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Binding of Kaposi's Sarcoma-Associated Herpesvirus K-bZIP to Interferon-Responsive Factor 3 Elements Modulates Antiviral Gene Expression▽

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Kaposi's sarcoma-associated herpesvirus encodes numerous regulatory proteins capable of modulating viral and cellular gene expression and affecting host cell functions. K-bZIP, a leucine zipper-containing transcription factor encoded by ORFK8, is one such protein. During infection, transcription of the ORFK8 early gene is turned on by the immediate-early replication and transcription factor activator (RTA). One described function of the K-bZIP nuclear protein is to interact with and repress RTA-mediated transactivation of viral promoters, including that of the *K8* gene. In the present work, we provide evidence that the expression of K-bZIP results in the activation of the *ifn*-β gene. Of interest, *ifn*-β gene activation by K-bZIP is independent of interferon (IFN)-responsive factor 3 (IRF-3) and nuclear factor κB (NF-κB) activation. Using a DNA binding affinity assay and electromobility shift assay, we report that K-bZIP binds efficiently to the PRDIII-I region of the beta IFN (IFN-β) promoter, and, in doing so, it prevents the attachment of activated IRF-3 but not that of NF-κB or ATF2/c-Jun to the IFN-β promoter sequence. As a consequence, *ifn*-β gene activation in response to IFN inducers such as Sendai virus infection or expression of retinoic acid-inducible gene I, mitochondrial antiviral signaling protein, or TANK-binding kinase 1 (TBK-1) is severely impaired (>90%) by the presence of K-bZIP. K-bZIP also prevents the activation of RANTES and CXCL11, whose promoters are also regulated by IRF-3. Lysine 158 (target for SUMO conjugation), threonine 111, and serine 167 (targets for phosphorylation) mutants of K-bZIP were equally effective as wild-type K-bZIP in mediating the repression of TBK-1-activated *ifn*-β gene expression. Lastly, the overexpression of CREB binding protein could not reverse the K-bZIP repression of TBK-1-activated *ifn*-β gene expression. In all, our results indicate that K-bZIP binds directly to the PRDIII-I region of the IFN-β promoter and, as a consequence, causes a low level of *ifn*-β gene transcription. In doing so, K-bZIP prevents IRF-3 from binding to the IFN-β promoter and precludes the formation of the enhanceosome, which is required for maximal *ifn*-β gene transcription. A new role for K-bZIP as a protein involved in immune evasion is therefore uncovered.

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 is an oncogenic gammaherpesvirus that is tightly linked with the development of Kaposi's sarcoma, primary effusion lymphomas, and multicentric Castleman's disease (3, 4, 6, 42). As with all herpesviruses, KSHV infection proceeds in a temporally regulated fashion with the sequential expression of viral genes. The first viral proteins to be expressed upon entry are those encoded by immediate-early (*ie*) genes. Among those, the expression of the replication and transcription factor activator (RTA) encoded by open reading frame 50 (ORF50) is both necessary and sufficient for the lytic infectious cycle and reactivation from latency (28, 30, 44, 50). RTA regulates and activates the expression of several viral genes involved in the lytic replication process (8, 28, 44, 45). For two such genes, *orf57* and *k8*, RTA activates transcription through the direct binding of a common 12-base palindromic sequence found within the promoters of these genes (5, 27). Transcription initiating from the K8 promoter yields several distinct spliced variants from which two major proteins are translated: K-bZIP (also known as K8α), a homodimerizing protein of 237 amino acids with a prototypic bZIP domain at the C terminus, and a truncated version of K-bZIP, K8β, lacking the C-terminal leucine zipper (26, 51, 53). K-bZIP is a nuclear, phosphorylated, and sumoylated protein that colocalizes with PML oncogenic domains and viral replication compartments (16, 47). Not surprisingly, a role for K-bZIP in viral DNA replication was reported (1, 22, 47). Through its ZIP motif, K-bZIP also binds to and negatively regulates the activity of RTA on the K8 and ORF57 promoters but not that of the Pan promoter (18, 21). Thus, the expression of K-bZIP and its binding to RTA result in a negative autoregulation mechanism. Sumoylation of lysine 158 and phosphorylation of threonine 111 also play important roles in the transcriptional repression activity of K-bZIP (16, 17). K-bZIP physically interacts with p53 and functionally represses p53 transcriptional activity, with increased cellular survival as a possible outcome (32). The expression of K-bZIP causes cell cycle G_1 arrest through the accumulation of the cyclin-dependent kinase inhibitor p21 (48, 49) and binding to the cyclin-CDK2 complex (19). Details on the functionality of K8β are very limited but suggest that this protein acts as a functional antagonist of K-bZIP (51).

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Viral proteins carried by virions or that are expressed early after infection often display activities that interfere with the mounting of an effective immune response. In doing so, these viral proteins assist in the establishment of infection and help avoid recognition and elimination of the infected cells by the immune system. One potent antiviral defense mechanism targeted by viruses is the interferon (IFN) system. IFNs are a family of proteins that can be classified in three distinct subsets in humans: type I, type II, and type III, represented by alpha/beta IFN (IFN-α/β), IFN-γ, and IFN-λ, respectively. IFNs are cytokines that are rapidly produced and secreted in response to viral infections and that limit the spread of infection by preventing viral protein synthesis. Not surprisingly, viruses have evolved mechanism to counteract the IFN pathway. In the case of KSHV, a recent report indicates that proteins present within the KSHV virion can efficiently prevent IFN-responsive factor 3 (IRF-3) activation (33), a key transcription factor involved in the synthesis of type I IFNs (IFN-α and IFN-β) (12, 36). One such virion-associated protein, encoded by ORF45, binds IRF-7, inhibits its phosphorylation, and blocks the activation of both IFN-α/β promoters in response to Sendai virus (SeV) infection (54, 55). Through its ubiquitin ligase activity, the RTA protein also targets the IRF-7 protein and causes its degradation (52). KSHV also encodes several other proteins capable of antagonizing the IFN pathway (for a recent review, see reference 35).

In the present work, we were interested in determining whether the K-bZIP protein of KSHV modulates the type I IFN pathway. Our results indicate that K-bZIP expression triggers low-level activation of the *ifn*-β gene. This activation is independent of IRF-3 and NF-κB and includes the direct binding of K-bZIP to the IFN-β promoter. In doing so, K-bZIP prevents the proper activation of the *ifn*-β gene in response to SeV infection or the expression of IFN-β inducers, including retinoic acid-inducible gene I (RIG-I), mitochondrial antiviral signaling protein (MAVS), and TANK-binding kinase 1 (TBK-1). K-bZIP also prevents the activation of the *rantes* gene by a mechanism similar to that observed for the *ifn*-β gene. These results highlight another mechanism utilized by KSHV to curtail the mounting of an effective antiviral response.

MATERIALS AND METHODS

Cell culture. A549 cells (ATCC) were cultured in F12K/Ham medium (HyClone) containing 10% heat-inactivated fetal bovine serum. HEK 293T cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (Megacell) containing 3% heat-inactivated fetal bovine serum.

Plasmids and constructs. The sequence of full-length K-bZIP (residues 1 to 239) was cloned into a modified pCMV2N3T vector (a gift of Didier Trouche) in which the two nuclear localization signals were removed (pCMV3T). K8 was amplified from the genomic DNA of BCBL-1 with the following primers: K8 forward (5′-GTGGATCCCTGCAAATGCC-3′) and K8 reverse (5′-AATCTAGATTCAACATGGTGGG-3′). The PCR product was cloned into pCMV3T digested with BamHI and XbaI in frame with the three hemagglutinin (HA) tags. Mutants were generated by site-directed mutagenesis (Stratagene, La Jolla, CA) using primers K-bZIPK158R forward (5′-CTGTAGTTAGGGCCGAAG-3′), K-bZIPK158R reverse (5′-CTTCGGCCCTAACTACAG-3′), K-bZIPT111A forward (CTCTCTCACGCACCACCAAGAG), K-bZIPT111A reverse (CTCTTGGTGGTGCGTGAGAGAG), K-bZIPS167A forward (CAGTCACATGCTCCCACGCGAAAG), and K-bZIPS167A reverse (CTTTCGCGTGGGAGCATGTGACTG) using HA-K8 as a template. pIFN-βLuc was obtained from Tom Maniatis (Harvard University, Cambridge, MA) (7). Expression vectors for RIG-I, TBK-1, IRF-3, IRF-3–green fluorescent protein, and a dominant negative form of IRF-3 (IRF-3–DN) were obtained from J. Hiscott and Rongtuan Lin (Lady Davis Institute, McGill University, Montreal, Canada) (25, 41). The MAVS expression vector was obtained from Zhijian Chen (University of Texas Southwestern Medical Center, Dallas, TX) (39). The plasmid encoding a histidine-tagged K-bZIP fusion protein (His-K8) was a generous gift from Henri Gruffat (34).

