THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 37, pp. 26976–26984, September 15, 2006

IKK-i/IKKε Controls Constitutive, Cancer Cell-associated NF-κB Activity via Regulation of Ser-536 p65/RelA Phosphorylation*

Received for publication, April 3, 2006, and in revised form, June 19, 2006 Published, JBC Papers in Press, July 13, 2006, DOI 10.1074/jbc.M603133200

Mazhar Adli and Albert S. Baldwin[1]

From the Lineberger Comprehensive Cancer Center, Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599

Nuclear factor κB (NF-κB) has been studied extensively as an inducible transcriptional regulator of the immune and inflammatory response. NF-κB activation downstream of lipopolysaccharide or cytokine stimulation is controlled by the IκB kinase complex, which contains IKKα and IKKβ. Significantly, the constitutive activity of NF-κB has been implicated as an important aspect of many cancer cells, but mechanisms associated with this activity are poorly understood. An inducible kinase, IKK-i/IKKε, related to the catalytic forms of the IκB kinase, has been studied as an anti-viral, innate immune regulator through its ability to control the activity of the transcription factors IRF-3 and IRF-7. Here, we demonstrate that IKK-i/IKKε is expressed in a number of cancer cells and is involved in regulating NF-κB activity through its ability to control basal/constitutive, but not cytokine-induced, p65/RelA phosphorylation at Ser-536, a modification proposed to contribute to the transactivation function of NF-κB. Knockdown of IKK-i/IKKε or expression of a S536A mutant form of p65 suppresses HeLa cell proliferation. The data indicate a role for IKK-i/IKKε in controlling proliferation of certain cancer cells through regulation of constitutive NF-κB activity.

The transcription factor nuclear factor-κB (NF-κB)[2] plays a pivotal role in controlling the expression of a diverse set of genes that contribute to a variety of biological functions, including cell survival, cell proliferation, and immune and inflammatory responses (1). The classic form of NF-κB is composed of a heterodimer of the p50 and p65 subunits, which is preferentially localized in the cytoplasm as an inactive complex with inhibitor proteins of the IκB family. Following exposure of cells to a variety of stimuli, including inflammatory cytokines and LPS, IκBs are phosphorylated by the IKKα/β complex, polyubiquitinated, and subsequently degraded by the 26 S proteasome complex (1–3). Released NF-κB complexes then accumulate in the nucleus, where they transcriptionally regulate the expression of genes involved in the immune and inflammatory responses (3).

Based on a number of observations, it was assumed that virtually all inducers of NF-κB lead to the activation of a single classic IKKα/β/γ complex. However, recent studies demonstrated the existence of distinct IKK complexes that do not contain IKKα, -β, or -γ (4). One of these complexes was described as a PMA-inducible IκB kinase complex, with a critical component being an IKK-related kinase designated IKKε (5), which is identical to a kinase named IKK-i identified via its induction downstream of LPS-induced signaling (6). IKKε in turn is closely related to another recently discovered IKK-related kinase designated as TBK1 (TANK-binding kinase 1) (7) or NAK (NF-κB activating kinase) (8). TBK1, which is highly homologous to IKKε, binds to TANK and TRAF and may form an alternative IKK complex consisting of IKKε and TBK1 (7).

IKKε and TBK1 are enzymatically distinct from the homologous enzymes IKKα and IKKβ (9) and have been shown to play important roles in the innate immune response. These kinases function as critical components of the interferon regulatory factor 3 (IRF3) and IRF7 signaling pathways involved in responses to viral infection or dsRNA treatment (10, 11). Recent studies demonstrated that embryonic fibroblasts (MEFs) derived from TBK1-deficient ($TBK1^{-/-}$) mice show impaired production of NF-κB-dependent (12) as well as IRF3-dependent (13) gene expression. It has also been shown that IFN-β and IFN-inducible gene expression is defective in TBK1 knock-out cells in response to LPS, poly(I:C), or viral infection (14–16).

The relationships of IKKε and TBK1 with NF-κB activation remain enigmatic. Although recent studies defined their roles in IRF3 and IRF7 transcriptional activation (10, 11, 13) and suggested their involvement in NF-κB activation (7–9, 12, 17, 18), the exact molecular mechanism of NF-κB activation by these kinases is not clearly understood. One report (19) indicated that IKKε plays a key role integrating signals induced by pro-inflammatory stimuli by activating CAAT/enhancer-binding protein δ whose expression is regulated by NF-κB. There is a recent report suggesting that IKKε and TBK1 are among the

* This work was supported by National Institutes of Health Grants AI35098, CA73756, and CA75080 (to A. S. B.) and by the Waxman Cancer Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: 22-000 Lineberger Cancer Center, CB #7295, University of North Carolina School of Medicine, Chapel Hill, NC 27599. Tel.: 919-966-3652; Fax: 919-966-0444; E-mail: abaldwin@med.unc.edu.

[2] The abbreviations used are: NF-κB, nuclear factor κB; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; IRF, interferon regulatory factor; MEF, embryonic fibroblast; IFN, interferon; TNF, tumor necrosis factor; GFP, green fluorescent protein; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; EMSA, electrophoretic mobility shift assay; siRNA, small interference RNA; shRNA, small hairpin RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DKO, IKKε/TBK1 doubly deficient MEFs; WT, wild type; KM, kinase mutant.

kinases that mediate inducible phosphorylation of p65 at Ser-536 (19), an event proposed to stimulate inherent p65 transactivation function (20). In this model, TBK1 and IKKε would control NF-κB at a level distinct from the traditional IKK-mediated control of IκB degradation.

Here, we show that IKKε is expressed in a variety of cancer cell lines. Based on this, we have investigated a role for IKKε as related to constitutive, cancer-associated NF-κB activity. Our experiments reveal an important role for IKKε in controlling the activation of Ser-536 phosphorylation of the RelA/p65 subunit and functional NF-κB activity in several cancer cell lines and in 293T cells.

MATERIALS AND METHODS

Reagents and Materials—All cells were cultured in Dulbecco's modified Eagle's medium, complemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin. Generation of wild-type, IKKε single, and IKKε-TBK1 double knock-out cells was described previously (15), and they were the kind gift of S. Akira. A monoclonal antibody against FLAG (M2) was obtained from Sigma. An antibody to IKKε and to phospho-specific NF-κB p65 (Ser-536) were obtained from Cell Signaling. Antibodies to β-tubulin and to IκBα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to IKKα and IKKβ were obtained from Upstate Biotechnology Inc. LPS (L6529, Sigma) was used at a final concentration of 1 μg/ml. Recombinant human TNF-α (Promega) was used at a final concentration of 10 ng/ml. Recombinant mouse interleukin-1β (Roche Applied Sciences) was used at a final concentration of 10 ng/ml. Effectene transfection reagent obtained from Qiagen was used according to the manufacturer's protocol.

Plasmids and Constructs—3X-κB luciferase reporter construct contains 3 κB consensus DNA binding sites from the major histocompatibility complex class I promoter fused upstream of firefly luciferase. Wild-type and kinase mutant forms of FLAG-tagged IKKε (IKKε K38) have been described previously (5). Wild-type and kinase mutant forms of TBK1 (TBK1 K38) have been described previously (11) and were the kind gift of J. Hiscott. pLuc-110 IFNβ reporter constructs have been described previously (10) and were the kind gifts of T. Maniatis.

Transfections and Reporter Assays—For transient transfections, the indicated cell lines were seeded in 6-well plates at 30–50% density and transfected the next day with the indicated expression vectors for 48 h using Effectene (Qiagen) transfection reagent according to the manufacturer's instruction. For reporter assays, 2×10^5 cells were seeded in 24-well plates and co-transfected the next day with the indicated luciferase reporter genes and a β-galactosidase reporter gene as an internal control. The total amount of transfected DNA (500 ng of DNA) in each well was adjusted by adding empty plasmid vector (pcDNA3.1). Luciferase activity of whole cell lysates was measured by using a luciferase assay kit (Promega). β-Galactosidase activity was measured by liquid β-galactosidase assay with chlorophenolred-β-D-galactopyranoside substrate. Relative luciferase activity was calculated by normalizing the assay results to β-galactosidase expression values. Luciferase -fold induction was calculated by normalizing the results to control treatment, which was assumed as 1 -fold induction.

