse-labeled for 60 min, lysed in detergent, and immunoprecipitated with antibody specific for a peptide representing either the N terminus or the C
terminus of the 65-kDa subunit of NF-κB (Fig. 2A). The NF-κB p65 N-terminal peptide antibody immunoprecipitated a 65-kDa protein from uninfected cells and a second protein at 37-kDa shown below to be IκBα (Fig. 2A, lane 3) (a nonspecific band is seen in all lanes at 50 kDa). Significantly, when the immunoprecipitation was repeated for infected cells, a third protein was seen at 32 kDa (Fig. 2A, lane 4). The 32-kDa protein comigrated with the higher molecular weight form of A238Lp immunoprecipitated from the cell lysates with N1 anti-A238Lp antibody (Fig. 2A, lane 1). A 28-kDa protein was not seen in the NF-κB immunoprecipitate, even though substantial levels of the 28-kDa form of A238Lp could be recovered from the lysates using the p28-specific antibody, C1 (Fig. 2A, lane 2). An antibody recognizing the C terminus of p65 immunoprecipitated only two proteins, NF-κB p65 and IκBα, at 37 kDa from both uninfected (Fig. 2A, lane 5) and infected cells (Fig. 2A, lane 6).

The proteins at 65, 37, and 32 kDa were identified as NF-κB p65, IκBα, and A238L p32 by immunoprecipitation followed by Western blot analysis (Fig. 2B). Vero cell lysates that had been immunoprecipitated with either anti-IκBα antibody (lane 1) or anti-p65 antibody (lane 2) or an irrelevant control antibody of anti-IκB kinase antibody (lane 3) or the anti-A238Lp antibody N2 (lane 4) were transferred onto nitrocellulose. The upper half of the membrane was probed with an anti-p65 antibody, while the lower half was probed with either a monoclonal antibody to IκBα or the anti-A238Lp antibody, N2. Western blot analysis confirmed that the 37-kDa protein was IκBα, which co-precipitated a 65-kDa protein detected with anti-NF-κB p65 antibody (lane 1). In a reciprocal manner, immunoprecipitation with NF-κB p65 antisum (lane 2) followed by Western blot analysis with NF-κB p65 antibody (upper panel) and IκBα antibody (middle panel) also identified the 65-kDa/37-kDa complex as NF-κB p65 and IκBα. When immunoprecipitates of p65 were generated from ASFV-infected cells and analyzed by Western blot using antibody N2, which detects both forms of A238Lp, a 32-kDa band was seen (lane 2, bottom panel), thus identifying the 32-kDa protein as A238L p32. A control immunoprecipitation with anti-IκB kinase antibody (Fig. 2B, lane 3) demonstrated that the Western blotting was specific. When immunoprecipitates of A238Lp were generated and transferred to nitrocellulose and the membrane was probed with the N2 anti-A238Lp antisum, both A238L p28 and A238L p32 were seen (lane 4, bottom panel); however, Western blotting of the A238L immunocomplex with anti-p65 did not detect NF-κB p65 (lane 4, top panel). Interestingly, therefore, this result suggests that either N2 did not recognize A238Lp complexed to p65 or that the antibody displaced p65 from A238L p32.

A sequential reimmunoprecipitation approach was taken to confirm the identity of the 32-kDa protein associated with NF-κB p65 in infected cells (Fig. 2C). Lysates from an equal number of infected cells were immunoprecipitated with either N1 anti-A238Lp antisum (lane 1) or anti-p65 antisum (lane 2). The complex of three proteins (p65, IκBα, and p32) in the anti-p65 immunoprecipitate were dissociated at room temperature with 1% deoxycholate and reprecipitated with the N1 antibody specific for the 32-kDa form of A238Lp (Fig. 2C, lane 4). A single band at 32 kDa was detected, confirming the identity of the viral protein precipitating with p65 as the higher molecular weight form of A238Lp. A control using normal rabbit serum to reimmunoprecipitate the complex is shown (lane 5).