Transfection. Subconfluent A549 cells were transfected using the TransiT-LT reagent (Mirus Corporation, Madison, WI) according to the manufacturer's instructions and analyzed 24 h posttransfection. Transfection of HEK 293T cells was performed using calcium phosphate precipitation procedures. Cells were plated at 100,000 cells/well (12-well plate) the day prior to transfection. Cells were transfected with 0.1 μg of reporter plasmid and up to 2 μg of expression vector per well and brought to a total of 2.5 μg of DNA per well for each condition with the pCMV3T control plasmid. Cells were lysed 48 h after transfection, and luciferase activity was measured on an MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA).

RT-QPCR. Total RNA was extracted from 293T-transfected cells or A549-transfected cells using TRIzol reagent (Invitrogen, Ontario, Canada). All RNA samples were treated with DNase I to eliminate residual genomic DNA prior to amplification. cDNA was synthesized, and real-time quantitative PCR (RT-QPCR) analysis was performed using a Rotorgene apparatus (Corbett Research) with SyBRGreener technology (Invitrogen). The specificity of amplification was assessed for each sample by melting curve analysis. All transcripts were normalized according to *gapdh* housekeeping gene expression. The following primer pairs were used: *gapdh* forward (5′-CGAGATCCCTCCAAAATCAA-3′) and *gapdh* reverse (5′-TTCACACCCATGACGAACAT-3′), *ifn*-β forward (5′-AAACTCATGAGCAGTCTGCA-3′) and *ifn*-β reverse (5′-AGGAGATCTTCAGTTTCGGAGG-3′), *cox-2* forward (5′-TGCATTCTTTGCCCAGCACT-3′) and *cox-2* reverse (5′-AAAGGCGCAGTTTACGCTGT-3′), *rantes* forward (5′-GAGGCTTCCCCTCACTATCC-3′) and *rantes* reverse (5′-CTCAAGTGATCCACCCACCT-3′), and *isg15* forward (5′-CATGGGCTGGGACCTGACG-3′) and *isg15* reverse (5′-CGCCAATCTTCTGGGTGATCTG-3′).

Cell extracts. HEK 293T were transfected with Myc–IRF-3 and HA–K-bZIP expression vectors using calcium phosphate in 10-cm plates. One day after transfection, the cells were treated for 16 h with SeV. Cell pellets were resuspended in 400 μl of buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride) and then incubated for 10 min on ice. Twenty-five microliters of NP-40 (10%) was added, and nuclei were sedimented by centrifuging the samples at 12,000 × *g* for 30 s. The nuclear pellets were resuspended in 50 μl of buffer B (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) and incubated on a rocker for 15 min at 4°C. The suspensions were clarified by centrifugation at 13,000 × g for 5 min. The nuclear extracts (supernatants) were isolated, and the protein content was estimated using the BCA protein assay kit (Pierce).

DNA affinity binding assay. The DNA affinity binding assay method was described previously by Severa et al. (40). Briefly, a biotinylated oligonucleotide containing the IFN-β promoter sequence (5′-CCCCCCAAATGACATAGGAAAACTGAAAGGGAGAAGTGAAAGTGGGAAAATTCC-3′) or the IFN-stimulated response element (ISRE) region of RANTES (5′-CCCCCCCTATTTCAGTTTTCTTTTCCGTTTTGT-3′) was annealed with the corresponding antisense oligonucleotide in 1× SSC buffer (0.15 M NaCl plus 15 mM sodium citrate). Biotinylated DNA oligonucleotides were mixed with 150 μg of nuclear extracts in 500 μl of binding buffer containing 20 mM Tris-HCl (pH 7.5), 75 mM KCl, 1 mM DTT, and 5 mg/ml bovine serum albumin in presence of 13% glycerol and 20 μg of poly(dI-dC) and incubated for 25 min at room temperature (RT). Streptavidin magnetic beads (Roche), washed three times with 400 μl of 1× binding buffer, were then added to the reaction mixture and incubated for 30 min at 4°C and for 10 min at RT with mixing by rotation. The beads were collected using a magnet and washed three times with 500 μl of binding buffer. The bound proteins were eluted from the beads by boiling in Laemmli sample buffer and were resolved by 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting using anti-K-bZIP (a gift from H. Gruffat), anti-IRF-3, anti-p50, anti-ATF2, and anti-cJun/AP1 (Santa Cruz Biotechnology) antibodies. Equal amounts of nuclear extracts used in the assay were confirmed by immunoblotting using anti-PARP1 antibody.

Protein expression and purification. The His-K8 fusion protein was expressed and isolated from *Escherichia coli* BL21(DE3) cells following 3 h of induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at RT. The His-K8 protein was purified on a Ni-nitrilotriacetic column (QIAGEN).

Electromobility shift assay (EMSA). Various quantities (50 to 200 ng) of recombinant K-bZIP protein were assayed for DNA binding by gel shift analysis using ^{32}P-labeled double-stranded (DS) oligonucleotide corresponding to the IFN-β promoter (5′-AAATGACATAGGAAAACTGAAAGGGAGAAGTGA

AAGTGGGAAATTCC-3′). The binding mixture (20 μl) contained 10 mM HEPES (pH 8), 60 mM KCl, 4 mM $MgCl_2$, 100 μM EDTA, 100 μg of bovine serum albumin (BSA) per ml, 250 μM DTT, 15% glycerol, and 0.5 μg of poly(dI-dC). After 15 min of incubation at RT with the probe, samples were loaded onto a 5% polyacrylamide gel (29:1) prepared in 0.5× Tris-borate-EDTA buffer. After 2 h at 150 V, the gel was dried and exposed to Kodak film at −80°C overnight. To identify the location of the binding more precisely, a 100-fold molar excess of unlabeled double-stranded oligonucleotides corresponding to the PRDIV region of the IFN-β promoter (5′-AAATGTAAATGACATAGG-3′), the PRDIII-I region of the IFN-β promoter (5′-GAAAACTGAAAGGGA GAAGTGAAAGTG-3′), the PRDII region of the IFN-β promoter (5′-AGTG GGAAATTCCTCTGA-3′), and the ISRE region of the RANTES promoter (5′-CTATTTCAGTTTTCTTTTCCGTTTTGT-3′) were added to the binding mixture before the labeled probe was added.

IRF-3 dimerization assay. Native PAGE gels were used as described previously (15). Briefly, 10 μg whole-cell extract in native sample buffer (62.5 mM Tris-HCl [pH 6.8], 15% glycerol, and bromophenol blue) was resolved by electrophoresis on a 7.5% acrylamide gel (without SDS) prerun for 30 min at 40 mA using 25 mM Tris and 192 mM glycine (pH 8.4) with and without 1% deoxycholate in the cathode and anode chambers, respectively. After transfer onto a nitrocellulose membrane, the IRF-3 monomer and dimer were detected by immunoblotting using an IRF-3 antibody (a kind gift from M. David).

