Distinct Roles of the IκB Kinase α and β Subunits in Liberating Nuclear Factor κB (NF-κB) from IκB and in Phosphorylating the p65 Subunit of NF-κB*

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Phosphatidylinositol 3'-kinase (PI3K) and the serine/threonine kinase AKT have critical roles in phosphorylating and transactivating the p65 subunit of nuclear factor κB (NF-κB) in response to the pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF). Mouse embryo fibroblasts (MEFs) lacking either the α or β subunit of IκB kinase (IKK) were deficient in NF-κB-dependent transactivation following treatment with IL-1 or TNF. However, in contrast to IKKβ-null MEFs, IKKα-null MEFs were not substantially defective in the cytokine-stimulated degradation of IκBα or in the nuclear translocation of NF-κB. The IKK complexes from IKKα- or IKKβ-null MEFs were both deficient in PI3K-mediated phosphorylation of the transactivation domain of the p65 subunit of NF-κB in response to IL-1 and TNF, and constitutively activated forms of PI3K or AKT did not potentiate cytokine-stimulated activation of NF-κB in either IKKα- or IKKβ-null MEFs. Collectively, these data indicate that, in contrast to IKKβ, which is required for both NF-κB liberation and p65 phosphorylation, IKKα is required solely for the cytokine-induced phosphorylation and activation of the p65 subunit of NF-κB that are mediated by the PI3K/AKT pathway.

The NF-κB family of transcription factors consists of binary complexes of subunits with related promoter-binding and transactivation properties. The p65/RelA, RelB, and c-Rel subunits stimulate transcription, whereas the p50 and p52 subunits serve primarily to bind to DNA (1). The prototypical NF-κB complex is the p65-p50 heterodimer (2). NF-κB is sequestered in a latent form in the cytoplasm through its interaction with the inhibitory IκB proteins. In response to signals, IκB kinase is activated, and IκB is phosphorylated and degraded, releasing NF-κB, which enters the nucleus and binds to DNA (2–5). However, the phosphorylation and degradation of IκB and the consequent liberation of NF-κB are not sufficient to activate NF-κB-dependent transcription, which also relies on a second pathway, which leads to the stimulus-induced phosphorylation of the p65/RelA or RelB, and c-Rel subunits of NF-κB (6–15).

Our laboratory (13) and others (7, 14) have shown that the pro-inflammatory cytokines IL-1 and TNF induce the phosphorylation and activation of the p65 subunit of NF-κB, a pathway distinct from the one leading to IκB degradation and NF-κB nuclear translocation. Additionally, phosphatidylinositol 3’-kinase (PI3K) and the serine/threonine kinase AKT play critical roles in this pathway (13, 16, 17). Recently, an additional function for IL-1-stimulated PI3K/AKT activation has been reported: phosphorylation of the NF-κB p50 subunit in response to these kinases increases the DNA-binding capacity of the NF-κB complex (18).

Targeted gene disruptions have demonstrated that IKKβ (but not IKKα) is largely responsible for cytokine-induced IκB degradation and NF-κB nuclear translocation (19–24). However, IKKα-null mouse embryo fibroblasts (MEFs) are deficient in inducing several NF-κB-dependent mRNAs in response to IL-1 and TNF (21). Activated AKT interacts with IKKα upon cytokine stimulation and induces the phosphorylation of threonine 23 (25). These findings raise the interesting possibility that, although IKKα is dispensable for IκBα degradation and NF-κB nuclear translocation, it may be required in the PI3K/ AKT pathway that leads to the phosphorylation and activation of NF-κB. Therefore, we have investigated the roles of the IKK α and β subunits in the IL-1- and TNF-mediated phosphorylation and activation of the p65 subunit of NF-κB.

