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African Swine Fever Virus IAP-Like Protein Induces the Activation of Nuclear Factor Kappa B

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African swine fever virus (ASFV) encodes a homologue of the inhibitor of apoptosis (IAP) that promotes cell survival by controlling the activity of caspase-3. Here we show that ASFV IAP is also able to activate the transcription factor NF-κB. Thus, transient transfection of the viral IAP increases the activity of an NF-κB reporter gene in a dose-responsive manner in Jurkat cells. Similarly, stably transfected cells expressing ASFV IAP have elevated basal levels of c-*rel*, an NF-κB-dependent gene. NF-κB complexes in the nucleus were increased in A224L-expressing cells compared with control cells upon stimulation with phorbol myristate acetate (PMA) plus ionomycin. This resulted in greater NF-κB-dependent promoter activity in ASFV IAP-expressing than in control cells, both in basal conditions and after PMA plus ionophore stimulation. The elevated NF-κB activity seems to be the consequence of higher IκB kinase (IKK) basal activity in these cells. The NF-κB-inducing activity of ASFV IAP was abrogated by an IKK-2 dominant negative mutant and enhanced by expression of tumor necrosis factor receptor-associated factor 2.

The NF-κB transcription factors are a ubiquitously expressed family of proteins involved in the regulation of many immune and inflammatory functions. NF-κB is composed of homo- or heterodimers of Rel family members (3, 10). In most cells, NF-κB factors are present in an inactive form in the cytoplasm of resting cells, retained through complex formation with a family of inhibitors, the IκB proteins (IκBα, IκBβ, and IκBε) (3, 10).

NF-κB activation results from the phosphorylation of two conserved serines at the N terminus that renders IκB susceptible to proteolysis via the ubiquitin proteosome pathway (31, 40). After the degradation of IκB, NF-κB can translocate to the nucleus to activate gene expression. The signal-induced phosphorylation of IκB is accomplished by an IκB kinase (IKK) complex that contains two kinases, IKK-1 and IKK-2, as well as several scaffolding proteins (19, 42). The IKKs are activated by signals derived from the tumor necrosis factor receptor (TNF-R), which may involve other kinases, such as NIK (18), MEKK-1, -2, and -3 (17), and PK-Cζ (30).

TNF is a pleiotropic cytokine inducing a variety of biologic responses (for a review, see reference 38). Binding of TNF to its receptors TNF-R1 and TNF-R2 generates two types of signals, one that initiates programmed cell death and another that leads to activation of the transcription factor NF-κB (1). TNF-induced cell death is mediated primarily by TNF-R1 due to the presence of an 80-amino-acid region known as the death domain, which is absent in TNF-R2 (32, 35), whereas TNF-R2 is mostly involved in cell activation, cell survival, and antiapoptotic functions (34). Both TNF-R1 and TNF-R2 can stimulate NF-κB activation (9, 15, 16, 36). It is generally accepted that the overall outcome of death versus survival depends on the balance of NF-κB and apoptotic signals.

Thus, activation of NF-κB can inhibit apoptotic cell death (2) and appears to play a protective role in TNF-mediated apoptosis by activating the transcription of several antiapoptotic genes, including the inhibitor of apoptosis (IAP) (12), A20 (14), and Bcl-X and Bcl-2 (5, 33, 43).

Cellular proteins of the family of baculovirus IAPs, including several human IAP relatives, have been shown to interfere with apoptotic processes (8). Part of this ability is mediated by their direct inhibition of caspase activity, but they can also mediate their antiapoptotic activity by their ability to activate NF-κB (6). c-IAP1 and c-IAP2 specifically inhibit the active forms of caspase-3 and caspase-7 and also block the etoposide-induced processing of caspase-3. c-IAP2, whose expression is activated by NF-κB, cannot prevent TNF-mediated apoptosis in cells in which NF-κB is inhibited (6). It is also known that v-Rel, the viral homologue of the NF-κB family member c-Rel, induces expression of an avian form of IAP (41). Furthermore, c-IAP1 and c-IAP2 can associate with TNF-R2, although they cannot bind it directly. They are recruited to the receptor by binding to TNF receptor-associated factor 1 (TRAF-1) and TRAF-2 heterocomplexes, being therefore involved in NF-κB activation by TNF (27).