Immunoblot analysis. Whole-cell extracts were prepared as previously described (9), resolved by SDS-PAGE, and transferred onto a nitrocellulose membrane (Bio-Rad, Mississauga, Ontario, Canada). The membrane was blocked in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 5% nonfat dry milk for 1 h and incubated with primary antibody, anti-IRF-3, anti-IRF-3–phospho-Ser396 (described previously in reference 38), anti-TBK-1/IKKε (Imgenex, San Diego, CA), and anti-actin (Chemicon, Temecula, CA) in PBS containing 0.05% Tween 20 and 5% nonfat dry milk or 5% BSA for phosphospecific antibodies. After five 5-min washes in PBS containing 0.05% Tween 20, the membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin G (KPL laboratories, Gaithersburg, MD) in blocking solution. Immunoreactive proteins were visualized by chemiluminescence using the Supersignal Picowest substrate (Pierce, Rockford, IL).

Immunofluorescence. For immunofluorescence analyses, A549 cells were grown on coverslips and transfected with the indicated expression plasmids. At 24 h posttransfection, cells were fixed in 4% paraformaldehyde for 15 min at RT, washed in PBS, and permeabilized using PBS containing 0.5% Triton for 15 min at RT. After blocking in PBS containing 10% normal goat serum, cells were incubated with anti-IRF-3 and anti-HA (Sigma) in PBS containing 3% BSA at RT for 3 h. The cells were then subjected to three washes in PBS and incubated with Alexa488-labeled anti-rabbit (Molecular Probes) or Alexa568-labeled anti-mouse (Molecular Probes) antibodies for 1 h at RT in the dark. After two washes in PBS, the cells were incubated in 1 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI) diluted in PBS for 5 min. After five washes in PBS and a wash in water, coverslips were mounted onto slides using Gel-Mount anti-fading mounting medium (Biomeda, Burlingame, CA). Fluorescence was visualized using an epifluorescence Axiovert Zeiss microscope.

Statistical analysis. Statistical analysis was performed with the aid of the Graph Pad In Stat software using the unpaired Student *t* test. Statistical significance was achieved when the *P* value was <0.05.

RESULTS

K-bZIP was previously reported to act as a transcriptional repressor of gene expression (16, 18). To determine whether K-bZIP influences *ifn*-β gene expression, we transfected 293T cells with increasing doses of a K8 expression plasmid encoding full-length K-bZIP (K8α) and monitored endogenous *ifn*-β gene expression levels by RT-QPCR. As shown in Fig. 1A, a dose-dependent increase in IFN-β mRNA was detected 48 h posttransfection. We typically observed up to a 10-fold increase in IFN-β mRNA in response to K-bZIP expression. The expression of K-bZIP also led to IFN-β gene activation in A549 cells (Fig. 1B) or HeLa cells (not shown), suggesting that this effect is not restricted to 293T cells. *ifn*-β gene induction following the expression of K-bZIP was somewhat specific, considering that mRNA levels of other genes such as *rantes* (Fig. 1C), *isg15* (Fig. 1D), and *cox-2* (Fig. 1E) were either very weakly activated or not modulated in response to K-bZIP.

The *ifn*-β gene is regulated by a series of activated transcription factors, including IRF-3, NF-κB, and ATF-2/c-Jun, that bind to the promoter to form the IFN-β enhanceosome and promote transcription (29). First, to determine whether K-bZIP activates the *ifn*-β gene by inducing the nuclear translocation of IRF-3, A549 cells were transfected with the pCMV3T-HA-K8 expression vector and analyzed by immunofluorescence. Twenty-four hours posttransfection, cells were stained for IRF-3 and K-bZIP. As presented in Fig. 2, K-bZIP is detected in the nucleus of pCMV3T-HA-K8-transfected cells but not in control cells. When analyzed for IRF-3, K-bZIP-expressing cells exhibited cytoplasmic IRF-3 localization, suggesting that K-bZIP does not induce IRF-3 translocation. SeV-infected cells (Fig. 2) or TBK-1-transfected cells (data not shown) were used as a positive control for IRF-3 translocation. In such cells, immunoreactive IRF-3 locates primarily to the nucleus. These results suggest that *ifn*-β gene activation by K-bZIP occurs in the absence of IRF-3 nuclear accumulation.

To confirm that K-bZIP turns on *ifn*-β gene transcription in the absence of IRF-3 activation, A549 cells were transfected with increasing quantities of pCMV3T-HA-K8, and the phosphorylation status of IRF-3 was monitored by Western blot analysis. As shown in Fig. 3A, despite the detection of IRF-3 (forms I and II), no serine 396-phosphorylated forms (forms III and IV) of IRF-3 could be detected upon K-bZIP expression. The endogenous expression levels of TBK-1 and IKKε, responsible for IRF-3 phosphorylation, were similar in the absence and in the presence of K-bZIP. Lastly, we analyzed the formation of an IRF-3 dimer in the presence of K-bZIP. As shown in Fig. 3B, K-bZIP expression was not associated with IRF-3 dimer formation, in contrast to SeV infection. Beside IRF-3, we also studied the activation of NF-κB, another important transcription factor involved in the activation of the *ifn*-β gene. As shown in Fig. 3A, despite constant levels of p65, the expression of K-bZIP did not result in the phosphorylation of this NF-κB subunit. Furthermore, the expression of K-bZIP did not cause p65 nuclear translocation, as determined by immunofluorescence (data not shown). In these experiments, SeV infection was used as a positive control for IRF-3 phosphorylation and dimerization (Fig. 3B). These results suggest that neither IRF-3 nor NF-κB is activated following K-bZIP expression.

Next, to further confirm that the K-bZIP-mediated activation of the IFN-β promoter is independent of IRF-3 and NF-κB activation, functional assays were performed. 293T cells were cotransfected with IFN-β activators (TBK-1 or IKKβ) and K-bZIP in the absence or in the presence of dominant negative forms of IRF-3 (IRF-3ΔN) or IκBα (IκBα DN), and IFN-β promoter activation was measured using the firefly luciferase reporter. As shown in Fig. 4A, TBK-1 efficiently activated the IFN-β promoter, and this activation was strictly dependent on IRF-3, as IRF-3ΔN completely blocked the effects of TBK-1. Conversely, K-bZIP efficiently activated the IFN-β promoter, but unlike TBK-1, the effects of K-bZIP were unaffected by the expression of IRF-3ΔN. Similar experiments conducted using IKKβ indicate that this effector is capable of activating the IFN-β promoter through an NF-κB-dependent