**EXPERIMENTAL PROCEDURES**

**Biological Reagents and Cell Culture**—Recombinant human IL-1β was from NEC, National Institutes of Health. Recombinant human TNF was from Preptech (Rocky Hill, NJ). LY 294002 was from Sigma. Polyclonal anti-IKKα, anti-IKKβ, anti-IKKγ, anti-p65/RelA, and anti-IκBα antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-AKT, anti-AKT, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-JNK1, and anti-JNK antibodies were from Cell Signaling Technologies (Beverly, MA). Protein A-Sepharose and glutathione-agarose beads were from Amersham Biosciences, Inc. (Buckinghamshire, United Kingdom). Wild-type and IKKα- and IKKβ-null MEFs, kindly provided by Dr. Inder Verma (21), were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin. For all experiments, unless otherwise indicated, cells at 80% confluence on 100-mm dishes were preincubated with the PI3K inhibitor LY 294002 (20 μM) for 30 min at 37°C prior to stimulation with IL-1 (2 ng/ml) or TNF (25 ng/ml) at 37°C for the indicated time periods. All results shown are typical of at least three independent experiments.

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1 The abbreviations used are: NF-κB, nuclear factor κB; IκB, interleukin; TNF, tumor necrosis factor; PI3K, phosphatidylinositol 3’-kinase; IκB, IκB kinase; MEFs, mouse embryo fibroblasts; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; RSK, ribosomal S6 kinase; JNK, c-Jun NH2-terminal kinase; IP10, interferon-inducible protein 10; EMSA, electrophoretic mobility shift assay; TAD, transactivation domain; GST, glutathione S-transferase.

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Northern Analyses—The cells were stimulated with either IL-1 or TNF for 4 h or, where indicated, preincubated with LY 294002 for 30 min prior to stimulation with IL-1 or TNF for 4 h. Total RNA was isolated using the TRIzol reagent (Invitrogen). RNA was fractionated by electrophoresis on a formaldehyde gel and transferred to Hybond-N, a positively charged nylon membrane, according to the procedures provided by Amersham Biosciences, Inc. cDNA probes for murine IL-6, murine interferon-inducible protein 10 (IP10), and human glyceraldehyde-3-phosphate dehydrogenase mRNAs were made using the random priming kit from Amersham Biosciences, Inc. Probe hybridization and washing were performed according to procedures provided by Amersham Biosciences, Inc., and signals were visualized by autoradiography. The murine probes for IL-6 and IP10 were kindly provided by Dr. Thomas Hamilton (Cleveland Clinic Foundation).

Transfection and Reporter Assay—The NF-κB-dependent reporter plasmid p5XIP10B, a kind gift of Dr. Bryan Williams (Cleveland Clinic Foundation), contains five tandem copies of the NF-κB site from the IP10 gene. For reporter assays, wild-type and IKKα- and IKKβ-null MEFs were stably transfected using Lipofectin (Invitrogen) with 10 μg of p5XIP10B and 1 μg of pBABEPuro. Pools of stably transfected cells were selected with and maintained in puromycin. In separate experiments, the NF-κB reporter cells were transiently transfected using Lipofectin with 0.5 μg of pSv2-βgal and 5 μg of either vector or construct expressing wild-type or lipid phosphatase-deficient mutants of PTEP δ-β, kindly provided by Dr. Kenneth Yamada (26); constitutively activated p110δ/δ, kindly provided by Drs. Doreen Cantrell and Karin Reif (27); constitutively activated AKT, kindly provided by Dr. Julian Downward (28); wild-type p65/RelA, kindly provided by Dr. Dean Ballard (29); wild-type IKKα, kindly provided by Dr. David Donner (25); or wild-type IKKβ, kindly provided by Dr. Zhaodan Cao (30). Cells were divided into the appropriate number of plates for treatment 6 h following transfection. After 24 h, the cells were harvested. The cells were stimulated with either IL-1 or TNF for 4 h or, where indicated, preincubated with LY 294002 for 30 min prior to stimulation with IL-1 or TNF for 4 h. Luciferase or galactosidase activity was determined with the luciferase activity or chemiluminescent reagents (both from Promega, Madison, WI). Luciferase activity was normalized to β-galactosidase activity to control for transfection efficiency. The viability of each transfected cell population was measured at the time of harvesting by trypan blue exclusion.