Surprisingly, a comparison of lanes 1 and 2 of Fig. 2C showed that more A238L p32 was recovered from lysates with anti-p65 antibody (lane 2) than when the same lysate was immunoprecipitated with excess N1 antibody specific for A238L p32 (lane 1). The results suggested that most of the A238Lp in cells was bound to NF-κB p65 and that the epitope on A238Lp recognized by the anti-N-terminal peptide antibody was masked by NF-κB p65. This was consistent with two observations: first, that increased levels of A238L p32 could be recovered from the complex with p65 if they were first dissociated in deoxycholate and then reprecipitated (comparing Fig. 2C, lanes 1 and 4); second, p65 is not detected by Western blot of an N2 immunoprecipitate (Fig. 2B, lane 4).

An experiment was performed to investigate whether a trimolecular complex existed between p65, IκBα, and A238L p32 (Fig. 2D). Lysates from Vero cells infected for 8 h with ASFV were immunoprecipitated with an excess of anti-IκBα antiserum, revealing proteins at 37 kDa (IκBα), 65 kDa (p65), and 70 kDa (perhaps other Rel proteins) but not at 32 kDa (Fig. 2D, lane 1). This result indicated IκBα bound p65 separately from A238L p32. The supernatant from this IκBα-depleted lysate
was then immunoprecipitated with NF-κB p65 antiserum and showed two major proteins at 65 and 32 kDa and a reduced amount of IκBα at 37 kDa (Fig. 2D, lane 2). NF-κB p65-IκBα complexes were much reduced but not completely absent from this sample, probably due to low affinity of the IκBα antibody (as seen in Fig. 2B, lane 1). These results indicate that A238L p32 bound to NF-κB p65 that was not complexed with IκBα. In infected cells, therefore, NF-κB p65 exists as two pools, either complexed to IκBα or to A238L p32 (Fig. 2D, lane 3).

Cytoplasmic Localization of the NF-κB p65-A238L p32 Complex—The localization of the NF-κB p65-A238L p32 complex was determined in infected cells after pulse labeling and fractionation into cytoplasm and nuclei. Nuclear or cytoplasmic fractions were immunoprecipitated with either N1 anti-A238L antiserum (lane 1) or anti-NF-κB p65 antiserum (lane 5), identifying the 32-kDa protein as A238L p32. The same immunocomplex was reimmunoprecipitated with normal rabbit serum as a control (lane 5). D, A238L p32 binds to NFκB p65 that is not complexed to IκBα. Vero cells infected for 8 h with ASFV were metabolically labeled, and the lysate was immunoprecipitated with excess anti-IκBα antiserum (lane 1). The supernatant from this IκBα-depleted lysate was then immunoprecipitated with anti-p65 antiserum (lane 2). Lysate immunoprecipitated with anti-p65 antiserum alone is shown in lane 3. A238L p32 does not co-immunoprecipitate with IκBα that is complexed to NF-κB p65.