African swine fever virus (ASFV) is a large DNA virus that infects different species of suids, causing an acute and frequently fatal disease (37). Infection by ASFV is characterized by the absence of a neutralizing immune response, which has prevented the development of a conventional vaccine. The analysis of the complete 170-kb DNA sequence of this virus has revealed several genes capable of modulating host response (39). Among these, A224L encodes a 27-kDa protein homologous to IAP members (4) which inhibits caspase-3 activation both in IAP-transfected cells and during viral infection by interaction with the catalytic fragment of caspase-3 (20). Using stably or transiently transfected cells, we show here that

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ASFV IAP (A224L) is also able to activate NF-κB, its effect being dependent on IKK activity.

This is the first description of a viral IAP-like gene that is able to activate NF-κB that not only helps explain the ability of ASFV to modulate the immune response by regulating NF-κB activation either positively or negatively (23, 24) but also helps clarify the molecular mechanisms of NF-κB activation.

MATERIALS AND METHODS

Cells and reagents. Jurkat cells were obtained from the American Type Culture Collection and grown in RPMI 1640 medium (Gibco, Rockville, Md.), supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco, Rockville, Md.) and containing 2 mM L-glutamine plus 100 μg of streptomycin and 100 U of penicillin per ml and nonessential amino acids, at 37°C in a 7% CO_2 in air saturated with a water vapor incubator.

Recombinant TNF-α was purchased from Genzyme (Cambridge, Mass.). Phorbol myristate acetate (PMA) and calcium ionophore A23187 were purchased from Sigma (St. Louis, Mo.).

Antisera from rabbits hyperimmunized with peptides derived from human c-Rel (antibody 265), anti-p65 antibody, and NF-κB essential modulator (NEMO) antibody 3328 were kindly provided by Nancy Rice (National Cancer Institute Cancer Research and Development Center [NCI-FCRDC], Frederick, Md.).

Plasmids. The pNF3TK Luc reporter plasmid contains a 3× tandem repeat of the NF-κB-binding motif of the *H-2k* gene upstream of the thymidine kinase minimal promoter and the luciferase reporter gene The AP-1-Luc plasmid includes the AP-1-responsive (−73 to +63 bp) region of the human collagenase promoter fused to the luciferase gene.

Expression plasmids encoding either wild-type or a dominant negative mutant of IKK-2 were kindly provided by J. Moscat (Centro de Biología Molecular Severo Ochoa, Madrid, Spain), and the TRAF-2 expression vector plasmid was kindly provided by B. Seed (21). pcDNA-IAP was generated by cloning the A224L open reading frame into the pcDNA3 mammalian expression vector (Invitrogen).

Transfections. Jurkat T cells were transfected with 1 μg of specific plasmid per 10^6 cells using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were treated or not with TNF-α (10 ng/ml) or PMA (10 ng/ml) plus calcium ionophore (1 μM) for 6 h. They were then lysed with passive cell culture lysis reagent (Promega, Madison, Wis.) and microcentrifuged at full speed for 5 min at 4°C, and 20 μl of each supernatant was used to determine firefly luciferase activity in a Monolight 2010 luminometer (Analytical Luminiscence Laboratory). Results were expressed as the fold increase in luminescence relative to the value obtained with the nonstimulated control after normalization of protein concentration, determined by the bicinchoninic acid (BCA) spectrophotometric method (Pierce, London, United Kingdom). For normalization of transfection efficiency, cells were cotransfected with 100 ng of the reporter plasmid pTK-Renilla (Promega, Madison, Wis.), and luciferase activity was recorded using the dual luciferase assay (Promega, Madison, Wis.). Results are always expressed as values normalized to *Renilla* activity.

For G418 selection, cells were transfected with 0.5 μg of pcDNA3 or pcDNA3-A224L as described above. Two days later, antibiotic selection was applied (1 mg of G418 [Sigma] per ml). Cells were refed with fresh medium every 3 days until colonies were apparent (2 to 3 weeks).

RT-PCR analysis of A224L mRNA expression. Total RNA was isolated by using the Trizol reagent (Gibco) following the manufacturer's recommendations. First-strand cDNA was obtained from 1 μg of total RNA (previously denatured by heating for 2 min at 65°C and placed on ice) with 10 μl of reverse transcriptase reaction mixture containing 10× Moloney murine leukemia virus reverse transcription (RT) buffer (Epicentre), 10 mM dithiothreitol, 0.5 mM oligo(dT), a 50 μM concentration of each of the four deoxyribonucleoside triphosphates, 12.5 U of Moloney murine leukemia virus reverse transcriptase, and 20 U of RNasin (Promega). The reaction mixtures, in a final volume of 50 μl with RNase-free water, were incubated for 1 h at 37°C. For PCR, a variable amount of the cDNA obtained (5 μl) was used in a total volume of 50 μl of a PCR mixture. PCR amplification was carried out with 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 90 s, and extension at 72°C for 2 min. PCR products were electrophoresed on 1% agarose gels containing ethidium bromide.