Gel Electrophoretic Mobility Shift Assays—For electrophoretic mobility shift assays (EMSAs), where indicated, the cells were preincubated with LY 294002 prior to stimulation with IL-1 or TNF for 20 min. The NF-κB-binding site (5′-GAGCAGAGGGAAATTCCGTAACTT-3′) from the NF-κB site was used as a probe. Binding reactions contained 0.5% Nonidet P-40 lysis buffer as described previously (31). The binding reaction was carried out with nuclear extracts containing equal amounts of protein at room temperature for 30 min in a total volume of 20 μl. DNA-nuclear complexes were separated on 5% polyacrylamide gels by electrophoresis in low ionic strength Tris borate/EDTA buffer. The gels were dried, and the labeled complexes were visualized by autoradiography.

Immunoblotting and Immunoprecipitation—Cells were washed once with phosphate-buffered saline and lysed for 30 min at 4 °C in 1 ml of 0.5% Nonidet P-40 lysis buffer as described previously (32). Cellular debris was removed by centrifugation at 16,000 × g for 15 min. For immunoblotting, cell extracts were fractionated directly by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblot analysis was performed with the indicated primary antibodies, which were visualized with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse immunoglobulins using the ECL Western blotting detection system (PerkinElmer Life Sciences). For immunoprecipitations, cell extracts were incubated with 3 μg of primary antibody for 4 h, followed by incubation for 1 h with 50 μl of protein A-Sepharose beads (50% suspension). The beads were washed three times with lysis buffer, and samples were analyzed by SDS-PAGE and autoradiography.

Analysis of the Phosphorylation of the NF-κB-dependent Transcription Domain and IκBα(1-54)—A fragment of p65 residues 354–51 representing the transcription activation domain (TAD) was used (12). The IκBα fragment (residues 1–54) was kindly provided by Dr. Joseph DiDonato (Cleveland Clinic Foundation). These proteins were expressed as glutathione S-transferase (GST) fusions in bacteria and purified using glutathione-agarose beads after sonication at 4 °C in 0.5% Nonidet P-40 lysis buffer (33). The GST fusion proteins were eluted from the beads in kinase buffer (20 mM HEPES (pH 7.6), 20 mM MgCl2, 2 mM dithiothreitol, 20 μM ATP, 20 mM β-glycerophosphate, 20 mM dithiothreitol, 0.1 mM sodium orthovanadate, 3 μCi of [γ-32P]ATP, and 10 mM reduced glutathione). The cells were stimulated with either IL-1 or TNF for 20 min, or where indicated, preincubated with LY 294002 for 30 min prior to stimulation with IL-1 or TNF for 20 min. Nuclear and cytoplasmic extracts were prepared as described previously (31); or the cells were lysed, and anti-IKKβ antibody was used to immunoprecipitate the IKK complex from each sample. In vitro phosphorylation was performed using ~1 μg of the p65 TAD/GST or IκBα(1-54)/GST fusion protein as a substrate with either cytoplasmic or nuclear extracts (5 μg of protein) or the immunoprecipitated IKK complex as the kinase in kinase buffer at 30 °C for 30 min (12). Following the kinase reaction, phosphorylation of either substrate was analyzed by SDS-PAGE, followed by autoradiography. In separate experiments, wild-type MEFs were transiently transfected using Lipofectin with 5 μg of either vector or construct expressing constitutively activated AKT (28). The transfected cells were divided into three plates for treatment 8 h following transfection. After 24 h, the cells were stimulated with either IL-1 or TNF for 20 min. The cells were lysed, and anti-IKKα antibody was used to immunoprecipitate the IKK complex from each sample. In vitro phosphorylation of the p65 TAD/GST fusion protein was performed as described above with the immunoprecipitated IKK complex.

