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

Mazhar Adli and Albert S. Baldwin

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) 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 or IKKβ (4). One of these complexes was designated 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 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
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 IKKe would control NF-κB at a level distinct from the traditional IKK-mediated control of IkB degradation.

Here, we show that IKKe is expressed in a variety of cancer cell lines. Based on this, we have investigated a role for IKKe as related to constitutive, cancer-associated NF-κB activity. Our experiments reveal an important role for IKKe 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, IKKe single, and IKKe-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 IKKe 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 instructions for 48–72 h. Additionally, Silencer® pre-designed siRNA targeting IKKα 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.

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—IKKe mRNA was knocked down with the GeneSupressor System (IMGENEX). Plasmids encoding control shRNA and IKKe shRNA were transfected by Effectene transfection reagent (Qiagen) according to manufacturer’s instructions for 48–72 h. Additionally, Silencer® pre-designed siRNA targeting IKKe 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

IKKe Is Expressed in a Number of Cancer Cells and in SV40 Large T-immortalized 293 Cells—To address a potential role for IKKe in controlling NF-κB activity, we explored the expression of IKKe in a variety of cell lines. Immunoblotting of extracts of several cancer cell lines revealed constitutive expression of IKKe 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 IKKe expression and phosphorylated Ser-
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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.

IKKe or TBK1 Activates an NF-κB-dependent Reporter in a Kinase-dependent Manner—To investigate a potential role for IKKe 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 IKKe is expressed and is potentially active in these cells. Both IKKe 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 IKKe on activation of the 3X-κB luciferase reporter showed that -fold induction of luciferase activity is proportional to the IKKe plasmid concentration, whereas there was no significant induction with the vector control or with the kinase mutant (KM) form of IKKe (Fig. 2C).

IKKe Expression Induces NF-κB DNA Binding Activity—We next investigated whether IKKe expression can induce NF-κB binding to a consensus DNA target sequence. FLAG-tagged IKKe was transiently expressed in HEK 293T cells for ~48 h, and nuclear extracts were prepared for EMSAs. As shown in Fig. 3A, IKKe 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 IKKe-overexpressing cells led to more DNA binding activity, but this increase appears to be an additive effect of TNF-α and IKKe rather than a synergistic effect. Western blot analysis of cytoplasmic extracts, shown in the lower panel, demonstrates expression levels of IKKe. We next aimed to investigate the
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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. 3A was incubated with the indicated antibody and electrophoresed on a separate gel (Fig. 3B). The number at the top of the figure indicates the lane numbers (lanes 1–4) from the reactions used in Fig. 3A. 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. 3B). 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. 4A). 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. 4A), 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. 4B, 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. 4B).

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

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**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.” The NF-κB complex is indicated 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 TNF-α 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.

**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 induce phosphorylation of 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 S01 position (Thr-S01 → Ala), which was thought to control IKKε activity; however, the mutation did not change its activity in terms of phosphorylating p65.
translational modification on p65 might be defective in IKKe-deficient MEFs in response to NF-κB inducers. To test our hypothesis, IKKe-deficient and IKKe and TBK1 doubly deficient MEFs (DKO) were stimulated with TNF-α, a well known NF-κB inducer, and compared with similarly treated WT MEFs. As seen in Fig. 5A, p65 was phosphorylated at the Ser-536 position in response to TNF-α as early as 5-min post-stimulation. Interestingly, in all of the stimuli tested, there was no significant difference in terms of either p65 phosphorylation pattern or IκBα degradation kinetic in IKKe-deficient or DKO MEFs compared with WT MEF cells. Analysis of β-tubulin levels confirmed that loading was essentially equivalent in all lanes.

determine if the loss of TBK1 together with IKKe would effect the phosphorylation of p65 and IκBα degradation, WT MEFs and MEFs deficient for both IKKe 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. 5B). This result indicated that NF-κB activation, as measured by p65 phosphorylation and IκBα degradation in response to TNF-α, is independent of IKKe and TBK1.

We next tested the effect of IL-1β on p65 phosphorylation as well as IκB degradation (Fig. 5C). 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
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was observed in 10 min. It is interesting again to observe that both p65 phosphorylation at Ser-536 and IkBα degradation were normal in IKKe-deficient cells compared with WT cells. In parallel studies, experiments were also performed in cells where both IKKe and TBK1 were deleted (DKO), and we observed no significant difference from WT cells relative to Ser-536 p65 phosphorylation or IkBα degradation after stimulation with TNF-α or IL-1β (Fig. 5D). These results suggest a minimal role of IKKe and TBK1 in cytokine-induced p65 phosphorylation and IkBα 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 IKKe and TBK1 kinases (11, 13, 15). In our experimental system, LPS stimulation of WT MEFs and IKKe-deficient cells showed similar levels of inducible p65 phosphorylation (Fig. 5E). 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 IKKe-deficient cells. In addition to p65 phosphorylation, IkBα 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 IKKe-deficient MEFs (Fig. 5F).