Western blot analysis. Cells were washed twice with phosphate-buffered saline (PBS) and lysed in TNT buffer (20 mM Tris-HCl [pH 7.6], 200 mM NaCl, 1% Triton X-100) supplemented with protease inhibitor cocktail tablets (Roche). Protein concentration was determined by the BCA method. Cell lysates (50 μg of protein) were separated in a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel, blotted onto an Immobilon extra membrane (Amersham), and reacted with specific primary antibodies. Membranes were exposed to horseradish peroxidase-conjugated secondary antibodies (Dako), followed by chemiluminescence (ECL; Amersham) detection by autoradiography.

Solid-phase in vitro phosphorylation kinase assay. The fusion proteins glutathione *S*-transferase (GST)-IκB-α(1–70) and mutant S32A-S36A-GST-IκB-α(1–70) were expressed from plasmids kindly provided by Nancy Rice (NCI-FCRDC, Frederick, Md.).

We used 0.5 μg of GST-IκB-α or mutant GST-IκB-α as the substrate for in vitro phosphorylation in which whole-cell extracts from stimulated pcDNA and pcDNA A224L Jurkat cells were assayed. Each 25 μl of whole-cell extract was made from 10^6 Jurkat cells, stimulated with 20 ng of PMA plus 1 μg of ionomycin for 15 min. After stimulation, cells were washed in PBS and lysed in TNT buffer supplemented with phosphatase inhibitors (1 mM $NaVO_3$, 10 mM NaF, and 10 mM Na_2MoO_4) and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin, 2 μg of leupeptin, and 2 μg of aprotinin per ml).

Cleared extracts were incubated for 12 h with 1 μl of specific NEMO antiserum to immunoprecipitate IKKs. Immunoprecipitates were finally resuspended in kinase buffer containing 20 mM HEPES (pH 7.6), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 μM ATP, and 1 μCi of [^{32}P]ATP (specific activity, 3,000 Ci/mol) supplemented with phosphatase inhibitors and mixed with the recombinant GST-IκB-α or recombinant mutated GST-IκB-α immobilized on Sepharose beads. After 20 min at 30°C, the kinase reaction was terminated by washing with TNT buffer. Phosphorylated proteins were separated in an SDS–10% PAGE gel, dried and developed by autoradiography.

Electrophoretic mobility shift assay. Nuclear extracts from Jurkat cells were prepared as previously described (22). Briefly, cells were collected by centrifugation, washed twice with PBS, and resuspended in 400 μl of ice-cold buffer A (10 mM HEPES [pH 7.6], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM Na_2MoO_4, 1 μg of pepstatin, 2 μg of leupeptin, and 2 μg of aprotinin per ml). After 15 min on ice, Nonidet P-40 was added to reach a final concentration of 5% (wt/vol), and cells were vortexed and centrifuged for 30 s at 15,000 × *g*. The nuclear pellet was extracted with 50 μl of buffer C (20 mM HEPES [pH 7.6], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM Na_2MoO_4, 1 μg of pepstatin, 4 μg of leupeptin, and 4 μg of aprotinin per ml) for 30 min on a rocking platform and further centrifuged at 15,000 × *g* for 10 min. Protein concentrations were determined by BCA assay.

The DNA-binding assays were performed essentially as reported previously (24) using as the labeled probe the double-stranded κB element of the interleukin-2 receptor alpha promoter (5′-GCAGGGGAATCTCCCTCT-3′). For antibody-specific supershifts, 1 μl of anti-p65 antibody, kindly provided by Nancy Rice (NCI-FCRDC, Frederick, Md.), was incubated with the nuclear extract for 20 min on ice before incubation with the radioactive probe. Bound complexes were separated in a 5% acrylamide gel, and their specificity was determined by competition with a 50-fold molar excess of the same unlabeled oligonucleotide.

RESULTS

ASFV IAP (A224L) activates NF-κB. Some cellular IAPs have been shown to activate NF-κB. Therefore, we tested the effect of the ASFV IAP-like molecule in NF-κB activation by transiently transfecting the gene (A224L) into Jurkat cells together with an NF-κB reporter gene. As shown in Fig. 1, ectopic expression of A224L was able to activate the reporter activity in a dose-responsive manner.