RESULTS

IL-1- and TNF-induced NF-κB-dependent Transcription Is Deficient in Both IKKα- and IKKβ-null MEFs, but Only IKKβ-null MEFs Are Deficient in NF-κB Liberation and Nuclear Translocation—The complete activation of NF-κB requires two pathways leading to the liberation of NF-κB and to the activation of the transcription function of NF-κB. Studies from our laboratory (13) and by Madrid et al. (16, 34) have demonstrated a critical role of the PI3K/AKT pathway in activating NF-κB in response to the pro-inflammatory cytokines IL-1 and TNF by phosphorylation of the carboxyl-terminal transactivation do-
main of p65. IKKα-null MEFs were reported to be defective in NF-xB-dependent transcription following stimulation with IL-1 and TNF (21), even though IKKα seemed to be dispensable for cytokine-induced IκB degradation and NF-xB nuclear translocation (19–24). We have now investigated the roles of both IKKα and IKKβ in the activation of NF-xB through the PI3K/AKT pathway. Both IKKα- and IKKβ-null MEFs were deficient in IL-1- and TNF-stimulated induction of the NF-xB-dependent endogenous genes IL-6 and IP10 (Fig. 1). Both were also deficient in activating an NF-xB-dependent reporter (Fig. 1B), confirming that the defects are at the level of NF-xB function. To explore why IL-1 and TNF fail to activate NF-xB in IKKα- and IKKβ-null MEFs, we investigated known pathways leading to NF-xB activation and the signaling deficiencies responsible for causing the null MEFs to be unresponsive to cytokine stimulation. Wild-type and IKKα- and IKKβ-null MEFs were exposed to either IL-1 or TNF, and total cell extracts were analyzed for phosphorylated AKT, ERK, JNK, p90 RSK, and p38 (Fig. 2). No defects were detected in any of these pathways in IKKα- or IKKβ-null MEFs. WT, wild-type MEFs.

![Graph](image1.png)

**Fig. 2.** IL-1- and TNF-stimulated phosphorylation and activation of AKT, ERK, JNK, p90RSK, and p38 are intact in both IKKα- and IKKβ-null MEFs. Cells were exposed to either IL-1 or TNF for the times indicated. Total cell extracts were prepared, and equal amounts of protein were analyzed by SDS-PAGE and Western blotting for activated, phosphorylated AKT (pAKT), ERK (pERK), JNK (pJNK), p90RSK (pRSK), and p38 (p38). No defects were detected in any of these pathways in IKKα- or IKKβ-null MEFs. WT, wild-type MEFs.

**Fig. 3.** IKKβ-null MEFs (but not IKKα-null MEFs) are substantially deficient in IL-1- and TNF-stimulated IκBα degradation, p65 nuclear translocation, and NF-xB DNA binding. **A**, IκBα degradation. Cells were exposed to either IL-1 or TNF for the times indicated, and total cell extracts were prepared. Equal amounts of protein were analyzed by SDS-PAGE and Western blotting. B, EMSAs. Cells were exposed to either IL-1 (IL) or TNF (T) for 20 min, and nuclear extracts were prepared. Equal amounts of each extract were analyzed by EMSA for the ability of NF-xB to bind to a labeled NF-xB consensus site from the IP10 gene. WT, wild-type MEFs; C, control.