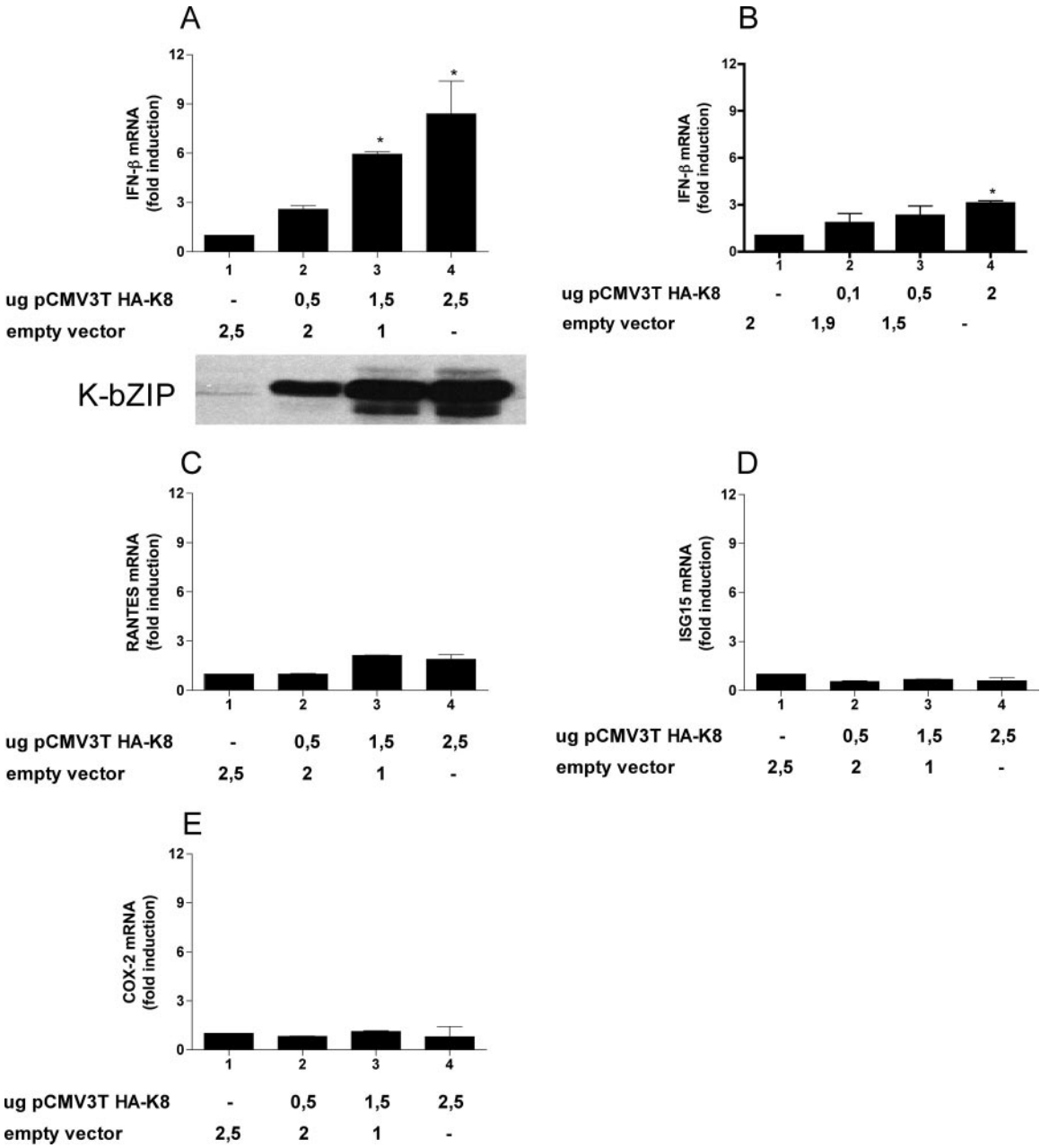

FIG. 1. K-bZIP activates *ifn*-β gene transcription. 293T (A and C to E) or A549 (B) cells were transfected with increasing quantities of an expression vector (pCMV3T HA-K8) coding for K-bZIP. Empty pCMV3T vector was use to keep DNA amount constant. Forty-eight hours posttransfection, cells were harvested, and total RNA was extracted using TRIzol reagent. RNA samples were treated with DNase I and analyzed for IFN-β (A and B), RANTES (C), ISG15 (D), and Cox-2 (E) mRNA levels by RT-QPCR. Results are expressed as the increase (*n*-fold) relative to control (empty vector) after normalization with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels. Results are expressed as mean (triplicate) induction (*n*-fold) in mRNA ± standard deviation (SD) and are representative of at least two experiments. K-bZIP expression (A, bottom) was determined by Western blotting using anti-K-bZIP rabbit antiserum. *, $P < 0.05$.

mechanism since these effects were completely blocked by IκBα DN (Fig. 4B). On the contrary, the activation of the IFN-β promoter by K-bZIP was refractory to IκBα DN. These results indicate that IFN-β promoter activation by K-bZIP is independent of IRF-3 and NF-κB activation.

The results obtained so far suggest that K-bZIP activates the *ifn*-β gene independently of IRF-3 and NF-κB. To determine how this activation might occur, we next tested whether K-bZIP could bind to the IFN-β promoter. Nuclear extracts of resting and SeV-infected cells cotransfected with IRF-3 and K-bZIP expression vectors were made and analyzed by Western blotting for K-bZIP, IRF-3, p50 (NF-κB), ATF2/c-Jun, and PARP1, used as controls to show equal input of nuclear extracts (Fig. 5A). Double-stranded oligonucleotides containing the entire IFN-β promoter were incubated with the nuclear extracts, and the nuclear proteins bound to the IFN-β pro-

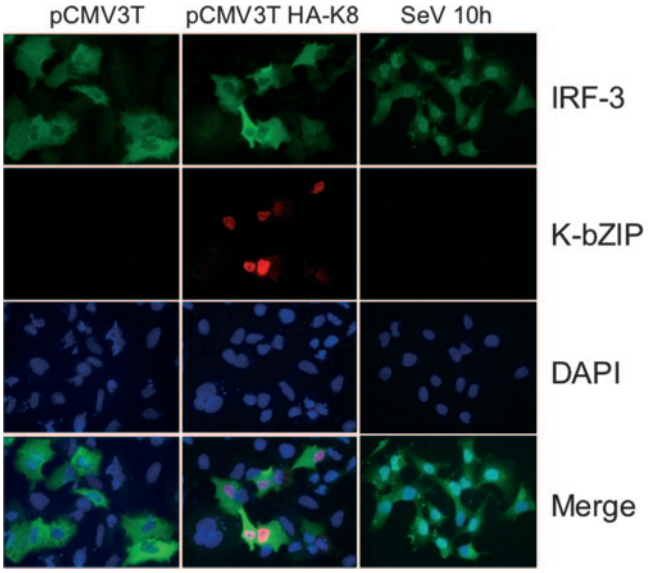

FIG. 2. K-bZIP does not induce IRF-3 nuclear translocation. A549 cells were transfected with pCMV3T or pCMV3T HA-K8 expression vector. Twenty-four hours after transfection, cells were processed for immunofluorescence analysis using rabbit anti-IRF-3 or mouse anti-HA (K-bZIP) antibodies followed by Alexa488-labeled anti-rabbit or Alexa568-labeled anti-mouse antibodies. Infection of cells by SeV was used as a positive control. Nuclei were counterstained blue by using DAPI. Fluorescence was visualized using an Axiovert Zeiss epifluorescence microscope.

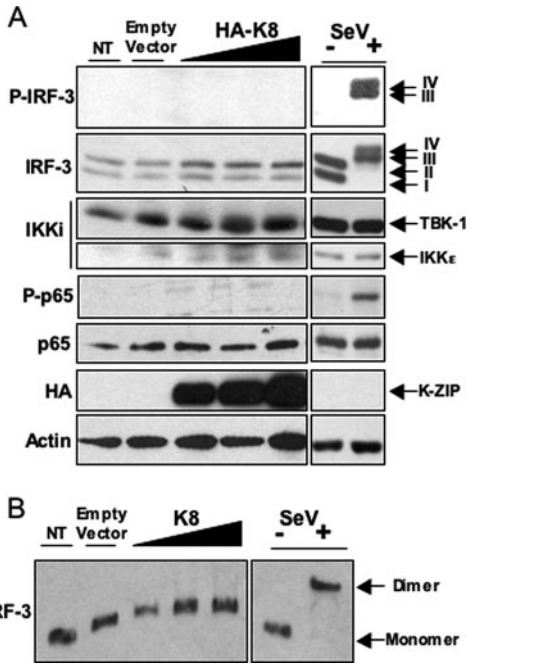

FIG. 3. K-bZIP does not activate IRF-3 or NF-κB. (A) A549 cells were either nontransfected (NT), transfected with control (pCMV3T), or transfected with increasing quantities of pCMV3T HA-K8 expression vectors. Twenty-four hours later, whole-cell extracts were made, and proteins were analyzed by Western blotting using specific antibodies. SeV infection was used as a positive control for IRF-3 and NF-κB phosphorylation. Actin was used as a control to show that similar amounts of proteins were loaded. (B) Same experiment shown in A except that whole-cell extracts were analyzed for IRF-3 monomer and dimer formation after migration of samples through a native nondenaturing gel. Following transfer to a membrane, IRF-3 was detected using anti-IRF-3 antibodies. SeV infection was used as a positive control for IRF-3 dimer formation.

moter were analyzed by Western blotting. As shown in Fig. 5A, K-bZIP bound the IFN-β promoter in a dose-dependent manner, with marginal effects of SeV infection. On the other hand, the efficient binding of IRF-3 to the IFN-β promoter was dependent on the presence of SeV. It is noteworthy that at the highest dose tested, the coexpression of K-bZIP was associated with much reduced IRF-3 binding to the IFN-β promoter. Conversely, the binding of the p50 subunit of NF-κB was induced by SeV infection but was not affected by K-bZIP. Similarly, K-bZIP had no effect on the ability of ATF-2/c-Jun to bind the IFN-β promoter sequence. These results indicate that K-bZIP does not promote the binding of IRF-3 to the IFN-β promoter. Furthermore, the data indicate that K-bZIP is capable of binding, directly or indirectly, to the IFN-β promoter and impairs the recruitment of IRF-3 when activated by an inducer such as SeV. We next tested the ability of K-bZIP to bind to the ISRE of the RANTES promoter, which is also activated by IRF-3 (23, 24). As shown in Fig. 5B, K-bZIP bound the ISRE of the RANTES promoter, with SeV having no effect. Again, we demonstrated that K-bZIP affects the binding of IRF-3 to the promoter, indicating that the effects of K-bZIP are not restricted to the IFN-β promoter.