To better understand the mechanism by which A224L was activating NF-κB, we generated Jurkat cell lines that stably express the ASFV A224L gene after selection with G418 (Fig. 2). As control cells, we used pooled populations of clones stably expressing the empty plasmid (pcDNA3). In agreement with the transient transfection assays, pooled populations of Jurkat clones stably expressing the A224L gene have greater basal luciferase activity (about twofold) than the control pcDNA3-transfected cells, after transfection with an NF-κB-

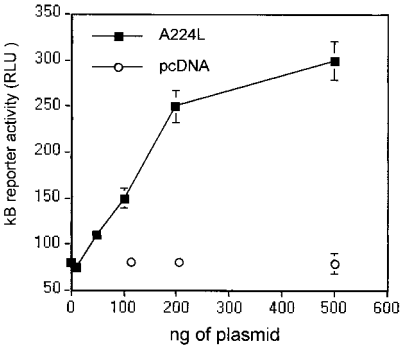

FIG. 1. A224L increases NF-κB-driven transcription. Jurkat T lymphocytes were transfected with the control vector pcDNA3 or graded doses of pCMV-A224L plasmid, together with the luciferase NF-κB reporter construct. After 14 h, luciferase activity was measured in cell extracts. Shown are the means ± standard error (SE) of relative light unit (RLU) values per 0.1 μg of protein from transfected cells, obtained from three independent experiments.

luciferase reporter gene (Fig. 3A, left panel). Interestingly, those cells also responded better to potent stimuli of NF-κB activation, such as PMA plus ionophore or TNF-α (data not shown), than the control cells. This effect was specific, since A224L had no effect on basal or PMA plus ionophore AP-1-dependent transcription (Fig. 3A, right panel).

Next, we assayed whether this elevated NF-κB activation was associated with a higher level of active NF-κB in the nucleus of cells stably expressing the viral IAP. As shown in Fig. 3B, unstimulated cells showed almost undetectable nuclear complexes bound to NF-κB oligonucleotides, as detected by electrophoretic mobility shift assays. A224L-expressing cells have barely detectable active NF-κB complexes. Interestingly, upon stimulation with PMA plus ionophore, more active NF-κB nuclear complexes were detected in A224L cells than in control pcDNA3-transfected cells. Those complexes represent active NF-κB, as demonstrated by supershifting by p65-specific antibodies (Fig. 3B).

Extracts of ASFV IAP-expressing cells also showed higher levels of NF-κB, as detected by Western blot (Fig. 3C) using a specific anti-c-Rel antibody, further confirming that NF-κB is up regulated in A224L-expressing cells and indicating that, as expected, the enhancement of NF-κB activity found in A224L-expressing cells stimulates the synthesis of c-Rel, since c-*rel* is a κB-dependent gene.

ASFV IAP activity depends on IKK activity. NF-κB activity is the result of Iκβ-α phosphorylation by IKKs and posterior degradation. Thus, to investigate the mechanism of NF-κB activation mediated by the viral IAP, we tested whether IKKs were activated in A224L-expressing cells. For this, cell extracts from both pcDNA- and A224L-expressing cells were prepared and immunoprecipitated with anti-IKK-specific antiserum (NEMO). In parallel, and as a control, an irrelevant anti-c-Rel antiserum was also used to immunoprecipitate extracts from A224L-expressing cells (Fig. 4A). The immunoprecipitates were assayed for in vitro IKK activity using both recombinant GST-Iκβ-α and mutant GST-Iκβ-α (which lacks serine residues Ser-32 and Ser-36 and consequently cannot be phosphorylated) as substrates, as described in Materials and Methods.

A higher amount of phosphorylated GST-IκB-α was obtained in A224L-transfected cells (Fig. 4B), and, as expected, no phosphorylation was observed with the immunoprecipitate obtained with the anti-c-Rel antibody (not shown) or when mutated GST-Iκβ-α was used as the substrate (Fig. 4C). In