Western Blot—After stimulation, cultured cells were lysed on ice for 5 min in radioimmune precipitation assay lysis buffer with freshly added protease and phosphatase inhibitor cocktails. Lysates were cleared by centrifugation at 4 °C for 15 min at 13,000 × *g*. The amount of total protein was measured, and equal amounts (20 μg) were fractionated by NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen) and electrotransferred to polyvinylidene difluoride membranes. Membranes were blotted with the indicated antibodies, and proteins were detected using an enhanced chemiluminescence detection system (Amersham Biosciences). Where indicated, membranes were stripped and re-probed with the indicated antibodies.

Electrophoretic Mobility Shift Assay—EMSAs were performed as previously described (21). Briefly, 4–5 μg of nuclear extracts prepared following cell stimulation was incubated with a radiolabeled DNA probe containing an NF-κB consensus site. For supershifts, 1 μl of anti-p65 antibody (Rockland) or 2 μl of anti-p50 antibody (Santa Cruz Biotechnology, SC-7178) was added, and the binding reaction was allowed to proceed for an additional 15 min. Protein-DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by autoradiography.

siRNA and shRNA Transfection—IKKε mRNA was knocked down with the GeneSupressor System (IMGENEX). Plasmids encoding control shRNA and IKKε shRNA were transfected by Effectene transfection reagent (Qiagen) according to manufacturer's instructions for 48–72 h. Additionally, Silencer® predesigned siRNA targeting IKKε and TBK1 have also been utilized and were transfected with a Silencer® siRNA Transfection II kit. Lysate preparation and Western blots were performed as described.

Cell Proliferation and MTT Assay—A cell proliferation assay has been performed as described by using the TACS MTT assay kit (R&D Systems). First, the optimal cell number, which was 1×10^4 for HeLa cells in our system, was determined. After transfection, an equal number of cells was seeded in a 96-well plate for the indicated times. 10 μl of MTT reagent was added to each well, including the blank wells, and the mixture was incubated for 4–5 h at 37 °C. Then 100 μl of detergent reagent was added, the mixture was incubated for at least 12 h at 37 °C, and absorbance values at 570 nm were measured with a reference wavelength of 650 nm.

RESULTS

IKKε Is Expressed in a Number of Cancer Cells and in SV40 Large T-immortalized 293 Cells—To address a potential role for IKKε in controlling NF-κB activity, we explored the expression of IKKε in a variety of cell lines. Immunoblotting of extracts of several cancer cell lines revealed constitutive expression of IKKε in breast cancer cell lines MDA MB 468, SK BR3, Sum 226, and MCF7; HeLa cells; PC3 and LNCaP prostate cancer cells; and 293T embryonic kidney cells (Fig. 1). As a marker for endogenous NF-κB activity, we immunoblotted the extracts from these cells with an antibody that recognizes only the RelA/p65 subunit phosphorylated at Ser-536. These data revealed a correlation between IKKε expression and phosphorylated Ser-

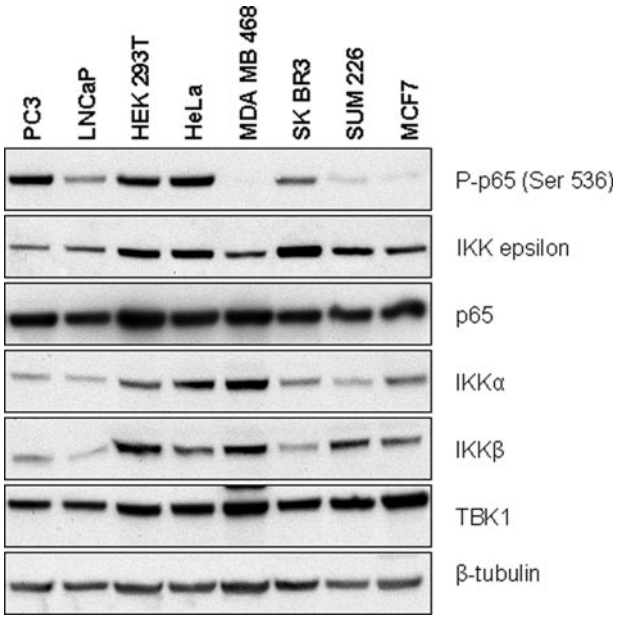

FIGURE 1. **IKKε expression is elevated in a number of cancer cells.** Total cell extracts from indicated cell lines were prepared and immunoblotted with the indicated antibodies. IKKε expression was elevated in many cancers cells and the level of the expression is well correlated with p65 phosphorylation at Ser-536. The levels of IKKα, IKKβ, and TBK1 are not consistently correlated with p65 phosphorylation level.

536 RelA/p65 in most of the cells analyzed. Notably, TBK1 was expressed in these cells but did not consistently correlate with RelA/p65 Ser-536 phosphorylation. The data also reveal that IKKε, although considered an inducible kinase, is found to be constitutively expressed at significant levels in most of the cell lines investigated.

IKKε or TBK1 Activates an NF-κB-dependent Reporter in a Kinase-dependent Manner—To investigate a potential role for IKKε and TBK1 in NF-κB regulation, experiments were initiated to analyze their potential involvement in controlling NF-κB-dependent promoters. Based on the results from Fig. 1, we focused these experiments on HEK 293T cells, because IKKε is expressed and is potentially active in these cells. Both IKKε and TBK1, but not their kinase mutant forms, activated the 3X-κB and IFN-β luciferase promoter constructs (Fig. 2, *A* and *B*). Unlike the 3X-κB promoter, the IFN-β promoter is considered a complex promoter regulated by coordinate actions of NF-κB and other transcription factors; therefore, it is not considered to be regulated exclusively by NF-κB. These results are similar to those of Shimada *et al.* (6). Interestingly, in all of the assays performed, TBK1 was observed to be a better activator of the reporters (Fig. 2, *A* and *B*). Analysis of the effects of different concentrations of IKKε on activation of the 3X-κB luciferase reporter showed that -fold induction of luciferase activity is proportional to the IKKε plasmid concentration, whereas there was no significant induction with the vector control or with the kinase mutant (KM) form of IKKε (Fig. 2*C*).

IKKε Expression Induces NF-κB DNA Binding Activity—We next investigated whether IKKε expression can induce NF-κB binding to a consensus DNA target sequence. FLAG-tagged IKKε was transiently expressed in HEK 293T cells for ~48 h, and nuclear extracts were prepared for EMSAs. As shown in