**Fig. 2.** NFκB p65 selectively coprecipitates the 32-kDa form of A238Lp. A, a protein of 32-kDa co-precipitates with NFκB p65 in ASFV-infected cells. Metabolically labeled Vero cell lysates were immunoprecipitated with antisera to either A238Lp (lanes 1 and 2) or NFκB p65 (lanes 3–6). At 8 hpi, A238L p32 was detected with N1 (lane 1), and A238L p28 was detected with C1 (lane 2). NFκB p65 was detected with anti-N-terminal p65 peptide antibody in uninfected cells (lane 3) and at 8 hpi (lane 4). NFκB p65 was detected anti-C-terminal p65 peptide antibody in uninfected cells (lane 5) and at 8 hpi (lane 6). A 32-kDa protein selectively co-precipitated with p65 with the N-terminal antisera at 8 hpi (lane 4) but not the C-terminal antisera (lane 6). This 32-kDa protein comigrated with A238L p32 seen in lane 1. B, identification of NFκB p65, IκBα, and A238L p32, respectively, using Western blot analysis. Vero cell lysates were immunoprecipitated with antibody to IκBα (lane 1), NFκB p65 (lane 2), anti-IκBα kinase antibody as an irrelevant control (lane 3), or anti-A238L antisera, N2 (lane 4), and then blotted onto nitrocellulose. Top, the membrane was probed with anti-NFκB p65 antiserum. Middle, Western blot with anti-IκBα. Bottom, Western blot with anti-A238L N2 antisera. C, identification of A238L p32 bound to NFκB p65 by sequential reimmunoprecipitation. Vero cells infected for 8 h with ASFV were pulse-labeled and lysed, and equal numbers of cells were immunoprecipitated with either N1 anti-A238L antisera (lane 1) or anti-NFκB p65 serum (lane 2). The sample from lane 2 was dissociated from the protein A beads with 1% deoxycholate (lane 3) and then reimmunoprecipitated with N1 anti-A238L antisera (lane 4), identifying the 32-kDa protein as A238L p32. The same immunocomplex was reimmunoprecipitated with normal rabbit serum as a control (lane 5). D, A238L p32 binds to NFκB p65 that is not complexed to IκBα. Vero cells infected for 8 h with ASFV were metabolically labeled, and the lysate was immunoprecipitated with excess anti-IκBα antisera (lane 1). The supernatant from this IκBα-depleted lysate was then immunoprecipitated with anti-p65 antisera (lane 2). Lysate immunoprecipitated with anti-p65 antisera alone is shown in lane 3. A238L p32 does not co-immunoprecipitate with IκBα that is complexed to NFκB p65.

**Fig. 3.** Subcellular localization of A238Lp and A238Lp bound to NFκB p65. Cells infected with ASFV for 8 h were fractionated into nuclei (N) and cytoplasm (C). Fractions were immunoprecipitated with N1, N2, or NFκB p65 antibodies, and proteins were separated by SDS-PAGE. Both forms of A238Lp were found in the cytoplasm. NFκB p65 was only found in the cytoplasm complexed to A238L p32.
pared with uninfected cells and were increased toward normal by proteasome inhibition. These results show that ASFV infection itself stimulates IkBa degradation by proteasomes. Significantly, therefore, the signal transduction pathway leading to IkB kinase activation and resulting in IkB phosphorylation is not blocked during ASFV infection, since signal-induced phosphorylation of IkB is a prerequisite for its degradation.

Synthetic rates of IkBa were measured in infected cells by metabolic labeling and immunoprecipitation with anti-IkB antiserum (Fig. 4A, top panel). IkBa synthesis decreased in cells after proteasome inhibition (lane 2), showing that the synthesis of IkBa is dependent on its own degradation (25). Interestingly, the results show that synthesis of IkBa was increased in ASFV-infected cells (lane 3) compared with uninfected cells (lane 1), and this increase could be blocked by proteasome inhibition (lane 4). Although ASFV inhibits p65 activity, other members of the Rlf family have been shown to up-regulate the IkBa promoter activity (18). IkBa is also transcriptionally regulated by transforming growth factor-β (19), a cytokine that has been shown to be up-regulated in ASFV infection (15). The results demonstrate a complex regulation of the IkBa gene, which is under further investigation. The high synthetic rate of IkBa together with its low steady-state level in infected cells indicates that ASFV infection provides a potent signal for IkBa degradation.