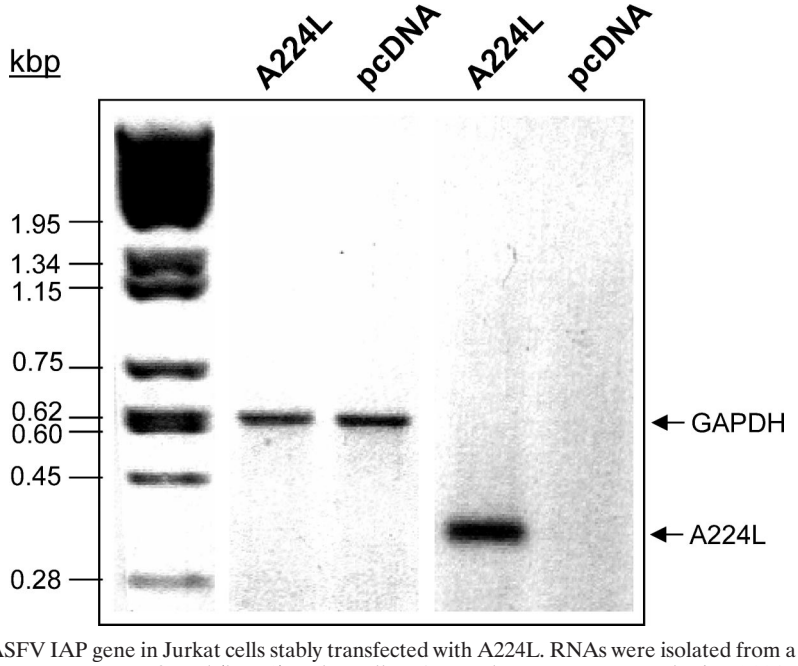

FIG. 2. Transcription of ASFV IAP gene in Jurkat cells stably transfected with A224L. RNAs were isolated from a pool of three different clones of A224L-transfected (lane 2) or pcDNA-transfected (lane 3) Jurkat cells. PCR products were generated using A224L-specific primers, and cDNAs were synthesized from RNAs and resolved in a 1.5% agarose gel. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control was used to control the RNA content and integrity. Positions of molecular size markers are included, and the sizes (in kilobase pairs) are indicated on the left.