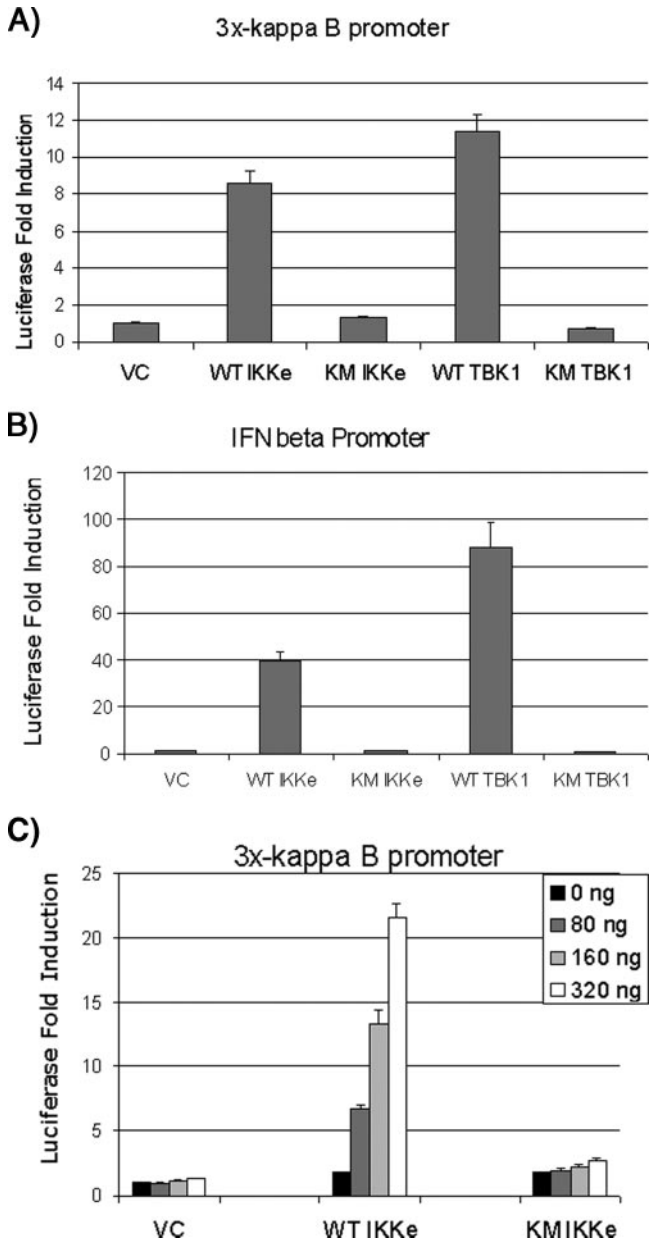

FIGURE 2. **IKKε and TBK1 but not their kinase mutant forms activate NF-κB-dependent reporter and IFNβ promoter.** HEK 293T cells seeded in 24-well plate were transiently co-transfected the next day with an expression vector for IKKε, TBK1, or their kinase mutant forms and vector encoding a reporter gene for 3x-κB reporter (*A*) and IFN-β promoter (*B*). Experiments were done at least twice in triplicate, and luciferase reporter gene activity was measured 24–48 h after transfection. *C*, IKKε activates NF-κB-dependent promoter in a concentration-dependent manner. The indicated concentration of IKKε and its kinase mutant form were co-transfected with 3x-κB reporter gene constructs in a 48-well plate.

Fig. 3*A*, IKKε effectively induced the DNA binding activity of NF-κB. TNF-α is included in this experiment for comparison purposes. It should be noted that there is basal NF-κB DNA binding activity in the VC lane (*lane 1*), which is better visualized given longer exposure times. TNF-α stimulation of IKKε-overexpressing cells led to more DNA binding activity, but this increase appears to be an additive effect of TNF-α and IKKε rather than a synergistic effect. Western blot analysis of cytoplasmic extracts, shown in the *lower panel*, demonstrates expression levels of IKKε. We next aimed to investigate the

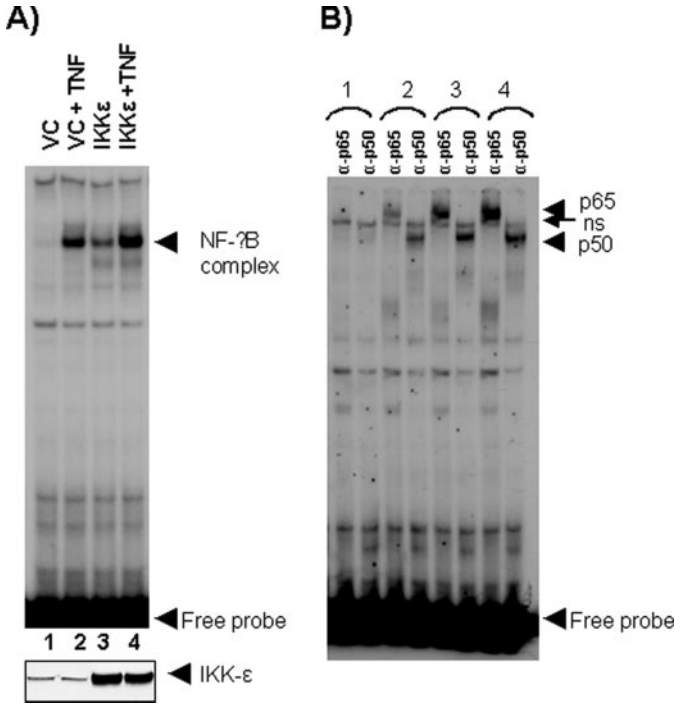

FIGURE 3. **IKKε induces significant NF-κB DNA binding activity.** *A*, HEK 293T cells seeded in 10-cm dishes were transfected with expression vector for IKKε or empty vectors for 48 h. The indicated cells were treated with 10 ng/ml TNF-α for 30 min. Protein-DNA complex was resolved by EMSA technique as described under "Materials and Methods." TNF-α is included in the experiment for control purposes. The NF-κB complex and the free probe are indicated by the *arrows*. Western blot analysis of cytoplasmic extracts is shown in the *lower panel* for the analysis of IKKε expression. *B*, NF-κB complex induced by IKKε and TNFα is mainly composed of p65 and p50 heterodimers. Each *lane* in *A* has been shifted with the indicated antibodies. The *numbers* at the *top* (*1–4*) show the lane numbers in *A*. RelA/p65 or p50 binding activity was assessed by incubation of 5 μg of nuclear extracts with either p65- or p50-specific antibodies followed by EMSA. In the lanes where an NF-κB complex is detected (*2–4*), there is a supershifted band with the p65 and p50 antibodies. *ns*, nonspecific binding.

nature of the major NF-κB subunits in this bound complex by gel shift assay. For this purpose each of the reactions used in Fig. 3*A* was incubated with the indicated antibody and electrophoresed on a separate gel (Fig. 3*B*). The number at the *top* of the figure indicates the lane numbers (*lanes 1–4*) from the reactions used in Fig. 3*A*. In the lanes where an NF-κB complex was detected (*lanes 2–4*), there was a positive response with the p65 and p50 antibodies. Therefore, we concluded that the complex that bound to this consensus binding site is composed predominantly of p65/p50 heterodimers (Fig. 3*B*). It should be noted that a single nucleotide change can lead to binding of different NF-κB subunits (22, 23); therefore, we cannot exclude the possibility that other subunits might also be activated and bind to slightly different NF-κB binding sites. TBK1 expression effects on DNA binding activity of NF-κB yielded very similar results (data not shown).

IKKε and TBK1 Expression Leads to Phosphorylation of Endogenous p65 at Ser-536—Recent studies have shown that post-translational modification of NF-κB subunits, such as p65, contribute significantly to NF-κB transactivation potential (reviewed in Ref. 3). Phosphorylation of p65 at Ser-536 is proposed to be a key modification that potentiates p65 transactivation function, hence NF-κB activation (20, 24). We next tested if IKKε and TBK1 affect p65 phosphorylation. We tested whether ectopically expressed IKKε leads to phosphorylation of endogenous p65 at Ser-536. Expression of GFP-IKKε leads to a significantly higher level of p65 phosphorylation at the Ser-536 position (indicated as P-p65 (Ser-536)) (Fig. 4*A*). Utilization of GFP-tagged IKKε expression vector allows for distinguishing between ectopically expressed IKKε (GFP-IKKε) and endogenous IKKε. This experiment reveals that IKKε induces higher levels of endogenous IKKε (Fig. 4*A*), which has been proposed to be regulated by NF-κB (25). This observation suggests that ectopically expressed IKKε is able not only to induce phosphorylation of endogenous p65 but also to induce NF-κB-dependent gene expression. Next we examined if TBK1, an IKKε homolog, will also induce Ser-536 phosphorylation of p65. As shown in Fig. 4*B*, WT forms of both IKKε and TBK1 induced p65 Ser-536 phosphorylation; however, their kinase mutant forms (Lys-38 → Ala) did not lead to phosphorylation of p65. Indeed when analyzed in detail, kinase mutant forms appear to inhibit the basal level of endogenous Ser-536 phosphorylation (compare *lane 1* to *lanes 4* and *6*). It is important to note that the phospho-p65 Ser-536 antibody specifically detected only the phosphorylated form of p65 and did not cross-react with unphosphorylated p65 (see Fig. 4*B*).