The observation that K-bZIP binds the IFN-β promoter and negatively affects the ability of IRF-3 to bind to the same promoter in response to SeV infection opened up the possibility that K-bZIP might be capable of suppressing *ifn*-β gene activation. To test this hypothesis, 293T cells were cotransfected with a pCMV3T-K8 expression vector together with expression vectors encoding known *ifn*-β gene inducers, RIG-I, MAVS, and TBK-1, and cells were analyzed for IFN-β mRNA levels by RT-QPCR. As presented above, K-bZIP increased IFN-β mRNA levels by eightfold (Fig. 6A). The expression levels of RIG-I, MAVS, and TBK-1 were effective in inducing IFN-β mRNA levels by 83-, 616-, and 93-fold, respectively. However, when K-bZIP was coexpressed with RIG-I, MAVS, or TBK-1, the level of IFN-β mRNA dropped by more than 90%. These results indicate that, by itself, K-bZIP is capable of activating *ifn*-β gene transcription minimally but that when K-bZIP is present, the induction of IFN-β in response to classical inducers such as RIG-I, MAVS, or TBK-1 is severely compromised. Similar results were obtained using A549 cells and SeV as the IFN-β inducer (Fig. 6B). We next tested the hypothesis that K-bZIP could interfere with RIG-I, MAVS, and TBK-1 activation of the *rantes* gene by analyzing by RT-QPCR mRNA from transfected 293T cells (Fig. 6C). As for the IFN-β, we demonstrated that K-bZIP affects the production of RANTES mRNA stimulated by RIG-I, MAVS, or TBK-1. We also demonstrated an inhibition by K-bZIP of the CXCL11 cytokine mRNA, which is also activated by IRF-3 in response of TBK-1 (data not shown). These results indicate that K-bZIP is capable of suppressing the activation of genes regulated through IRF-3.

In order to determine whether K-bZIP binds directly and, if so, to map the motifs required for the binding of K-bZIP to the

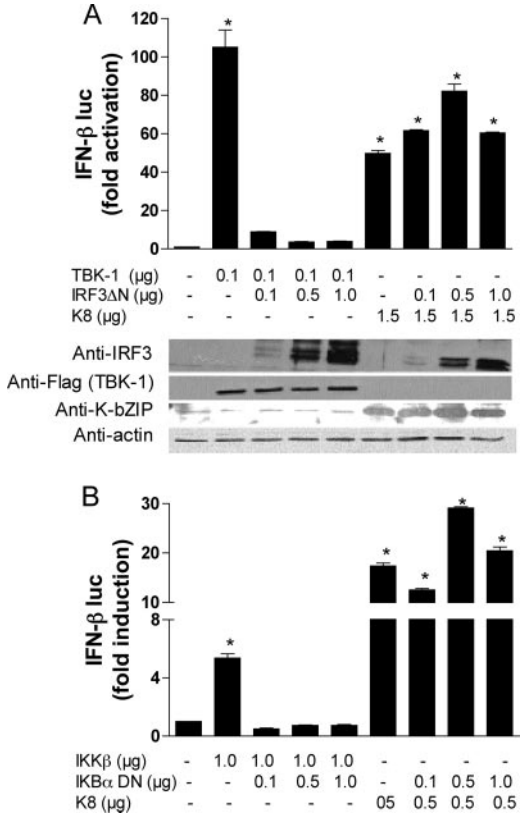

FIG. 4. K-bZIP activates *ifn*-β gene expression in the presence of dominant negative forms of IRF-3 and IKBα. (A) 293T cells were transfected with TBK-1 or HA-K8 expression vectors in the absence of in the presence of increasing quantities of a plasmid coding for a dominant negative form of IRF-3. (B) 293T cells were transfected with IKKβ or HA-K8 expression vectors in the absence or in the presence of increasing quantities of a plasmid coding for a dominant negative form of IκBα. Twenty-four hours later, cells were resuspended in lysis buffer, and luciferase activity was determined using a luminometer. Results are expressed as mean (triplicate) induction (*n*-fold) of IFN-β luciferase (luc) activity ± SD relative to cells transfected with a control plasmid and are representative of three independent experiments. ∗, $P < 0.05$.

IFN-β promoter, we first expressed and purified a His-K8 fusion protein and performed an EMSA using ^{32}P-labeled double-stranded oligonucleotides corresponding to the IFN-β promoter (Fig. 7). As a confirmation of the results shown in Fig. 5, we observed that recombinant K-bZIP bound the PRDIII-I region of the IFN-β promoter (Fig. 7B). Furthermore, K-bZIP binding occurred in the absence of other cellular or viral proteins, indicating a direct and dose-dependent attachment of K-bZIP to DNA (Fig. 7B, lanes 2 and 3). Using anti-His but not anti-HA antibodies, we could supershift the His-tagged K-bZIP–DNA complex (Fig. 7B, lanes 12 and 13). An excess of unlabeled double-stranded oligonucleotides corresponding to the PRDIII-I region of the IFN-β promoter efficiently competed with labeled probe for K-bZIP binding, as evidenced by the disappearance of the complex (Fig. 7B, lanes 6 and 7). In contrast, the incubation of K-bZIP in the presence of excess unlabeled double-stranded oligonucleotides corresponding to the PRDIV (Fig. 7B, lanes 4 and 5) or PRDII (lanes 8 and 9) region of the IFN-β promoter had marginal effects on the