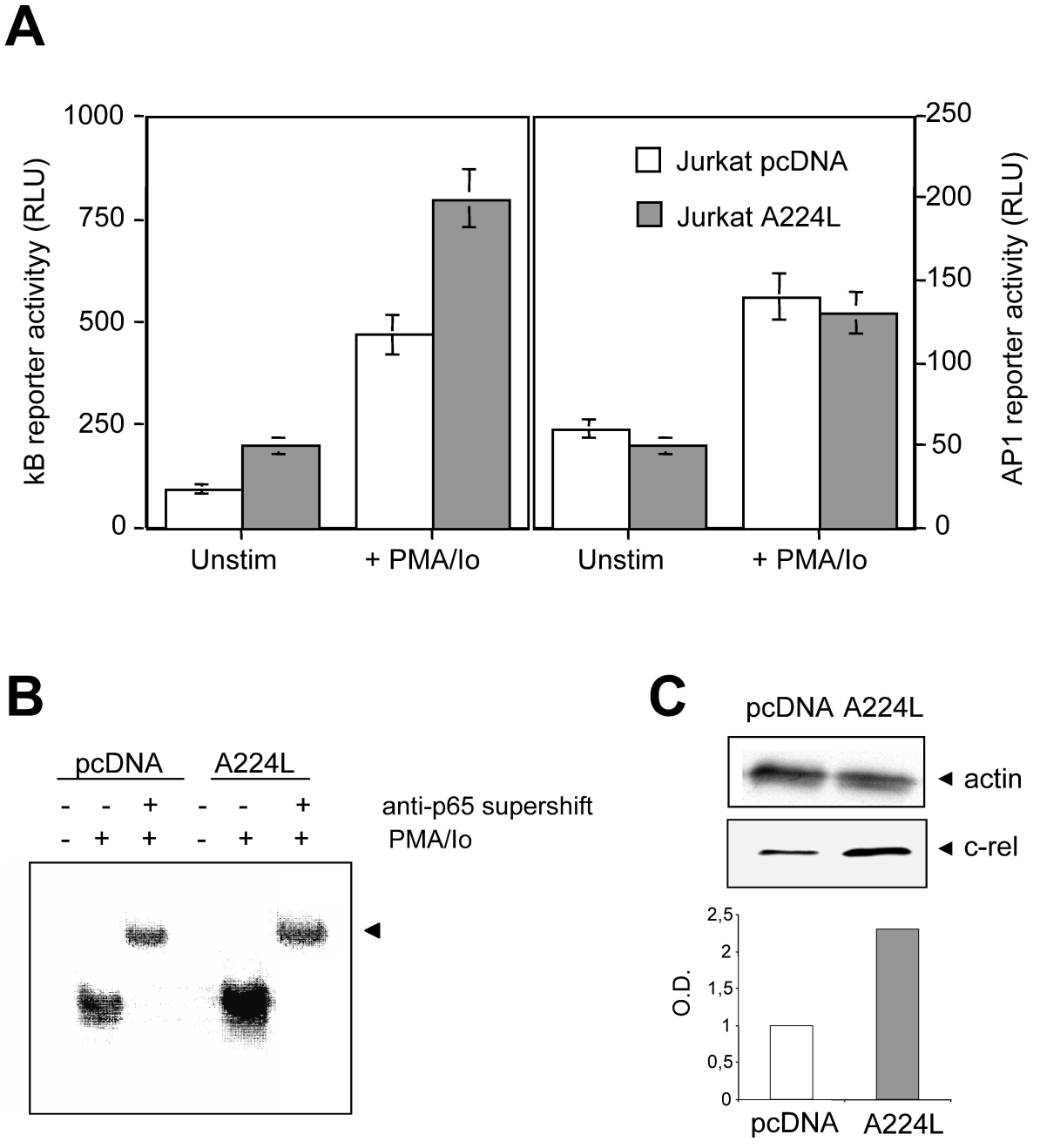

FIG. 3. Cells stably expressing A224L have elevated NF-κB activity. (A) Stimulation of NF-κB-directed transcription. Jurkat cells stably expressing A224L (shaded bars) or pcDNA as a control (open bars) were cotransfected with NF-κB (left panel) or AP-1 (right panel) luciferase reporter constructs. After 12 h of growth, the indicated cultures were exposed to PMA plus ionophore (Io) or left unstimulated. Whole-cell extracts were prepared at 24 h posttransfection and assayed for luciferase activity. Relative light units (RLU) per 0.1 μg of protein from triplicate transfections (mean ± SE) are shown. (B) A224L expression enhances NF-κB activity in the nucleus of stimulated cells. Supershift electrophoretic mobility shift assays for NF-κB DNA-binding activity were performed with nuclear extracts collected from cells not expressing or expressing A224L stimulated with PMA plus ionophore. The observed shift in mobility with specific anti-p65 antibody is indicated by an arrowhead. (C) Whole-cell extracts (50 μg) from Jurkat cells stably transfected with pcDNA or pcDNA-A224L were prepared, subjected to SDS-PAGE, and detected by immunoblotting with c-Rel-specific antibody. A representative experiment from three different assays is shown. A control using an antiactin antibody is also included to rule out differences in loading or transference. The increased amount of c-Rel in A224L-expressing cells was monitored by densitometry and is shown as optical density (O.D.).

agreement with the above results, we conclude that A224L-expressing cells have higher (about twofold) IKK activity in both unstimulated (not shown) and PMA-stimulated cells.

To further confirm the role of IKK in A224L activity, we transiently transfected A224L-expressing cells with an IKKβ (IKK2) plasmid or the pRK5 empty plasmid. As expected, the transfection of active IKKβ was able to activate the NF-κB reporter gene in pcDNA cells (Fig. 5). Furthermore, and more

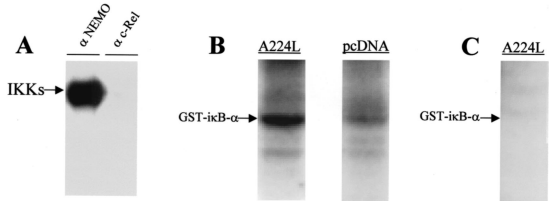

FIG. 4. Activation of IKKs by A224L. (A) Cleared extracts from 10^6 pcDNA-A224L stably transfected Jurkat cells were incubated and immunoprecipitated with 1 μl of NEMO antiserum or with anti-c-Rel antibody as a control. Immunoprecipitates were separated by SDS-PAGE and revealed with a monoclonal antibody against IKKs. (B and C) Cleared extracts from pcDNA- and A224L-transfected cells were immunoprecipitated with 1 μl of NEMO antiserum and used in an in vitro kinase assay as described in Materials and Methods, using (B) 0.5 μg of GST(1–115)-IκB-α or (C) 0.5 μg of mutated IκB-α as the substrate. Proteins were separated by SDS–10% PAGE and developed by autoradiography. Data were reproducible for at least three separate kinase assays performed under the same conditions.