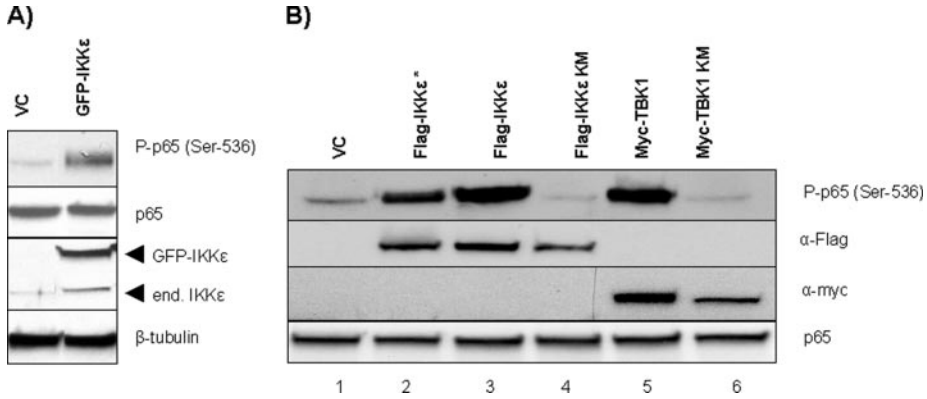

FIGURE 4. **IKKε and TBK1 expression leads to phosphorylation of endogenous p65 at Ser-536.** *A*, IKKε expression induces p65 phosphorylation and its own expression. HEK 293T cells were transiently transfected with 1 μg of the GFP-IKKε expression vector. Whole cell lysates were prepared 24 h after transfection and blotted with p65 phospho-specific antibody that detects phosphorylation at Ser-536 position (indicated as P-p65 (Ser-536)). Notably, endogenous IKKε expression is induced by ectopically expressed GFP-IKKε (indicated by *arrows*). The membrane was blotted with anti-p65 and anti-β-tubulin antibodies to demonstrate equal loading. *B*, WT IKKε and TBK1 but not their kinase mutant forms phosphorylate p65 at Ser-536 position. HEK 293T cells seeded in 6-well plate were transiently transfected with 1 μg of the WT FLAG-IKKε, KM FLAG-IKKε, WT myc-TBK1, and KM myc-TBK1 expression vectors. WT IKKε and TBK1 but not their kinase mutant forms induce p65 phosphorylation. Membranes were also blotted with anti-FLAG and anti-myc for IKKε and TBK1 expression. FLAG-IKKε* is WT IKKε with a mutation at 501 position (Thr-501 → Ala), which was thought to control IKKε activity; however, the mutation did not change its activity in terms of phosphorylating p65.

Analysis of Inducible p65 Ser-536 Phosphorylation in IKKε-deficient Cells—Observing that exogenous IKKε induces p65 phosphorylation, we hypothesized that this post-

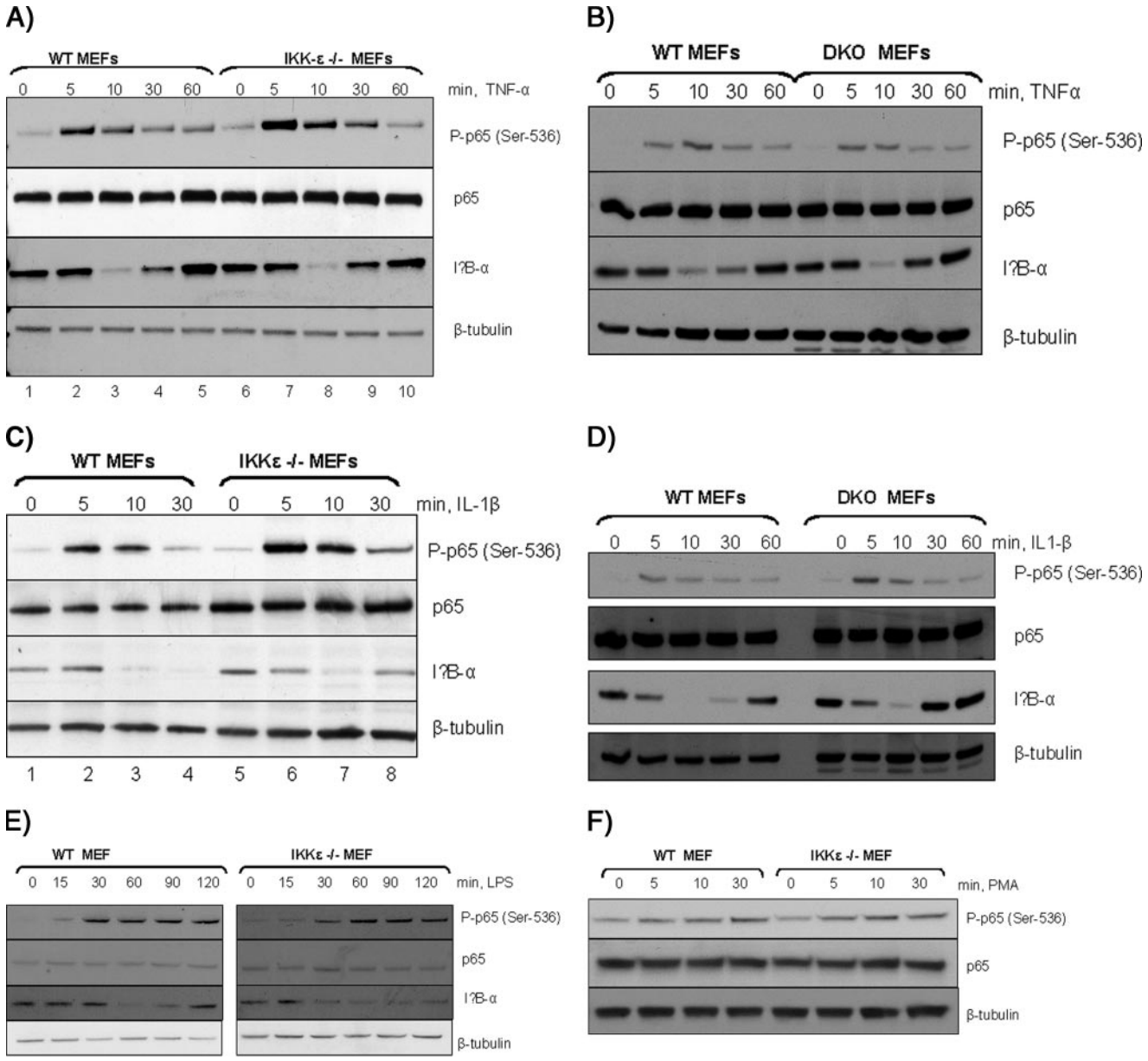

FIGURE 5. **p65 phosphorylation at Ser-536 position is normal in IKKε-deficient MEFs in response to different stimuli.** Cytokine induced p65 phosphorylation in IKKε singly deficient and IKKε and TBK1 doubly deficient MEFs is comparable to WT MEFs. Indicated MEF cells were seeded in 6-well plates, and at 70–80% confluency they were stimulated with 10 ng/ml TNFα (*A* and *B*), 10 ng/ml IL-1β (*C* and *D*), 1 μg/ml LPS (*E*), and 100 ng/ml PMA (*F*) for indicated time points. Whole cell extracts were prepared and blotted with indicated antibodies. Interestingly, in all of the stimuli tested, there was no significant difference in terms of either p65 phosphorylation pattern or IκBα degradation kinetic in IKKε-deficient or DKO MEFs compared with WT MEF cells. Analysis of β-tubulin levels confirmed that loading was essentially equivalent in all lanes.