One explanation for the observed suppression of NF-κB gene transcription by A238L protein could be the formation of a complex with A238Lp where the viral protein is resistant to proteasomal degradation. The relative stabilities of IkBa and the two forms of A238Lp in response to cell stimulation were therefore tested. Vero cells, either uninfected or infected with ASFV for 8 h, were incubated with cycloheximide for 1 h either in the presence or absence of PMA/LPS (Fig. 4B). Levels of both IkBa and A238Lp were detected by Western blotting with an anti-IkB antibody (top panel) and the N2 antibody that recognizes both forms of A238Lp (middle panel). In control experiments in uninfected cells, cycloheximide treatment showed that the half-life of IkBa was about 1 h (Fig. 4B, lanes 1 and 2), and IkBa was completely degraded after stimulation with PMA/LPS (Fig. 4B, lane 3) as expected (26). In infected cells, steady state levels of IkBa were lower than those in uninfected cells (Fig. 4B, lane 4), and IkBa was undetectable after the cycloheximide chase in the presence or absence of PMA/LPS (Fig. 4B, lanes 5 and 6) degradation. Both forms of A238Lp were stable during cell activation with PMA/LPS, indicating resistance to stimulus-induced degradation (Fig. 4B, middle panel, lanes 4–6). A control showing equal lane loading and transfer to nitrocellulose is shown with an abundant resident IkB protein, ERp60 (Fig. 4B, bottom panel).

A238Lp Replaces IkBa bound to NF-κB p65—The previous results point to the conclusion that the virus exploits signal-induced degradation of IkBa to expose NF-κB for binding to the IkB homologue. A238Lp then forms a stable complex with NFκB in the cytoplasm, preventing the transcription factor from moving into the nucleus and activating gene expression. In the next experiment, the requirement of proteasome activity for the replacement of IkBa with the viral A238L protein was tested by incubating cells with the proteasome inhibitor MG132 (Fig. 5). Cells were pulse-labeled, and lysates were immunoprecipitated with anti-p65 antibody (Fig. 5A, IP). Significantly, incubation of cells with MG132 decreased the levels of A238L p32 bound to p65. The immunoprecipitate also showed an apparent reduction in the amount of IkBa associated with p65 in MG132-treated cells, confirming the result in Fig. 4A, where immunoprecipitation with IkBa antibody detected decreased IkBa synthesis after proteasomal inhibition. The levels of IkBa bound to p65 were analyzed by Western blot of the complex precipitated with anti-p65 using an IkBa monoclonal antibody (Fig. 5A, WB). The Western blot gave the predicted result that there was more IkBa associated with p65 in MG132-treated cells.

To investigate whether MG132 had an inhibitory effect on A238Lp synthesis, metabolically labeled cell lysates were immunoprecipitated with the N1 anti-A238Lp antibody (Fig. 5B). In fact, levels of A238L p32 were slightly increased in the presence of MG132. It has been shown above (Fig. 2C) that the N1 epitope is masked in the p65-A238Lp complex. The result shown in Fig. 5B suggests that there was more free A238Lp
available in MG132-treated cells, supporting the idea that inhibition of IkBa degradation blocks formation of the A238Lp-p65 complex. Levels of another viral protein, vp30, an early viral gene product, were unchanged in the presence of MG132 (Fig. 5B). Taken together therefore, these results show that proteolytic removal of IkBa from p65 was required for the binding of A238Lp to p65.

**Analysis of the Post-translational Modification on A238L p32**—Throughout these experiments, we have shown that only the 32-kDa form of A238L bound to NF-kB p65. Clearly, the post-translational modification providing the extra 4 kDa was important for A238Lp to inhibit NF-kB p65. Given the functional and sequence homology between IkBa and A238Lp, we investigated the possibilities of phosphorylation, ubiquitination, and sumoylation (27) to explain the increased molecular weight seen for A238L p32.

For phospholabeling analysis, cells were incubated in phosphate-free medium containing [32P]orthophosphate for 5 h, and cell lysates were immunoprecipitated with either antibody N1 or antibody to vp30, the major phosphoprotein of ASFV as a positive control (Fig. 6). Antibody N1 did not detect any proteins labeled with [32P]orthophosphate in uninfected cells (lane 1) or 8-hpi cells (lane 2), although N1 readily detected A238Lp in cell lysates labeled with [35S]methionine/cysteine at 8 hpi (lane 3). In the control experiment, anti-vp30 mAb recognized the ASFV phosphoprotein from 32P-labeled ASFV-infected cell lysates (lane 4).