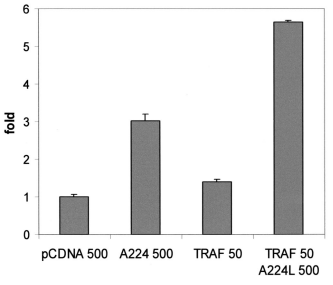

FIG. 6. ASFV IAP cooperates with TRAF-2 in increasing NF-κB activity. A total of 10^6 Jurkat T lymphocytes were transiently cotransfected with 1 μg of NF-κB luciferase reporter and 500 ng of pcDNA and pcDNA-A224L or 50 ng of the expression vector for TRAF-2. Values represent the fold induction of reporter gene activity for the respective transfections, which were done in triplicate. The value for pcDNA-transfected cells was 830 RLU and was considered 100% activity (onefold).

interestingly, in cells stably expressing A224L, the synergism in NF-κB reporter activation between IKKβ and A224L was evident. It is also important to note that transfection of IKKβ eliminated the ability of TNF-α to activate the reporter gene, indicating that IKK's activity is an event downstream of the TNF-α action.

To test the specificity of this effect, we transfected a kinase-dead dominant negative mutant of IKKβ into cells stably expressing A224L, which abrogated the enhanced activation of NF-κB reporter activity.

A224L cooperates with TRAF-2 in activation of NF-κB. Cellular IAP2 seems to act upstream of NF-κB in the TNF signaling cascade at a step leading to IκB-α degradation (36). Previous biochemical studies have suggested that the induction of NF-κB by TNF is mediated via TRAF-2 (27). Although recent evidence indicates that c-IAP2 interacts with TRAF-2 and this has been proposed as a mechanism by which c-IAP2 activates NF-κB (6), the precise role of c-IAP2 in this cytokine-signaling pathway remains unclear.

In order to investigate the mechanism operating in the viral IAP in relation to TRAF-2, we transiently transfected TRAF-2 and A224L genes into Jurkat cells, either separately or combined. As shown in Fig. 6, ectopic expression of the plasmid containing A224L induced the activation of NF-κB in Jurkat cells, as expected. The transfection of a plasmid containing TRAF-2 slightly enhanced the NF-κB activity. Interestingly, higher activation was obtained when we cotransfected cells with plasmids expressing A224L and TRAF-2, representing more than an additive effect on NF-κB activation. This last result suggests that A224L could activate IKKs by a mechanism involving the TRAF-2 molecule.

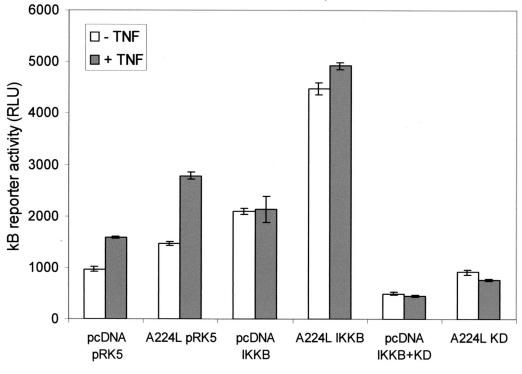

FIG. 5. Involvement of IKKs in ASFV IAP-mediated NF-κB activity. pcDNA3-transfected or A224L stably expressing Jurkat cells were cotransfected with 1 μg of the NF-κB-dependent promoter-luciferase reporter gene together with 1 μg of pRK5 empty plasmid or pRK5 IKKβ. After 24 h of culture, cells were either untreated or stimulated with TNF-α (50 ng/ml) for 16 h. The specificity of the effect was ensured by transfection of 1 μg of a dominant negative mutant of the IKKβ expression vector in pcDNA3-expressing cells or A224L-expressing cells. Relative luciferase values (RLU) per microgram are averages (mean ± SE) of two representative experiments in which each transfection was done in triplicate.

DISCUSSION

Here we describe for the first time a viral IAP-like molecule that is able to activate NF-κB. Similar results have been described previously for cellular XIAP, c-IAP1, and c-IAP2 (7), defining a new positive regulatory survival mechanism for IAPs, although the specific pathways involved are not fully understood. It was recently reported that the expression of XIAP in endothelial cells leads to increased nuclear translocation of the p65 subunit of NF-κB through a novel signaling pathway that involves the mitogen-activated protein kinase kinase kinase (MAP3K) TAK1 (11).

The viral IAP A224L was classified as an IAP homologue based on the existence of a single baculovirus internal repeat (BIR) motif, which defines the members of the IAP family. However, A224L does not have a canonical ring motif, which is present in many, although not in all, members of the IAP family and to which the ability to activate NF-κB has been mapped (6). A224L contains a sequence that may constitute a zinc finger domain of the four-cysteine type at the C-terminal region. In contrast to this, the zinc finger sequences in baculovirus IAPs and in cellular IAP genes shown to activate NF-κB are ring fingers of the C3HC4 type (7). Thus, it was somewhat surprising that, lacking this domain, A224L was able

to activate NF-κB, and this result could indicate that this domain is dispensable for NF-κB activation by some IAPs.