translational modification on p65 might be defective in IKKε-deficient MEFs in response to NF-κB inducers. To test our hypothesis, IKKε-deficient and IKKε and TBK1 doubly deficient MEFs (DKO) were stimulated with TNFα, a well known NF-κB inducer, and compared with similarly treated WT MEFs. As seen in Fig. 5*A*, p65 was phosphorylated at the Ser-536 position in response to TNF-α as early as 5-min post-stimulation. Interestingly, IKKε-deficient cells showed essentially the same pattern of phosphorylation kinetics in response to TNFα stimulation. In addition to p65 phosphorylation, IκBα degradation was also normal in IKKε-deficient cells compared with WT MEF cells. Analysis of β-tubulin levels confirmed that loading was essentially equivalent in all lanes (Fig. 5*A*). To determine if the loss of TBK1 together with IKKε would effect the phosphorylation of p65 and IκBα degradation, WT MEFs and MEFs deficient for both IKKε and TBK1 (double knock-out MEFs (DKO)) have been used under similar experimental conditions. Both p65 phosphorylation and IκBα degradation were normal in DKO MEFs (Fig. 5*B*). This result indicated that NF-κB activation, as measured by p65 phosphorylation and IκBα degradation in response to TNF-α, is independent of IKKε and TBK1.

We next tested the effect of IL-1β on p65 phosphorylation as well as IκB degradation (Fig. 5*C*). IL-1β is, like TNF-α, a well known inducer of NF-κB. In as early as 5 min, p65 was phosphorylated maximally, however, maximal IκBα degradation

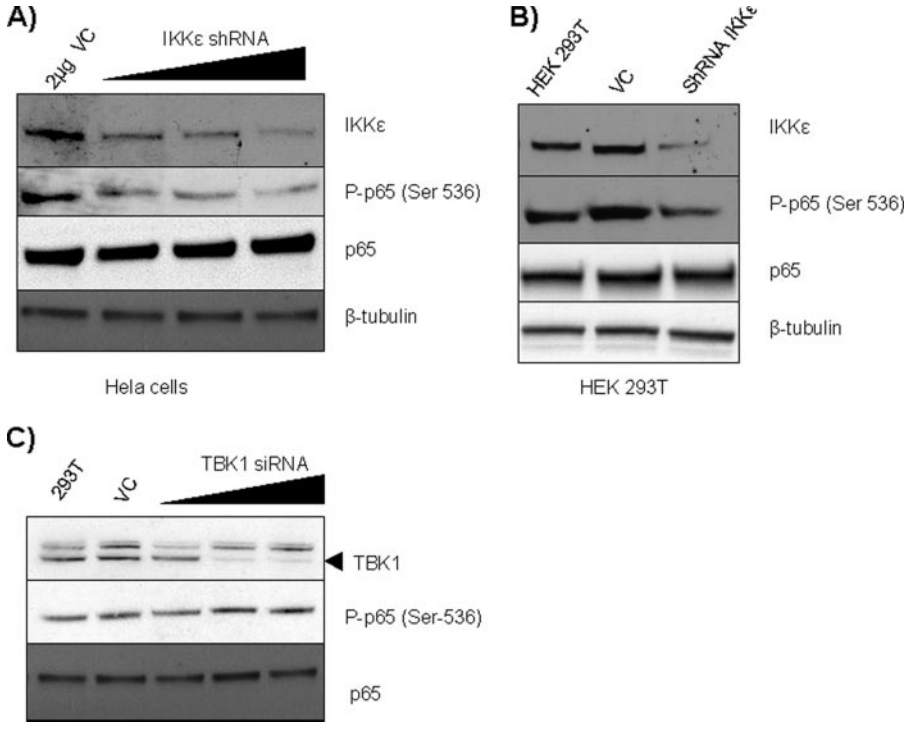

FIGURE 6. **Sustained knockdown of IKKε leads to impaired Ser-536 phosphorylation of p56.** *A* and *B*, vector encoding shRNA targeting IKKε or vector control (*VC*) alone was transfected in HeLa cells (*A*) in 6-well plate with increasing concentration of IKKε shRNA vector (1, 2, and 3 μg/well shRNA plasmid) or in HEK 293T cells (*B*) for 72 h. Lysate preparation and Western blots were done as described above. A significant knockdown in IKKε level was well correlated with reduction in p65 Ser-536 phosphorylation, whereas there is no change in total p65 level. β-tubulin level is shown to demonstrate equal loading in all lanes. *C*, siRNA targeting TBK1 was transfected in HeLa cells for 72 h. As shown, despite the significant reduction in TBK1 level, No significant change in p65 phosphorylation was observed.

was observed in 10 min. It is interesting again to observe that both p65 phosphorylation at Ser-536 and IκBα degradation were normal in IKKε-deficient cells compared with WT cells. In parallel studies, experiments were also performed in cells where both IKKε and TBK1 were deleted (DKO), and we observed no significant difference from WT cells relative to Ser-536 p65 phosphorylation or IκBα degradation after stimulation with TNF-α or IL-1β (Fig. 5*D*). These results suggest a minimal role of IKKε and TBK1 in cytokine-induced p65 phosphorylation and IκBα degradation.

The pathway to NF-κB activation in response to LPS has been characterized in molecular detail resulting in the discovery of a novel family of adapter proteins, which serve to regulate and polish up toll-like receptor responses. The first identified member of this adapter family was MyD88 (26). The importance of MyD88 in toll-like receptor signaling has been confirmed by the inability of MyD88-deficient mice to respond properly to a variety of toll-like receptor ligands, namely LPS, peptidoglycan, and bacterial CpG motifs (27, 28). Interestingly, analysis of MyD88-deficient cells in response to LPS demonstrated the existence of MyD88-independent, late NF-κB activation, and the induction of IRF-3-dependent genes, which has recently been verified to be regulated by IKKε and TBK1 kinases (11, 13, 15). In our experimental system, LPS stimulation of WT MEFs and IKKε-deficient cells showed similar levels of inducible p65 phosphorylation (Fig. 5*E*). As expected, the kinetics of the phosphorylation upon LPS stimulation is not as fast as TNF-α and IL-1β, but the pattern of phosphorylation is similar between WT and IKKε-deficient cells. In addition to p65 phosphorylation, IκBα degradation was also analyzed, and there was no defect in this process. We next tested the effect of PMA stimulation on p65 phosphorylation and did not observe any difference between WT and IKKε-deficient MEFs (Fig. 5*F*). Overall our data indicate that inducible Ser-536 phosphorylation is unaffected in IKKε-deficient cells and confirm that IKKε is not significantly involved in mechanisms associated with cytokine-, LPS-, or PMA-induced IκBα degradation.