Uninfected and ASFV-infected cell lysates, either immunoprecipitated with NF-κB p65 or the whole cell lysate, were separated by SDS-PAGE, blotted to nitrocellulose, and analyzed with anti-ubiquitin and anti-SUMO antibodies. Although many ubiquitinated and SUMO-modified proteins were detected in whole cell lysates, there was no specific band seen for A238Lp in infected cells (data not shown). Also, Western blotting of an NF-κB p65 immunoprecipitate that coprecipitated A238Lp did not detect any ubiquitinated or SUMO-containing protein at 32 kDa (data not shown). These experiments indicated that the modification of A238L p32 is not due to either ubiquitin, SUMO, or phosphate addition to A238L p28.

**A238Lp Inhibits NF-κB-dependent Cytokine Gene Expression in a Macrophage-like Cell Line**—A238Lp was first identified as a putative IkBa homologue because it inhibited an NF-κB-dependent reporter construct and NF-κB binding to DNA in a porcine kidney cell line (15). However, since the primary site of ASFV replication in vivo is the macrophage (14), a functional analysis of A238Lp expression was carried out in a macrophage cell line, RAW 264.7, using macrophage-specific cytokine promoter-reporter constructs co-transfected with A238Lp. This would more closely represent the effect on gene expression of A238Lp binding to p65 found in the infected host cell in vivo. TNFα, IL-8, and RANTES genes are rapidly up-regulated through the NF-κB signal transduction pathway in activated macrophages. The upstream promoters of these genes cloned upstream of reporter cDNAs were transfected into RAW 267 cells. A 2–3-fold increase from each of the IL-8, RANTES, and TNFα promoters was recorded when RAW cells were stimulated with PMA/LPS (Fig. 7A). Since macrophage cell lines are inherently difficult to transfect, with only 5–10% of cells ex-
pressing the transgene, reporter levels were lower than nor-
mally expected from other cell lines. When cells were co-
transfected with the A238Lp gene, basal and PMA/LPS-stimulated
activities were inhibited from IL-8, RANTES, and TNFα pro-
moters (Fig. 7A). The experiment shown is a representative of
the results obtained in three separate experiments. A plasmid
encoding NF-κB p65 co-expressed with the IL-8 promoter
transactivated gene expression, but when the A238L gene was
also expressed, transactivation was inhibited. This experiment
supports the findings that A238Lp influences p65 directly
rather than stepping upstream of NF-κB activation. As a con-
trol, an IL-8 promoter mutated at the NF-κB binding site
showed no activity, indicating that the increases were totally
dependent on the NF-κB binding to its cognate site. In a second
control of specificity of NF-κB inhibition, AP1-dependent tran-
scription was unaffected by A238L (Fig. 7A). These results
show that the viral IκB homologue specifically inhibited sev-
eral promoters containing NF-κB sites driving the expression
of macrophage-specific proinflammatory genes.

The previous experiments demonstrated that in infected
cells the endogenous IκBα associated with the p65 was re-
placed by the viral protein A238Lp (Fig. 5). It was also shown
that the viral homologue was resistant to activation-induced
degradation, suggesting that binding to p65 was long lived (Fig.
4B). Taken together, the results suggested that sustained bind-
ing of A238Lp to p65 would lead to prolonged inactivation of
the protein. The reversibility of inactivation of NF-κB by either
IκBα or A238Lp was therefore compared using the NF-κB-de-
pendent reporter assay (Fig. 7B). In the control, stimulation of
cells for 120 min led to a 2–3-fold increase in IL-8 promoter
activity. Activity was then measured in cells coexpressing the
IL-8 promoter plasmid with genes for IκBα (IL8 + IκBα) or A238L (IL8 + A238L). Cells were stimulated
with PMA/LPS for 0, 30, 60, or 120 min, and luciferase activity was measured (RLU). Increased luciferase activity demonstrated that inhibition
with IκBα was diminished after 60 min, whereas A238L inhibition was sustained over 2 h.