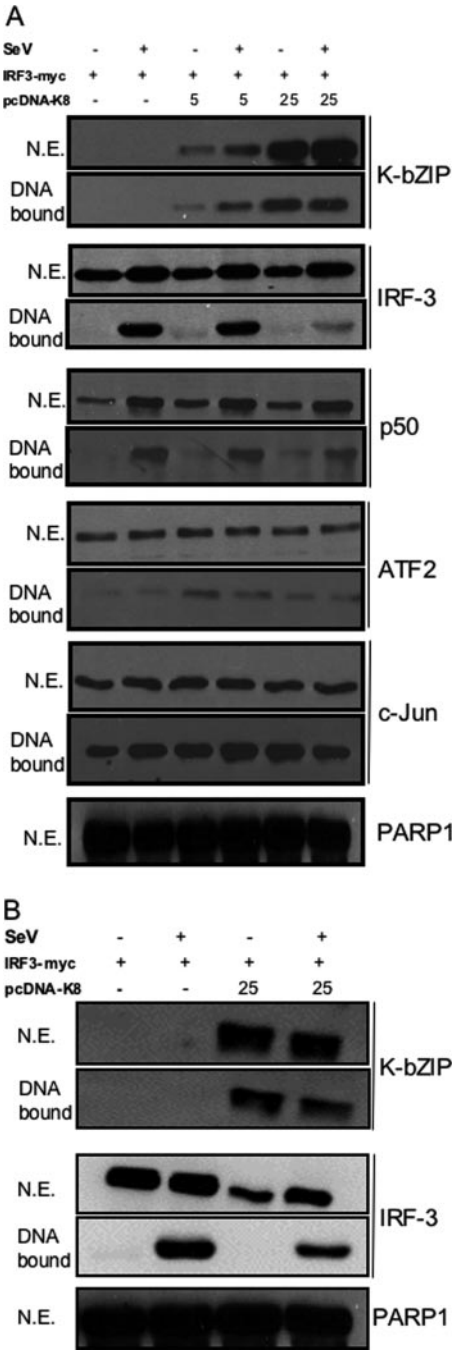

FIG. 5. K-bZIP binds and prevents IRF-3 from binding to the IFN-β and RANTES promoters. 293T cells were transfected with IRF-3–Myc and increasing amounts of K8 expression vectors. Twenty-four hours later, half of the cultures were infected with SeV for 18 h. Nuclear extracts (N.E.) were made and tested for binding to dsDNA oligonucleotides containing the IFN-β promoter sequence (A) and the ISRE region of the RANTES promoter (B). After the binding reaction, proteins were eluted and analyzed by Western blot using specific antibodies (left). Nuclear extracts were analyzed (5% of input used in the binding reaction) by Western blot to determine the amount of each protein in the nuclear fraction. PARP1 was used as a control to demonstrate that equal amounts of nuclear proteins were present in the various samples. The results are representative of three experiments.

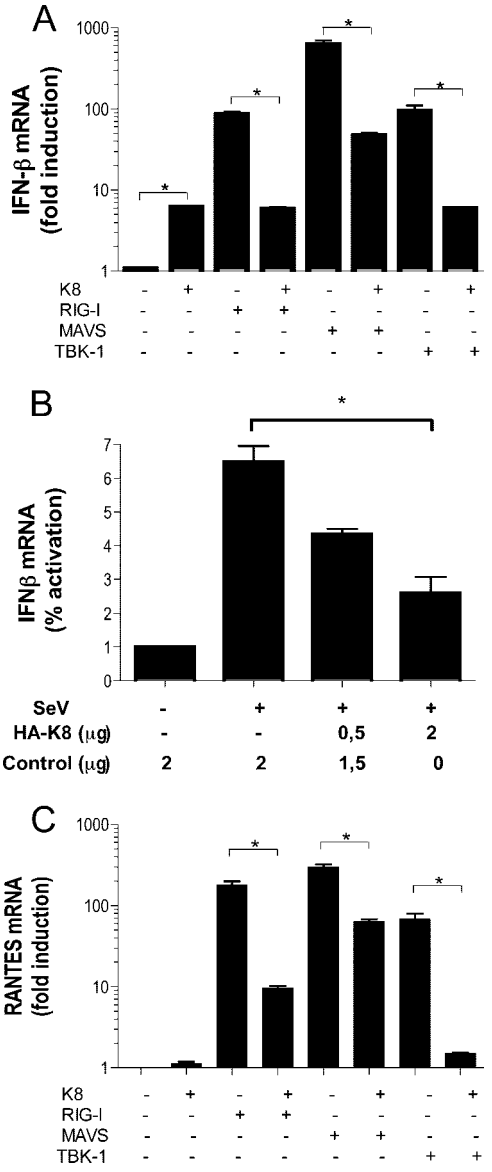

FIG. 6. K-bZIP represses *ifn*-β and *rantes* genes activation triggered by RIG-I, MAVS, and TBK-1. (A and C) 293T cells were transfected with expression plasmids coding for RIG-I, MAVS, or TBK-1 with or without pCMV3T-K8. Twenty-four hours posttransfection, total RNA was isolated and processed for IFN-β (A) and RANTES (C) mRNA by RT-QPCR as described in Materials and Methods. (B) A549 cells were transfected with increasing amounts of K8 expression vectors. Twenty-four hours later, the cultures were infected (or not) with SeV for 18 h, and total RNA was isolated and processed for IFN-β RT-QPCR. Results are expressed as mean (triplicate) induction (*n*-fold) ± SD relative to pCMV3T control transfected cells after normalization of samples with GAPDH mRNA expression. Results are representative of three independent experiments. *, $P < 0.01$.

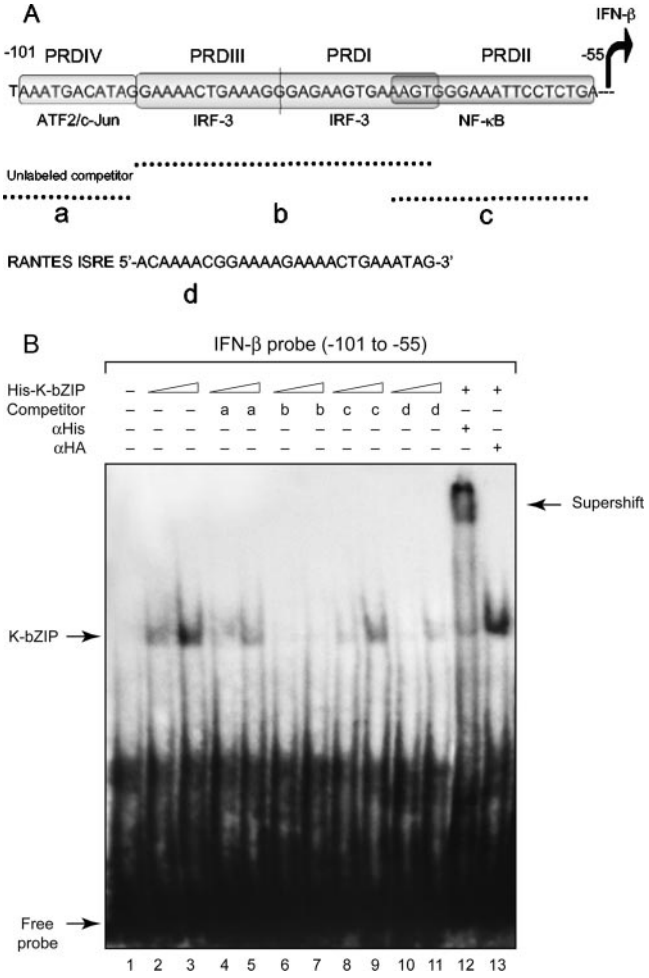

FIG. 7. K-bZIP binds directly to the PRDIII-I region of the IFN-β promoter. (A) Schematic representation of the region from positions −101 to −55 of the IFN-β promoter. Various PRD domains, transcription factor binding sites, and probes used in competition assays are presented. (B) EMSA for binding to a ^{32}P-labeled oligonucleotide probe containing the region of the IFN-β promoter from positions −101 to −55. Lanes: 1, free probe; 2 and 3, increasing amounts of His–K-bZIP binding to the IFN-β probe; 4 and 5, heterologous competition using an increasing dose of His–K-bZIP and unlabeled oligonucleotides corresponding to the PRDIV region (a) of the IFN-β promoter; 6 and 7, homologous competition using an increasing dose of His–K-bZIP and unlabeled oligonucleotides corresponding to the PRDIII-I region (b) of the IFN-β promoter; 8 and 9, heterologous competition using an increasing dose of His–K-bZIP and unlabeled oligonucleotides corresponding to the PRDII region (c) of the IFN-β promoter; 10 and 11, heterologous competition using an increasing dose of His–K-bZIP and unlabeled oligonucleotides corresponding to the ISRE region (d) of the RANTES promoter; 12, supershift of k-bZIP bound to PRDIII-I using anti-His antibodies; 13, lack of supershift of K-bZIP bound to PRDIII-I using anti-HA antibodies.

binding of K-bZIP to the IFN-β promoter-labeled probe. These experiments suggest that K-bZIP binds directly to the PRDIII-I region of IFN-β promoter and, in doing so, prevents the binding of IRF-3 to this promoter region, altering the correct formation of the enhanceosome complex. An excess of unlabeled oligonucleotides corresponding to the ISRE region of the RANTES promoter also competed for K-bZIP binding to the IFN-β promoter-labeled probe (Fig. 7B, lanes 10 and 11), suggesting that K-bZIP is also capable of directly binding to the RANTES promoter and interfering with its activation.