IKKε Controls Constitutive p65 Ser-536 Phosphorylation—It was surprising to observe that IKKε and TBK1 expression led to the phosphorylation of endogenous p65 but that MEFs deficient for these kinases depict normal phosphorylation patterns. Because we had observed a correlation between IKKε and Ser-536 in certain cancer cells, we therefore hypothesized that these kinases might be involved in basal or constitutive p65 phosphorylation. Because the basal or constitutive level of p65 phosphorylation is quite low in MEF cells, the potential that IKKε could contribute to basal/constitutive levels of Ser-536 phosphorylation was investigated in HeLa and HEK 293T cells. These cells have higher levels of IKKε expression, constitutive p65 phosphorylation, and NF-κB activation compared with MEFs. To knock down IKKε, both plasmid-based shRNA and normal siRNA technologies have been utilized against IKKε mRNA. Additionally, an identical control plasmid, which contains a scrambled sequence with no homology to any known human gene product, has been utilized. Extracts from control-treated and siRNA-treated HeLa cells were analyzed for IKKε knockdown as well as for endogenous p65 Ser-536 phosphorylation. Our results indicated that transfection of shRNA against IKKε leads to sustained effective knockdown of IKKε. Furthermore, the reduction in IKKε protein level is well correlated with significant reduction in the basal level of Ser-536 phosphorylation in HeLa cells as compared with vector control-treated cells (Fig. 6*A*). Quantitative real-time-PCR analysis showed more than a 70% reduction in *ikkε* mRNA in HeLa cells when transfected with 2 μg of shRNA plasmid (data not shown). To show that this is not a cell line-specific observation, similar experiments have been performed in HEK 293T cells. In cells where the shRNA plasmid was transfected, IKKε level were significantly reduced. Again, a significant reduction in Ser-536 phosphorylation of p65 was observed, whereas vector control-treated cells exhibited no change in IKKε levels or Ser-536 phosphorylation (Fig. 6*B*). These results demonstrate that IKKε has a significant role in controlling the basal/consti-

tutive p65 phosphorylation at Ser-536 position in two cell lines. Interestingly, knockdown of TBK1 with siRNA did not show a significant change in p65 phosphorylation at Ser-536 position (Fig. 6*C*). This result indicates the differential role of IKKε and TBK1 in terms of controlling basal/constitutive p65 phosphorylation, at least in the cell types analyzed.

IKKε Knockdown in HeLa Cells Results in Reduced Constitutive Activity of an NF-κB-dependent Promoter—To better elucidate the role of IKKε in controlling NF-κB activity, and to determine if reduction in p65 phosphorylation upon IKKε knockdown is correlated with reduced constitutive NF-κB activity, we have analyzed NF-κB-dependent luciferase reporter assays in HeLa cells. These cells were transfected with either negative control siRNA and or with IKKε siRNA for 48 h followed by transfection with the 3x-κB luciferase promoter construct. Importantly, knockdown of IKKε in a dose-dependent manner resulted in reduced promoter activity as compared with the control construct (Fig. 7). This result indicates that IKKε controls a significant portion of NF-κB-dependent activity in HeLa cells, presumably through its ability to control Ser-536 p65 phosphorylation.

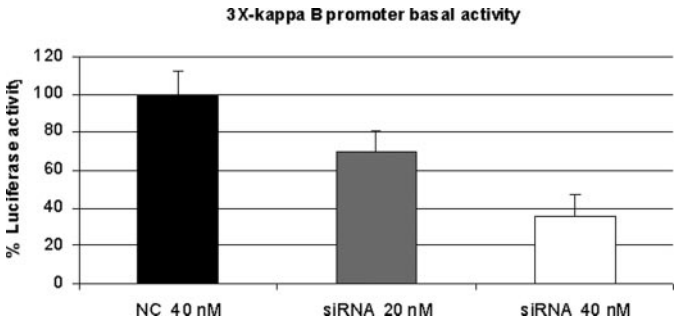

FIGURE 7. **IKKε knockdown in HeLa cells leads to reduction in basal activity of NF-κB dependent promoter.** HeLa cells seeded in 6-well plates were transfected by siRNA targeting IKKε or negative control (*NC*) siRNA at the indicated concentrations for 48 h. Then cells were split into 24-well plates and were transfected with 3X-κB promoter and *Renilla* luciferase promoter as an internal control. siRNA was also included in this transfection complex to give a second hit for IKKε knockdown. At indicated concentrations of siRNA (20 and 40 nM), knockdown efficiency of IKKε mRNA was 58 and 79%, respectively, as measured by quantitative real-time-PCR (data not shown). Reduction in IKKε level is well correlated with the reduction in basal activity of an NF-κB-dependent promoter.

IKKε and p65 Phosphorylation Positively Mediate HeLa Cell Proliferation—After observing that IKKε controls basal p65 phosphorylation and NF-κB activity in certain cancer cells, we asked if IKKε provides cell growth/survival functions. For this purpose, MTT cell proliferation assays were performed in HeLa cells transfected with shRNA against IKKε or with a non-phosphorylatable form of p65 (S536A) expression construct. For the data presented in Fig. 8 (*A* and *B*), HeLa cells were seeded in 6-well plates and were transfected the next day with the indicated plasmids. 24 h after transfection, cells were reseeded on 96-well plates, and the MTT proliferation assay was performed 24 and 48 h later. When IKKε was knocked down by shRNA, a significant reduction in cell proliferation was observed at the 48-h time point as compared with control cells transfected with the scrambled shRNA plasmid (Fig. 8*A*). In a similar experimental setting, we tested the effect of IKKε-mediated p65 phosphorylation on cell proliferation. For the purpose, IKKε was transfected with WT or a p65 mutant (S536A) that cannot be phosphorylated at the Ser-536 position. MTT assays read after 24 and 48 h shows that cells with mutant p65 do not proliferate efficiently when compared with cells expressing WT p65. This experiment indicates that p65 phosphorylation at Ser-536 is important for HeLa cell proliferation.

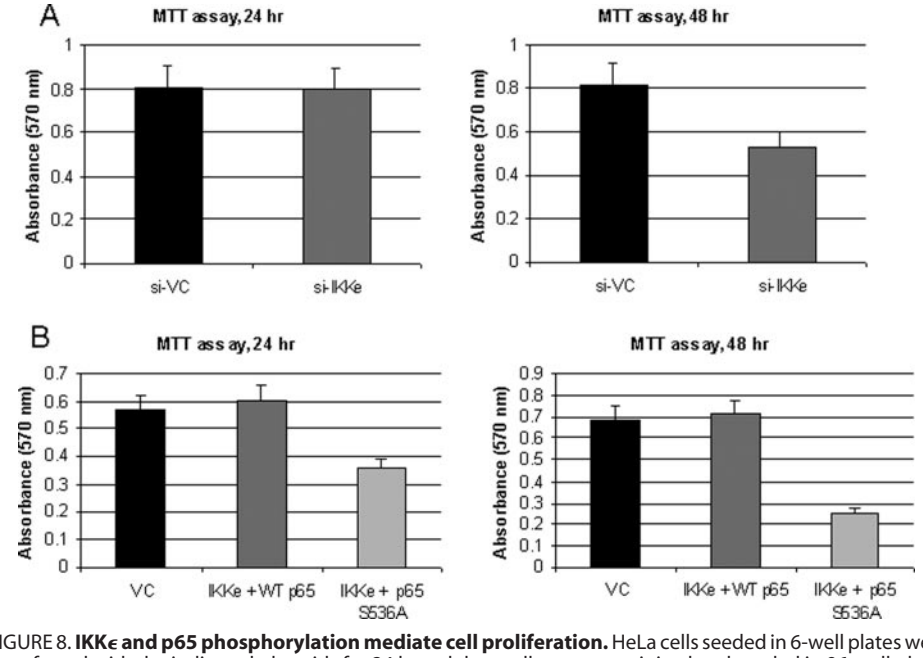

FIGURE 8. **IKKε and p65 phosphorylation mediate cell proliferation.** HeLa cells seeded in 6-well plates were transfected with the indicated plasmids for 24 h, and then cells were trypsinized and seeded in 96-well plates in triplicates. After the indicated time points, an MTT assay was performed. Results are representative of two independent experiments done in triplicates. When the IKKε level was modified by shRNA, cell proliferation was not as efficient as control shRNA-transfected cells. IKKε knockdown with shRNA after 48 h reduced cell proliferation significantly (*A*). *Si-VC* and *Si-IKKε* represent vector control shRNA and shRNA against IKKε, respectively. IKKε was also overexpressed together with WT and S536A mutant version of p65 to test the significance of IKKε-mediated p65 phosphorylation (*B*). Although IKKε- and WT p65-transfected cells proliferated slightly better than vector control-transfected cells, when IKKε was overexpressed with the S536A mutant version of p65, which cannot be phosphorylated at the Ser-536 position, there was significantly less cell proliferation. These data suggest that both IKKε level and p65 phosphorylation status are important for proper cell proliferation.