DISCUSSION

This study has probed the molecular mechanism for the
inhibition of the pro-inflammatory response of macrophages
during infection by African swine fever virus. We show that the
ASFV-encoded protein, A238Lp, with homology to IκBα, binds
directly to the p65 subunit of NF-κB. Immunoprecipitation and Western blotting studies showed that infected cells synthesize two forms of A238Lp: a smaller form that migrated at 28 kDa, a size predicted from the A238L reading frame, and a larger form that migrated at 32 kDa. We assume that the larger form is post-translationally modified, because transfection of the cDNA for A238L into cells results in expression of two proteins, but we do not know the nature of the modification. Significantly, the post-translational modification activated A238Lp, since only the 32-kDa form of the viral protein bound to NF-κB. Furthermore, the virus activates the NF-κB signal transduction pathway, and this is exploited by the virus to replace the endogenous IκBα with its own IκB homologue.

During activation of cells, IκB is targeted for degradation, allowing transport of the NF-κB into the nucleus (28, 29). The simplest model for the action of the viral IκB homologue would be for the viral protein to bind NF-κB but be resistant to signal-induced degradation. Essentially, the protein would act as a dominant negative inhibitor of NF-κB by retaining the protein in the cytoplasm. Several lines of evidence support this model. First, both forms of A238Lp were stable in cells activated by PMA and LPS, conditions that stimulated the rapid degradation of IκBα. The viral protein was not therefore subject to stimulus-induced degradation. Interestingly, since both forms of A238Lp were stable, resistance to degradation is an inherent property of the viral protein and does not result from the post-translational modification that promotes binding of A238Lp to NF-κB p65. Second, the cytoplasmic localization of the A238L-p65 complex suggests that A238Lp prevents translocation of p65 into the nucleus in a manner analogous to IκBα. Functional evidence for compromised nuclear translocation of NF-κB by A238Lp comes from gel shift experiments, demonstrating that in cells expressing the protein, levels of NF-κB DNA binding activity are reduced following stimulation (15).

Unlike IκBα, which can also inhibit NF-κB activity by enhancing export of nuclear NF-κB (30, 31), our experiments suggest that A238Lp does not act in the nucleus.

This study showed that, in common with many viruses, ASFV infection activated the NF-κB signal transduction pathway. Interestingly, when degradation of IκBα was blocked by MG132, greatly reduced levels of A238Lp were bound to NF-κB. We think that the virus exploits the signal-induced degradation of IκBα to expose NF-κB for binding to the viral IκB homologue. Immunoprecipitation of infected cell lysates with anti-IκBα antiserum failed to detect a trimolecular complex involving IκBα, p65, and A238Lp, supporting the idea that a separate pool of NF-κB p65 exists bound as a heterodimer with A238Lp. It also suggests that A238Lp binds to the same domain of RelA as IκBα, but this question needs to be addressed using deletion or point mutations of p65. It is also of interest that an anti-C-terminal p65 antibody did not precipitate the complex, suggesting that it might have displaced A238L, p32. It has been established that IκBα binds to NF-κB through ankyrin repeat domains in the central region together with a PEST domain in the C terminus of the molecule (32, 33). The central region of A238Lp shows the highest homology to the ankyrin repeats 3–6 in IκBα, and it may bind through this domain to p65.