TAD/GST). The results of this experiment indicated that the majority of the IL-1- and TNF-induced kinase activity for this substrate resided in the cytoplasmic fraction of wild-type MEFs and was absent in both IKKα- and IKKβ-null MEFs (data not shown). In view of these results, we assayed the ability of immunoprecipitated IKK complexes from wild-type and IKKα- and IKKβ-null MEFs to phosphorylate p65 TAD/GST. The cells were treated with IL-1 or TNF and, where indicated, incubated with LY 294002 before treatment to inhibit the cytokine-stimulated PI3K/AKT pathway. The composition of the immunoprecipitated IKK complex from each cell type is shown in Fig. 4A. The IKK complex from IKKα-null MEFs was unable to phosphorylate p65 TAD/GST efficiently (Fig. 4B, upper panel). Phosphorylation of p65 TAD/GST by the wild-type IKK complex depends on activation of the PI3K/AKT pathway by IL-1 and TNF, as preincubation with LY 294002 almost completely blocked phosphorylation in wild-type MEFs (Fig. 4B, upper panel). Interestingly, IKKβ-null MEFs were also deficient in phosphorylating p65 TAD/GST (Fig. 4B, upper panel). However, the IKK complex from IKKα-null MEFs was not defective...
in the IL-1- and TNF-stimulated phosphorylation of IκBα-(1–54)/GST, as was the IKK complex from IKKβ-null MEFs (Fig. 4B, lower panel) (21). Phosphorylation of IκBα-(1–54)/GST does not depend on activation of the PI3K/AKT pathway by IL-1 and TNF, as preincubation with LY 294002 had no effect on the phosphorylation of this substrate by the IKK complex (Fig. 4B, lower panel). To test whether IKK is capable of phosphorylating p65 in AKT-activated cells, the effect of overexpressing constitutively activated AKT on IKK phosphorylation of p65 TAD/GST was investigated. Wild-type MEFs were transiently transfected with either vector alone or activated AKT. 8 h after transfection, the cells were divided into three plates each. After 24 h, the cells were left unstimulated or were stimulated with IL-1 or TNF for 20 min. Cells were lysed; anti-IKK antibody was used to immunoprecipitate the IKK complex from each sample. A. Western analysis of the composition of the IKK complex from each cell type. B, *in vitro* phosphorylation. Approximately 1 μg of p65 TAD/GST (upper panel) or IκBα-(1–54)/GST (lower panel) was used as substrate, and the immunoprecipitated IKK complex was the kinase. Following the kinase reaction, phosphorylation of p65 TAD/GST was analyzed by SDS-PAGE, followed by autoradiography. C, *in vitro* phosphorylation of p65 TAD/GST. Wild-type MEFs (WT) were transiently transfected with 5 μg of either vector or activated AKT construct. After 8 h, transfected cells were divided into three plates each. After 24 h, each transfected sample was left unstimulated or was stimulated with either IL-1 or TNF for 20 min. Cells were lysed; anti-IKK antibody was used to immunoprecipitate the IKK complex from each sample; and the p65 TAD in *vitro* kinase assay was performed with the IKK complexes as described for B. The phosphorylation of p65 TAD/GST was analyzed by SDS-PAGE, followed by autoradiography. C, control.

**Fig. 4.** The IKK complex from IKKα- and IKKβ-null MEFs is deficient in both the IL-1- and TNF-stimulated phosphorylation of the transactivation domain of the p65 subunit of NF-κB, but only the IKK complex from IKKβ-null MEFs is defective in phosphorylating IκBα. Cells were stimulated with IL-1 (IL) or TNF (T) for 20 min or, where indicated, preincubated with LY 294002 (LY) for 30 min prior to stimulation with IL-1 or TNF for 20 min. Cells were lysed, and anti-IKK antibody was used to immunoprecipitate the IKK complex from each sample. A. Western analysis of the composition of the IKK complex from each cell type. B, *in vitro* phosphorylation. Approximately 1 μg of p65 TAD/GST (upper panel) or IκBα-(1–54)/GST (lower panel) was used as substrate, and the immunoprecipitated IKK complex was the kinase. Following the kinase reaction, phosphorylation of p65 TAD/GST was analyzed by SDS-PAGE, followed by autoradiography. C, *in vitro* phosphorylation of p65 TAD/GST. Wild-type MEFs (WT) were transiently transfected with 5 μg of either vector or activated AKT construct. After 8 h, transfected cells were divided into three plates each. After 24 h, each transfected sample was left unstimulated or was stimulated with either IL-1 or TNF for 20 min. Cells were lysed; anti-IKK antibody was used to immunoprecipitate the IKK complex from each sample; and the p65 TAD in *vitro* kinase assay was performed with the IKK complexes as described for B. The phosphorylation of p65 TAD/GST was analyzed by SDS-PAGE, followed by autoradiography. C, control.