One mechanism by which K-bZIP is known to cause transcriptional repression is through the sequestration of CBP (13). To determine whether the inhibitory effects of K-bZIP on the *ifn*-β gene likely involves CBP sequestration, we coex-

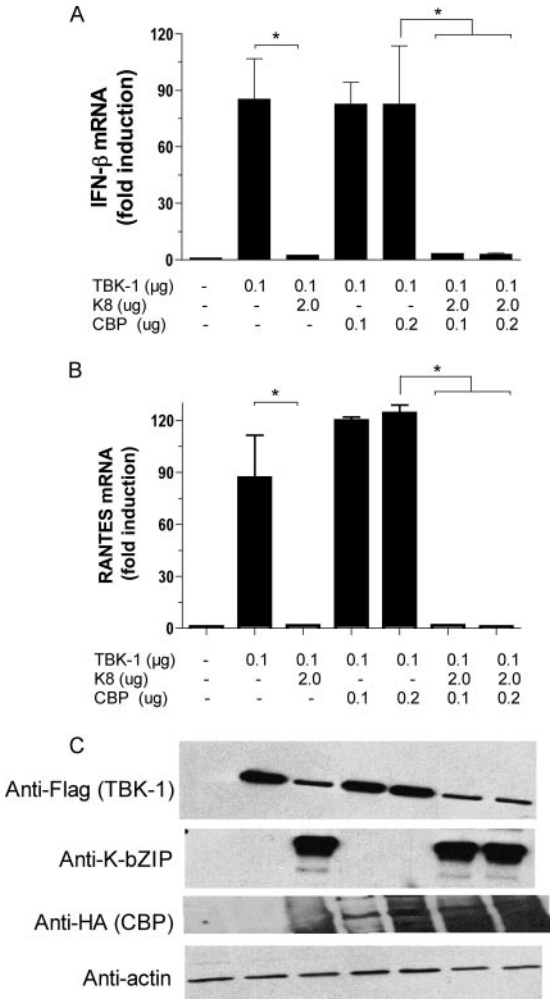

FIG. 8. Overexpression of CBP fails to reverse the repression of TBK-1-mediated *ifn*-β gene or *rantes* gene activation by K-bZIP. 293T cells were transfected with TBK-1 with or without pCMV3T-HA-K8 and CBP expression vectors. Total RNA and proteins were isolated 2 days after transfection, IFN-β or RANTES mRNA levels were determined by RT-QPCR, and protein levels were determined by Western blotting. Results are expressed as mean (triplicate) IFN-β induction (*n*-fold) ± SD relative to pCMV3T-transfected control cells after normalization of samples with GAPDH mRNA expression. Results are representative of two independent experiments. *, $P < 0.01$.

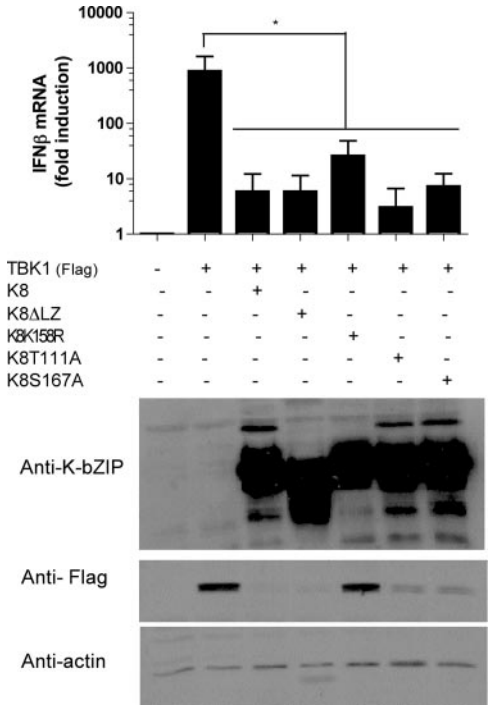

FIG. 9. The sumoylation-deficient K-bZIPK158R mutant and phosphorylation-deficient mutants inhibit TBK-1-mediated *ifn*-β gene activation. 293T cells were cotransfected with TBK-1 and pCMV3T-HA-K8, pCMV3T-Ha-K8K158R, pCMV3T-HA-K8ΔLZ, pCMV3T-HA-K8T111A, or pCMV3T-HA-K8S167A. Two days later, total RNA and proteins were isolated, the IFN-β mRNA level was determined by RT-QPCR, and protein levels were determined by Western blotting. Results are expressed as mean (triplicate) IFN-β induction (*n*-fold) ± SD relative to pCMV3T-transfected control cells after normalization of samples with GAPDH mRNA expression. Results are representative of three independent experiments. *, $P < 0.01$ relative to TBK-1-transfected cells.

pressed TBK-1 and K-bZIP together with an increasing amount of a CBP expression vector and monitored IFN-β and RANTES mRNA by RT-QPCR. As shown in Fig. 8, TBK-1 induced the transcription of the *ifn*-β (Fig. 8A) and *rantes* (Fig. 8B) genes very efficiently, with K-bZIP capable of repressing them by more than 90%. The addition of increasing quantities of a CBP expression plasmid had no impact on the ability of K-bZIP to repress TBK-1-induced *ifn*-β and *rantes* gene activation. The overexpression of CBP positively influenced TBK-1-induced *rantes* gene expression, indicating that CBP was functional. These results suggest that CBP sequestration by K-bZIP is likely not the mechanism by which K-bZIP represses *ifn*-β gene transcription. Interestingly, in this experiment, the protein expression levels of TBK-1 were reduced upon the concomitant expression of K-bZIP. This could be explained by the fact that K-bZIP is reported to act as a transcriptional repressor and therefore may negatively affect the expression of TBK-1 from the expression vector. However, the fact that K-bZIP did not influence the endogenous levels of TBK-1 (Fig. 3) and the fact that the K-bZIP K158R mutant had no impact on TBK-1 levels but inhibited TBK-1-mediated *ifn*-β gene activation as efficiently as wild-type K-bZIP (Fig. 9) suggest that the inhibitory effects of K-bZIP on TBK-1 cannot be accounted for by the observed drop in the TBK-1 protein level. Furthermore, when the amount of transfected pCMV3T-HA-K8 was reduced, K-bZIP had marginal effects on TBK-1 protein expression but retained its ability to inhibit TBK-1-induced IFN-β by >90% (data not shown).

The results shown in Fig. 6 indicate that K-bZIP is capable of repressing the induction of IFN-β triggered by RIG-I, MAVS, and TBK-1. A previous report suggested that the repressive activity of K-bZIP on RTA is linked to its sumoylation status (16). To determine whether the sumoylation of K-bZIP is linked to its ability to repress *ifn*-β gene activation, we tested the K158R mutant of K-bZIP that can no longer be conjugated to SUMO (16). As shown in Fig. 9, TBK-1 expression leads to a 750-fold increase in IFN-β mRNA. Wild-type K-bZIP efficiently blocked (>99%) TBK-1 activation of the *ifn*-β gene.

Similarly, the K158R sumoylation-negative mutant of K-bZIP, although somewhat less effective that wild-type K-bZIP, was still capable of suppressing (>95%) TBK-1-induced *ifn*-β gene activation (Fig. 9). Another report suggested that the phosphorylation of K-bZIP negatively impairs K-bZIP repression activity (17). As observed with the K158R mutant, the mutation of threonine 111 (T111A) and serine 167 (S167A) to alanine had no major impact on the ability of K-bZIP to modulate IFN-β mRNA levels. These results demonstrate that neither the sumoylation nor the phosphorylation status of K-bZIP is essential for its ability to repress the *ifn*-β gene when activated by TBK-1. Results shown in Fig. 9 also indicate that the other form of K-bZIP, K8β, behaves similarly to wild-type K-bZIP (K8α).