Overall our data indicate that inducible Ser-536 phosphorylation is unaffected in IKKe-deficient cells and confirm that IKKe is not significantly involved in mechanisms associated with cytokine-, LPS-, or PMA-induced IkBα degradation.

IKKe Controls Constitutive p65 Ser-536 Phosphorylation—It was surprising to observe that IKKe 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 IKKe 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 IKKe could contribute to basal/constitutive levels of Ser-536 phosphorylation was investigated in HeLa and HEK 293T cells. These cells have higher levels of IKKe expression, constitutive p65 phosphorylation, and NF-κB activation compared with MEFs. To knock down IKKe, both plasmid-based shRNA and normal siRNA technologies have been utilized against IKKe 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 IKKe knockdown as well as for endogenous p65 Ser-536 phosphorylation. Our results indicated that transfection of shRNA against IKKe leads to sustained effective knockdown of IKKe. Furthermore, the reduction in IKKe 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. 6A). Quantitative real-time-PCR analysis showed more than a 70% reduction in ikke 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, IKKe 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 IKKe levels or Ser-536 phosphorylation (Fig. 6B). These results demonstrate that IKKe has a significant role in controlling the basal/consti-
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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. 6C). 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.

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. 8A). 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.

**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 IKKe. This is interesting, because IKKe is normally considered a kinase that is induced quantitatively by LPS or cytokines. We have investigated a potential role for IKKe 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 IKKe- and TBK1-induced NF-κB-dependent promoter activation.

In agreement with previous results (10, 11), IKKe 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, IKKe 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 IKKe 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 IKKe and TBK1 may significantly contribute to the constitutive level of SS36 phosphorylation of p65. We have also observed that IKKe induces its own expression, which has been shown to be regulated by NF-κB. These data indicate that ectopically expressed IKKe induces p65 phosphorylation, NF-κB activation, and NF-κB-dependent gene expression. It also raises the possibility that IKKe functions in an autoregulatory loop, leading to its own expression.

It was interesting to observe that IKKe-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, IKKe-null cells (and DKO MEFs for both IKKe and TBK1) allowed inducible RelA/p65 phosphorylation to the same extent. Why is there no defect in inducible Ser-536 phosphorylation of p65 in IKKe-deficient cells? The first plausible explanation to this question is that the classic IKK signalsome complex is still intact in IKKe-deficient cells. Therefore, this complex likely compensates for the loss of IKKe. 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 IKKe-deficient cells. The third explanation to this question is that IKKe 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 IKKe, the potential that IKKe and TBK1 might be involved in constitutive p65 phosphorylation has been investigated in HeLa and HEK 293T cells that demonstrate higher levels of IKKe and constitutive p65 phosphorylation at Ser-536 position. IKKe was knocked down by plasmid-based shRNA technology in both HeLa cells and HEK 293T cells. Importantly, shRNA transfection leads to sustained knockdown of IKKe, and more importantly, reduction in the IKKe 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 IKKe 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 IKKe are not entirely orthologues at least in controlling basal phosphorylation of p65. As suggested earlier (15), IKKe and TBK1 might not be redundant in every signaling pathway that they affect. Thus the data presented here clearly show that IKKe 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 IKKe 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 IKKe contributes to the pathogenesis of breast cancer. Expression of a kinase-inactive form of IKKe blocked breast cancer cell colony formation. The results presented in that study are consistent with the findings presented here. We have shown that IKKe 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 IKKe or overexpression of mutated version of p65 (S536A) negatively effects the cell proliferation. These findings indicate an important role of IKKe and p65 phosphorylation in cancer cell proliferation.

There have been reports suggesting that IKKe 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 IKKe/TBK1. In other words, IKKe and TBK1 might have activated classic IKKs, which then lead to the phosphorylation of p65. To test this, we tried to detect activation of IKKB 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 IKKe 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
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has been clearly shown that stimulus-coupled IkB degradation and p65 nuclear translocation and DNA binding activity of NF-κB is normal in IKKe 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 IKKe- or TBK1-deficient cells, because there is normal IkB 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 IKKe and TBK1 under basal growth situations, at least in certain cells. If IKKe 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 IKKe 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 IKKe 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 IKKe, 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 IKKe functions separately from the classic IKK complex, or through distinct regulatory pathways. It is also unclear whether IKKe is the kinase that directly controls Ser-536 p65 phosphorylation. Nevertheless, the data indicate a potentially important role for IKKe in controlling at least part of the constitutive NF-κB activity generated in certain cancer cells, with subsequent downstream effects on cancer cell proliferation.

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