DISCUSSION

The majority of studies analyzing NF-κB activation have focused on induction of this transcription factor downstream of cytokine or LPS-dependent signaling. This response is generally dependent on the classic IKK complex, containing IKKα and IKKβ. Additional evidence has indicated that, besides the nuclear translocation of NF-κB, post-translational modifications, like p65 phosphorylation, are required to efficiently activate NF-κB-dependent gene transcription (20, 29–34). It is also well established that a number

of cells, particularly those of cancerous origin, exhibit significantly elevated levels of basal or constitutive NF-κB activity. In many cases, the origins of this activity remain unclear.

Here we show that several cancer cell lines, along with the SV40 large T-immortalized 293 cell line, exhibit relatively high levels of expression of IKKε. This is interesting, because IKKε is normally considered a kinase that is induced quantitatively by LPS or cytokines. We have investigated a potential role for IKKε and TBK1, kinases homologous to the catalytically active IKKα and IKKβ subunits, in controlling NF-κB activity, with the focus being phosphorylation of p65 at the Ser-536 position. Experiments were initiated to study IKKε- and TBK1-induced NF-κB-dependent promoter activation.

In agreement with previous results (10, 11), IKKε and TBK1, but not their kinase mutant forms, strongly activate NF-κB-regulated reporter constructs. It is important to note that, unlike the 3X-κB promoter, the IFN-β promoter is a complex promoter regulated by coordinate actions of NF-κB and other transcription factors, therefore it is not considered to be regulated exclusively by NF-κB. To confirm our reporter assays, gel shift assays have been performed. As expected, IKKε and TBK1 induced significant NF-κB DNA binding activity. Supershift assays identified p65 and p50 as main subunits of NF-κB complex. Recent studies have shown that post-translational modification of NF-κB subunits, such as p65 phosphorylation, contribute significantly to NF-κB activation. Phosphorylation of p65 at Ser-536 is proposed to be a key modification that potentiates p65 transactivation function, and hence NF-κB activation ability (20, 24, 32).

Recently, it has been reported that overexpression of IKKε or TBK1 together with p65, leads to the phosphorylation of ectopically expressed p65 at Ser-536 (19), however, this group has not analyzed the endogenous phosphorylation at Ser-536 of p65. Our results clearly support the hypothesis that the kinase activity of IKKε and TBK1 may significantly contribute to the constitutive level of S536 phosphorylation of p65. We have also observed that IKKε induces its own expression, which has been shown to be regulated by NF-κB. These data indicate that ectopically expressed IKKε induces p65 phosphorylation, NF-κB activation, and NF-κB-dependent gene expression. It also raises the possibility that IKKε functions in an autoregulatory loop, leading to its own expression.

It was interesting to observe that IKKε-deficient cells show a normal pattern of cytokine-inducible phosphorylation of p65 and IκBα degradation when compared with WT MEFs. We have tested a series of well known NF-κB inducers (IL-1β, LPS, and PMA) that are known to activate NF-κB by utilizing different signaling pathways. Compared with WT MEFs, IKKε-null cells (and DKO MEFs for both IKKε and TBK1) allowed inducible RelA/p65 phosphorylation to the same extent. Why is there no defect in inducible Ser-536 phosphorylation of p65 in IKKε-deficient cells? The first plausible explanation to this question is that the classic IKK signalsome complex is still intact in IKKε-deficient cells. Therefore, this complex likely compensates for the loss of IKKε. Secondly, there are other known and unknown kinases, in addition to IKK complex, that have been claimed to mediate Ser-536 phosphorylation of p65 (20, 34, 35), and in the same way, they may still induce phosphorylation in IKKε-deficient cells. The third explanation to this question is that IKKε and TBK1 are not involved in cytokine-induced p65 phosphorylation, but rather they are involved in the basal/constitutive level of p65 phosphorylation. In one set of experiments, this possibility has been investigated. Because MEF cells have low levels of basal p65 phosphorylation and low levels of IKKε, the potential that IKKε and TBK1 might be involved in constitutive p65 phosphorylation has been investigated in HeLa and HEK 293T cells that demonstrate higher levels of IKKε and constitutive p65 phosphorylation at Ser-536 position. IKKε was knocked down by plasmid-based shRNA technology in both HeLa cells and HEK 293T cells. Importantly, shRNA transfection leads to sustained knockdown of IKKε, and more importantly, reduction in the IKKε level is well correlated with significant reduction in the basal level of Ser-536 phosphorylation (Fig. 6), whereas in vector control-transfected cells there is no change in IKKε level and Ser-536 phosphorylation of p65. Interestingly knockdown of TBK1 did not reduce the basal level of p65 phosphorylation. Surprisingly these data suggest that TBK1 and IKKε are not entirely orthologues at least in controlling basal phosphorylation of p65. As suggested earlier (15), IKKε and TBK1 might not be redundant in every signaling pathway that they affect. Thus the data presented here clearly show that IKKε does not mediate cytokine-induced p65 phosphorylation at the Ser-536 position but has a significant role in basal and constitutive phosphorylation of p65 at least in certain cancer cells and in 293T cells. Basal p65 phosphorylation is well correlated with constitutive NF-κB activity, which has been implicated in the pathogenesis of many diseases, including cancer. The first evidence to our conclusion that IKKε mediates constitutive NF-κB activity came from a recent study published while this report was in preparation (36). In that study, Eddy *et al.*, provided evidences that IKKε contributes to the pathogenesis of breast cancer. Expression of a kinase-inactive form of IKKε blocked breast cancer cell colony formation. The results presented in that study are consistent with the findings presented here. We have shown that IKKε contributes to the basal/constitutive p65 phosphorylation and NF-κB activity as measured by EMSA and NF-κB-driven luciferase promoter activity. Furthermore, we have also shown that knockdown of IKKε or overexpression of mutated version of p65 (S536A) negatively effects the cell proliferation. These findings indicate an important role of IKKε and p65 phosphorylation in cancer cell proliferation.

There have been reports suggesting that IKKε and TBK1 may function as IκB kinase kinases (8, 37). Therefore they might function upstream of the classic IKK complex (IKKα/β/γ). So we questioned if the phosphorylation of p65 at Ser-536 is a direct or indirect effect of IKKε/TBK1. In other words, IKKε and TBK1 might have activated classic IKKs, which then lead to the phosphorylation of p65. To test this, we tried to detect activation of IKKβ and IKKα by probing the same blots in Fig. 4 with commercially available, phospho-IKKα/β antibodies; however, we could not detect any phosphorylation (data not shown). Although we cannot rule out that IKKε or TBK1 might function as IκB kinase kinases, our data support a model where these two kinases are direct effectors of p65 activation. There is evidence in the literature supporting this model. First of all, it

has been clearly shown that stimulus-coupled IκB degradation and p65 nuclear translocation and DNA binding activity of NF-κB is normal in IKKε and TBK1-deficient cells despite the fact that there is impaired NF-κB-dependent gene transcription (15, 16, 38). This evidence supports the fact that the activity of IKKα and IKKβ is normal in IKKε- or TBK1-deficient cells, because there is normal IκB degradation, normal p65 nuclear translocation, and normal DNA binding activity. We believe that our data, together with these findings, emphasize the RelA/p65 as the physiological target of IKKε and TBK1 under basal growth situations, at least in certain cells. If IKKε and TBK1 were upstream of classic IKKs, one would expect a deficiency in one of the above processes which are tightly regulated by IKKα and -β.