Interestingly, although this study has shown an increased degradation of IκBα during ASFV infection, it has also demonstrated an increased synthesis of IκBα in the presence of NF-κB p65 inhibition by A238Lp. An increase in IκBα expression following a decrease in NF-κB binding has been seen in other systems, for example, after transforming growth factor-β treatment of B cell lymphomas (19). ASFV infection has been previously shown to increase transforming growth factor-β synthesis (15), so this cytokine may account for IκBα transcriptional activation. Alternatively, during infection, there may be increases in other transcriptional activators of IκBα, such as heat shock proteins (20) or other Rel family members (18) that are not inhibited by A238Lp. Given the complexity of this posttranslational modification pathway, these possibilities are being investigated further.

One of the unique outcomes of this study is that we were unable to detect binding of the 28-kDa form of A238Lp to NF-κB. The viral protein, therefore, has homology with IκBα, yet binding to NF-κB requires post-translational modification by the host cell. Furthermore, it appears that the post-translational modification acts as a molecular switch whereby the virus can inhibit two signal transduction pathways with one protein. It has been shown recently, using A238Lp as bait in a yeast two-hybrid screen of a pig macrophage library, that the 28-kDa molecular mass form of A238Lp interacts with cyclophilin and the small subunit of calcineurin (protein phosphatase 2B) (34), thus inhibiting the nuclear factor of activated T cells pathway. A238Lp is therefore a remarkable viral protein with two forms affecting different signal transduction pathways, both of which have the potential to play an important role in immune evasion. The interaction between calcineurin and the NF-κB pathway has yet to be defined, but interestingly, a recent report showed that calcineurin activity suppresses NF-κB activation in macrophages (35).

Unfortunately, we have been unable to determine the nature of the modification. Given the structural and functional homology with IκBα, we investigated whether modifications known for IκBα were found on A238Lp. The presence of two lysines at positions 19 and 20 in the predicted amino acid sequence of A238Lp were similar in position to two lysine residues at amino acids 21 and 22 that are targets for ubiquitination in IκBα (36, 37). Western blot analysis with anti-ubiquitin antibodies did not, however, detect the 32-kDa form of A238Lp in lysates from infected cells, suggesting that A238Lp is not ubiquitinated. These results were consistent with the lack of degradation of A238Lp in cells stimulated by PMA and LPS. Phosphorylation of neighboring serine residues at positions 32 and 36 in IκBα by IκB kinase acts as a signal for ubiquitin ligase recognition (38). Notably, these serines are absent from analogous positions in A238Lp. This raised the possibility that A238Lp was modified by SUMO-1, a reaction that occurs at lysine 21 of IκBα (38). We were however unable to demonstrate a SUMO-1 modification to A238Lp. Analysis of the sequence of A238Lp predicts three possible consensus sites for phosphorylation: a casein kinase II site, a protein kinase C site, and one tyrosine phosphorylation site. Even so, phosphate labeling experiments and immunoprecipitation with isofrom-specific antibodies followed by phosphatase digestion showed that there was no phosphate modification.

Although we do not know the precise modification, it is possible to predict the mechanism of activation of A238Lp. Antibodies raised against the extreme C and N terminus of A238Lp were able to differentiate between the two forms of the protein. At first, this was difficult to reconcile with the prediction that anti-peptide antibodies should be conformation-independent and bind both forms of the protein. The selective binding of C-terminal specific antibody to the smaller 28-kDa form could be explained if the epitope were blocked by the post-translational modification. However, the selective binding of the N-terminal specific antibody to the 32-kDa form suggests that this epitope is masked in the 28-kDa protein. We favor a model where post-translational modification of A238Lp produces a large conformational change that exposes the N terminus of the protein for binding to NF-κB. This would explain why the
antibody raised against this region of the protein recognizes only A238L p32 not bound to p65.

It has been well established that activation of NF-κB protects cells from apoptosis (39, 40). One would therefore expect ASFV-infected cells that inhibit NF-κB to be more sensitive to activation-induced cell death (41). This would not be an advantage to the virus that needs to replicate and spread. However, the ASFV genome encodes two proteins with anti-apoptotic function, a Bcl2 homologue (42) and an Iap homologue (43), which may counteract the effect of NF-κB inhibition.

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