DISCUSSION

The KSHV genome encodes several proteins that interfere with the mounting of a rapid and efficient immune response. Apart from homologues of cellular cytokine (v-interleukin-6), chemokines (v-MIP-I, -II, and -III), and chemokine receptor (v-GPCR) that modulate angiogenesis and inflammatory responses, KSHV possesses several proteins dedicated to the blocking of IFN production and signaling. These include four proteins referred to as viral IRFs (for a recent review, see reference 31), K3, K5 (20), RTA (52), and ORF45 (55). We now add the K-bZIP transcription factor of KSHV to the long list of IFN-modulating proteins. K-bZIP is somewhat unique in the sense that its effect on IFN-β synthesis is dual, with transcriptional activation and repression observed depending on the experimental condition. First, when expressed by itself, K-bZIP activates the *ifn*-β gene as well as IFN-β promoter reporter constructs by approximately eightfold. *ifn*-β gene activation by K-bZIP occurs without a noticeable activation of IRF-3 and NF-κB transcription factors. Moreover, it remains as efficient in the presence of dominant negative forms of IRF-3 and IκBα, suggesting that the activation of these factors is not required for K-bZIP to activate *ifn*-β gene transcription. Thus, K-bZIP activates the *ifn*-β gene without the formation of the so-called *ifn*-β enhanceosome, which is made up of IRF-3, NF-κB, and ATF2/c-Jun. The detection of K-bZIP bound to the IFN-β promoter suggests that binding might be sufficient to activate the gene. Our data indicate that low-level binding of NF-κB and ATF2/c-Jun to the IFN-β promoter does occur in the presence of K-bZIP. Perhaps a tripartite complex composed of K-bZIP, NF-κB, and ATF2/c-Jun is sufficient to promote minimal transcriptional activation of the *ifn*-β gene. Second, in the presence of IFN-β inducers such as SeV, RIG-I, MAVS, or TBK-1, the K-bZIP protein behaves as a potent repressor of *ifn*-β gene activation. We routinely observed a reduction of greater than 90% in TBK-1-induced *ifn*-β gene activation in the presence of K-bZIP. One possible explanation for this effect relates to our data indicating that when K-bZIP is present, it efficiently prevents the binding of IRF-3 to the IFN-β promoter, precluding the formation of a functional enhanceosome and efficient gene transcription. The quantity of IRF-3 that reached to the nucleus in response to SeV infection is similar in the absence or in the presence of K-bZIP, indicating that the phosphorylation and translocation of IRF-3 occur normally and are unaffected by K-bZIP. Furthermore, we observed that K-bZIP not only affected the binding of IRF-3 on the IFN-β promoter but also prevented IRF-3 binding to the promoter of the *rantes* gene, coding for a cytokine with important roles in the recruitment of inflammatory cells. The direct binding of K-bZIP to DNA has not been formally proven, although chromatin immunoprecipitation assays suggest that K-bZIP associates with the viral episome near the replication origin and the early gene promoters (16, 22). To our knowledge, our results clearly show for the first time that K-bZIP is able to bind DNA directly (IFN-β and RANTES promoters), without the need of other cellular or viral factors.

Previous reports indicated that the sumoylation of K158 is linked with the repressive activity of K-bZIP (16). Our results obtained using the sumoylation-deficient K158R K-bZIP mutant indicate that this mutant was still very functional at suppressing IFN-β gene expression despite being approximately twofold less potent than wild-type K-bZIP. Sumoylation requires the physical interaction between ubiquitin-conjugating enzyme 9 (Ubc9) and the target protein (for a recent review, see reference 11). Although K158 lies outside the leucine zipper (residues 189 to 237), K-bZIP lacking the C-terminal zipper motif (K-bZIPΔLZ) no longer interacts with Ubc9 and does not appear to be sumoylated (16). K-bZIPΔLZ is also reported to be inapt at promoting transcriptional repression (16, 21). However, knowing that K-bZIP homodimerizes through its leucine zipper motif (26), it is also likely that K-bZIPΔLZ can no longer mediate transcriptional repression by its inability to dimerize. In contrast to the results reported previously by Liao et al. (21), our data indicate that K-bZIPΔLZ is as functional as wild-type K-bZIP at suppressing TBK-1-induced IFN-β gene expression. Several explanations could account for this apparent discrepancy. First, we studied cellular promoters, while Liao et al. studied the KSHV RTA promoter. Differences in regulatory elements between these promoters might account for the observed differences. Second, we measured endogenous gene expression, while Liao et al. used luciferase reporter genes. Third, we studied K-bZIP repressive activity using IFN-β inducers (RIG-I, MAVS, and TBK-1), while Liao et al. used KSHV RTA for the activation of gene expression. It is known that K-bZIP interacts and suppresses RTA activity through its leucine zipper motif (21). Although unlikely, given that K-bZIP suppresses several distinct IFN-β inducers, it is currently unknown whether K-bZIP physically interacts with these proteins. Our current working model is that K-bZIP directly binds to IRF-3 binding sites and occupies these sites, preventing the binding of activated IRF-3 dimers and causing impaired gene transcription. Our data also indicate that homodimerization is not essential for K-bZIP to mediate IFN-β gene repression, suggesting that binding to DNA can occur when K-bZIP is in a monomeric form.

K-bZIP is phosphorylated on threonine 111 and serine 167 (17). The major site of phosphorylation is threonine 111, and it can be phosphorylated by the cyclin-dependent kinase Cdk2 or the viral protein kinase vPK. In addition, the phosphorylation of T111 has a negative impact on K-bZIP repression activity (17). Our results indicating that the T111A, S167A, and K-bZIPΔLZ mutants are equally active at promoting transcriptional repression of TBK-1-mediated *ifn*-β gene activation compared to wild-type K-bZIP argue against a role for phosphorylation or dimerization under such circumstances. Current

studies to determine whether the different mutants of K-bZIP bind and prevent IRF-3 recruitment to the IFN-β promoter as efficiently as wild-type K-bZIP are ongoing.

Similarly, in the work reported previously by Liao et al., the overexpression of CBP could not reverse the K-bZIP-mediated repression of the TBK-1-activated *ifn*-β gene or the *rantes* gene (21), suggesting that this effect is independent of the sequestration or the inactivation of CBP by K-bZIP.

K-bZIP is not a universal transcriptional repressor. For instance, K-bZIP has no effect on the RTA-mediated activation of the PAN promoter (18). Furthermore, K-bZIP potentiates the transcriptional activation of p21 in cooperation with C/EBPα (49). K-bZIP therefore displays some specificity in its action. Our work indicates that K-bZIP can have an opposing effect on a gene depending on the cellular activation status. By interacting with the IFN-β promoter, K-bZIP causes the transcriptional activation of this gene. Although this may at first appear to be counterproductive for a virus to induce the secretion of a potent antiviral cytokine such as IFN-β, in doing so, K-bZIP strategically positions itself to prevent the binding of IRF-3 and counteract the assembly of functional transcriptional units on the *ifn*-β and *rantes* genes. The result is that less IFN and less RANTES are produced at early times after infection, when high levels of antiviral cytokines are susceptible to being made. One KSHV determinant that is susceptible to being recognized by viral sensing pathways is double-stranded viral genomic DNA. In fact, double-stranded DNA (dsDNA) detection by a recently identified Toll-like receptor-independent sensing pathway leads to substantial IFN-β production in an IRF-3-dependent manner (14, 43). In addition, herpesviruses have dsDNA genomes, and transcription originating on both DNA strands is reported to generate dsRNA molecules that are susceptible to being recognized by dsRNA sensors (2, 10, 37, 46). Thus, through its interaction with the IFN-β promoter, K-bZIP hinders the binding of IRF-3 that is activated in response to viral dsRNA or dsDNA and precludes efficient *ifn*-β gene activation.

Overall, our work indicates that the expression of K-bZIP causes the activation of the *ifn*-β gene. In doing so, K-bZIP ensures that robust *ifn*-β gene activation triggered by physiological inducers, such as dsRNA or dsDNA, is severely compromised. The result would be an infected cell undergoing active virus replication with minimal production and release of IFN-β. Our results add to the plethora of immune evasion mechanisms employed by KSHV to ensure its persistence within the infected host and help better understand the complex biology of human herpesviruses.

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