Very recently an article has been published indicating that IKKε mediates inducible phosphorylation of NF-κB p65 at serine 468 but not at serine 536 during T-cell co-stimulation (39). However, the outcome of the phosphorylation at Ser-468 by IKKε has not been shown, and the phosphorylation at this site by glycogen synthase kinase-3β has been claimed by the same group to be associated with negative NF-κB regulation (40).

To summarize, we have provided evidence that IKKε, and not TBK1, controls the constitutive NF-κB activity in certain cancer cells and in 293T cells. This evidence is supported by siRNA experiments and by associated reporter studies. It is presently unclear whether IKKε functions separately from the classic IKK complex, or through distinct regulatory pathways. It is also unclear whether IKKε is the kinase that directly controls Ser-536 p65 phosphorylation. Nevertheless, the data indicate a potentially important role for IKKε in controlling at least part of the constitutive NF-κB activity generated in certain cancer cells, with subsequent downstream effects on cancer cell proliferation.

Acknowledgments—We gratefully acknowledge Dr. John Hiscott (McGill University) for providing the TBK1 and the KM TBK1 (K38A) vectors; Dr. Tom Maniatis (Harvard University) for providing the IFNβ luciferase construct; and Dr. Shizuo Akira (Osaka University) for providing the IKKε and IKKε/TBK1 double null fibroblasts. We also acknowledge Dr. Raquel Sitcheran and Dr. Noel Hawke for carefully and critically reading the manuscript.

REFERENCES

1. Hayden, M. S., and Ghosh, S. (2004) *Genes Dev.* **18,** 2195–2224
2. Israel, A. (2000) *Trends Cell Biol.* **10,** 129–133
3. Ghosh, S., and Karin, M. (2002) *Cell* **109,** (suppl.), S81–S96
4. Peters, R. T., and Maniatis, T. (2001) *Biochim. Biophys. Acta* **1471,** M57–M62
5. Peters, R. T., Liao, S. M., and Maniatis, T. (2000) *Mol. Cell.* **5,** 513–522
6. Shimada, T., Kawai, T., Takeda, K., Matsumoto, M., Inoue, J., Tatsumi, Y., Kanamaru, A., and Akira, S. (1999) *Int. Immunol.* **11,** 1357–1362
7. Pomerantz, J. L., and Baltimore, D. (1999) *EMBO J.* **18,** 6694–6704
8. Tojima, Y., Fujimoto, A., Delhase, M., Chen, Y., Hatakeyama, S., Nakayama, K., Kaneko, Y., Nimura, Y., Motoyama, N., Ikeda, K., Karin, M., and Nakanishi, M. (2000) *Nature* **404,** 778–782
9. Kishore, N., Huynh, Q. K., Mathialagan, S., Hall, T., Rouw, S., Creely, D., Lange, G., Caroll, J., Reitz, B., Donnelly, A., Boddupalli, H., Combs, R. G., Kretzmer, K., and Tripp, C. S. (2002) *J. Biol. Chem.* **277,** 13840–13847
10. Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M., and Maniatis, T. (2003) *Nat. Immunol.* **4,** 491–496
11. Sharma, S., tenOever, B. R., Grandvaux, N., Zhou, G. P., Lin, R., and Hiscott, J. (2003) *Science* **300,** 1148–1151
12. Bonnard, M., Mirtsos, C., Suzuki, S., Graham, K., Huang, J., Ng, M., Itie, A., Wakeham, A., Shahinian, A., Henzel, W. J., Elia, A. J., Shillinglaw, W., Mak, T. W., Cao, Z., and Yeh, W. C. (2000) *EMBO J.* **19,** 4976–4985
13. McWhirter, S. M., Fitzgerald, K. A., Rosains, J., Rowe, D. C., Golenbock, D. T., and Maniatis, T. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 233–238
14. Takeuchi, O., Hemmi, H., and Akira, S. (2004) *J. Endotoxin. Res.* **10,** 252–256
15. Hemmi, H., Takeuchi, O., Sato, S., Yamamoto, M., Kaisho, T., Sanjo, H., Kawai, T., Hoshino, K., Takeda, K., and Akira, S. (2004) *J. Exp. Med.* **199,** 1641–1650
16. Perry, A. K., Chow, E. K., Goodnough, J. B., Yeh, W. C., and Cheng, G. (2004) *J. Exp. Med.* **199,** 1651–1658
17. Sankar, S., Chan, H., Romanow, W. J., Li, J., and Bates, R. J. (2005) *Cell Signal.* **18,** 982–993
18. Nomura, F., Kawai, T., Nakanishi, K., and Akira, S. (2000) *Genes Cells* **5,** 191–202
19. Buss, H., Dorrie, A., Schmitz, M. L., Hoffmann, E., Resch, K., and Kracht, M. (2004) *J. Biol. Chem.* **279,** 55633–55643
20. Sizemore, N., Leung, S., and Stark, G. R. (1999) *Mol. Cell. Biol.* **19,** 4798–4805
21. Mayo, M. W., Norris, J. L., and Baldwin, A. S. (2001) *Methods Enzymol.* **333,** 73–87
22. Leung, T. H., Hoffmann, A., and Baltimore, D. (2004) *Cell* **118,** 453–464
23. Natoli, G. (2006) *FEBS Lett.* **580,** 2843–2849
24. Fujita, F., Taniguchi, Y., Kato, T., Narita, Y., Furuya, A., Ogawa, T., Sakurai, H., Joh, T., Itoh, M., Delhase, M., Karin, M., and Nakanishi, M. (2003) *Mol. Cell. Biol.* **23,** 7780–7793
25. Wang, N., Ahmed, S., and Haqqi, T. M. (2005) *Gene* (*Amst.*) **353,** 118–133
26. Burns, K., Martinon, F., Esslinger, C., Pahl, H., Schneider, P., Bodmer, J. L., Di Marco, F., French, L., and Tschopp, J. (1998) *J. Biol. Chem.* **273,** 12203–12209
27. Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998) *Immunity* **9,** 143–150
28. Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) *Immunity* **11,** 115–122
29. Zhong, H., May, M. J., Jimi, E., and Ghosh, S. (2002) *Mol. Cell.* **9,** 625–636
30. Zhong, H., Voll, R. E., and Ghosh, S. (1998) *Mol. Cell.* **1,** 661–671
31. Wang, D., and Baldwin, A. S., Jr. (1998) *J. Biol. Chem.* **273,** 29411–29416
32. Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T., and Toriumi, W. (1999) *J. Biol. Chem.* **274,** 30353–30356
33. Madrid, L. V., Mayo, M. W., Reuther, J. Y., and Baldwin, A. S., Jr. (2001) *J. Biol. Chem.* **276,** 18934–18940
34. Madrid, L. V., Wang, C. Y., Guttridge, D. C., Schottelius, A. J., Baldwin, A. S., Jr., and Mayo, M. W. (2000) *Mol. Cell. Biol.* **20,** 1626–1638
35. Doyle, S. L., Jefferies, C. A., and O'Neill, L. A. (2005) *J. Biol. Chem.* **280,** 23496–23501
36. Eddy, S. F., Guo, S., Demicco, E. G., Romieu-Mourez, R., Landesman-Bollag, E., Seldin, D. C., and Sonenshein, G. E. (2005) *Cancer Res.* **65,** 11375–11383
37. Campbell, K. J., and Perkins, N. D. (2004) *Biochem. Soc. Trans.* **32,** 1087–1089
38. Kravchenko, V. V., Mathison, J. C., Schwamborn, K., Mercurio, F., and Ulevitch, R. J. (2003) *J. Biol. Chem.* **278,** 26612–26619
39. Mattioli, I., Geng, H., Sebald, A., Hodel, M., Bucher, C., Kracht, M., and Schmitz, M. L. (2006) *J. Biol. Chem.* **281,** 6175–6183
40. Buss, H., Dorrie, A., Schmitz, M. L., Frank, R., Livingstone, M., Resch, K., and Kracht, M. (2004) *J. Biol. Chem.* **279,** 49571–49574