Given the homology in the BIR sequence and the functional relationship between ASFV IAP and cellular IAPs in terms of inhibition of caspases (20), it is possible that the viral gene derives from a cellular gene. Genes that modulate apoptosis might be incorporated into viral genomes to sustain host cell viability (13). Gene products of several viruses control apoptosis by interacting with components of the conserved pathways that regulate cell death. Our results show that ASFV IAP is able to activate NF-κB, and therefore the ability to activate this transcriptional factor is not restricted to IAPs of cellular origin. Moreover, suppression of TNF-mediated cell death, which mainly involves inactivation of caspase-8 by c-IAP1 and c-IAP2, is thought to be mostly mediated by the ability of those IAPs to interact with TNF receptor proteins such as TRAF-2, resulting in NF-κB activation (6, 26). Although we were not able to demonstrate interaction of A224L with TRAF-2 in immunoprecipitation assays, the viral protein seems to be similar to c-IAP1 and c-IAP2, since it is able to functionally cooperate with TNF-R-associated protein TRAF-2 and to enhance NF-κB activation even in the absence of stimulus.

The involvement of IKKs in A224L-mediated NF-κB activation is supported by the fact that in A224L-transfected cells a constitutive activation of IKKβ was observed. In addition, the effect of A224L could be inhibited by a kinase-dead dominant negative mutant of IKKβ. In this regard, it is interesting that A224L is similar to XIAP (11). Therefore, our results indicate that A224L is able to directly activate NF-κB by inducing IKK activity. This is the first demonstration that any viral IAP induces IKK activity. Moreover, the activation of NF-κB was further enhanced when the cells were stimulated with TNF or PMA plus ionophore. This latter result is interesting because PMA plus ionophore activation does not activate TRAF as TNF does. This can be taken as an indication that A224L was able to activate NF-κB in a TNF-R-independent manner. Since A224L was able to activate NF-κB in the presence of PMA or TNF, this suggests that A224L should act downstream of where the PMA and TNF signals converge. In support of this is the fact that we were unable to coimmunoprecipitate A224L and TRAF-2, which suggests a possible role for A224L in both pathways.

c-IAP1 and c-IAP2 have been shown to physically interact with TNF-R via TRAF-1 and TRAF-2 association. The amino-terminal BIR-containing region of these IAPs is required for the interaction with TRAFs. The interaction of c-IAP1 and c-IAP2 with TRAF-1 and TRAF-2 appears to be specific in that these IAPs do not bind to TRAF-3, TRAF-4, TRAF-5, or TRAF-6, and other IAPs (XIAP and NIAP) reportedly fail to bind TRAFs altogether (28). Thus, TRAF binding is not a universal feature of IAP family proteins. In this regard, A224L is similar to XIAP, since we could not demonstrate an interaction of the viral protein with TRAF-2. However, XIAP may act by binding to some upstream kinases involved in IKK activation. Thus, it is tempting to speculate that A224L may behave like XIAP.

In addition to the effect on NF-κB, a study has provided evidence that some IAP family proteins can regulate the Jun amino-terminal kinase (JNK) pathway (29). Specifically, JNK1 activation induced by overexpression of pro-caspase-1 is reportedly augmented by coexpression of XIAP. However, we have not detected any effect on either basal or stimulated AP-1 activation in cells stably or transiently transfected with ASFV IAP.

Here we present evidence of a new type of strategy to evade host response, not described previously for mammalian viruses: the existence of a viral IAP that not only blocks caspase-3 activation (20) but also activates NF-κB. It is interesting that the virus encodes an IκB-like molecule (A238L) that behaves as a bona fide IκB inhibitor (24). However, A238L and A224L are expressed at different times during ASFV infection, A238L being an early protein and A224L a late protein in the viral cycle. As A224L induces, whereas A238L blocks, NF-κB activation, this may suggest that the virus requires a low NF-κB activity at early times of the viral cycle to avoid immune responses but a high activity at late times, probably to prevent apoptosis. The virus also encodes another apoptosis inhibitor, a Bcl-2-like molecule (25), which is an early protein in the viral cycle that could also act to inhibit programmed cell death at early times after infection.

Taken together, these results indicate that the control of apoptosis and survival in the host cell infected by ASFV is a tightly regulated process which must be of crucial importance both for replication of the virus and for viral evasion of the immune response.

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