IL-1- and TNF-dependent Activation of NF-κB through the PI3K/AKT Pathway Requires IKKα Independently of IκBα Degradation—We tested the ability of constitutively activated PI3K and AKT to enhance IL-1- and TNF-stimulated NF-κB-dependent promoter activation in wild-type and IKKα- and IKKβ-null MEFs. These cells, stably transfected with the NF-κB-dependent luciferase reporter construct p5XIP10κB, were transiently transfected with vector alone, activated p110, activated AKT, or wild-type p65. 8 h after transfection, the cells were divided into three plates each. After 24 h, the cells were left unstimulated or were stimulated with IL-1 (Fig. 5A) or TNF (Fig. 5B) for 4 h and assayed for luciferase activity. Transfection of wild-type p65 weakly increased both IL-1-induced (IKKα = 5.4-fold and IKKβ = 3.8-fold) and TNF-induced (IKKα = 3.9-fold and IKKβ = 3.0-fold) NF-κB-dependent promoter activation in IKKα- and IKKβ-null MEFs, but not nearly as well as in wild-type MEFs (IL-1 = 23.9-fold and TNF = 17.9-fold), over their respective untreated p65-transfected controls (Fig. 5, A and B). Transfection of p65 alone with no cytokine treatment resulted in an 8-fold increase in wild-type MEFs, a 3-fold increase in IKKα-null MEFs, and a 2-fold increase in IKKβ-null MEFs over their respective vector-transfected controls (data not shown), indicating that both basal and cytokine-induced p65-dependent transactivation is diminished in both IKKα- and IKKβ-null MEFs compared with wild-type MEFs. However, constitutively activated PI3K and AKT both
IKKα/IKKβ Mediate p65 Phosphorylation in Response to IL-1/TNF

failed to enhance IL-1- or TNF-stimulated NF-κB-dependent promoter activation in IKKα- and IKKβ-null MEFs in contrast to wild-type MEFs (Fig. 5, A and B). As we reported previously (13), neither activated PI3K nor AKT can induce NF-κB activation alone, presumably because a second signal is needed for IκBα degradation. As there was no defect in the cytokine-stimulated phosphorylation and degradation of IκBα in IKK-null MEFs, a major role of IKKα in cytokine-dependent signaling must be in the phosphorylation and activation of the p65 subunit of NF-κB. Blockade of IL-1- and TNF-induced p38 and IKKα activity by LY 294002 inhibited IL-1- and TNF-stimulated induction of IL-6 and IP10 mRNA (Fig. 6A) as well as NF-κB-dependent promoter activation (Fig. 6D). Despite dramatically inhibiting NF-κB-dependent endogenous gene transcription and promoter activation (Fig. 6, A and D), neither IL-1- or TNF-induced IκBα degradation (Fig. 6B, upper panel) nor NF-κB DNA binding (Fig. 6C) was affected by inhibiting PI3K. However, the phosphorylation and activation of AKT in response to IL-1 and TNF were dramatically inhibited by PI3K blockade (Fig. 6B, lower panel). The IL-1- and TNF-stimulated phosphorylation of ERK, JNK, p38βδ (35), and p38 was unaffected by pretreatment with LY 294002 (data not shown). The production of the phospholipid second messenger phosphatidylinositol 3,4,5-trisphosphate by PI3K could be inhibited directly by its endogenous antagonist, the tumor suppressor PTEN, a dual-specificity phosphatase that dephosphorylates phosphatidylinositol 3,4,5-trisphosphate. We explored the effects of inhibiting IL-1- and TNF-stimulated PI3K activity by LY 294002 as well as by expressing PTEN on NF-κB-dependent promoter activation in wild-type MEFs stably transfected with the NF-κB-dependent luciferase reporter p5XIP10oxB. The cells were transiently transfected with either vector or wild-type PTEN. After 48 h, cells were preincubated with either vehicle or LY 294002 for 30 min prior to stimulation with IL-1 or TNF, and luciferase activity was measured (Fig. 6D). Blockade of IL-1- and TNF-induced PI3K activity with either wild-type PTEN or LY 294002 inhibited both IL-1 and TNF stimulation of the NF-κB-dependent promoter. We also compared the effects of wild-type PTEN with those of three different mutants that lack lipid phosphatase activity. Only wild-type PTEN suppressed NF-κB activation (data not shown). Therefore, the ability of PTEN to suppress NF-κB-dependent transcription driven by IL-1 and TNF depends on its lipid phosphatase activity. These data confirm our previous results showing that the PI3K/AKT pathway functions to stimulate p65 activation independently of the degradation of IκBα and activation of the ability of NF-κB to bind to DNA (13).

DISCUSSION

IKKα and IKKβ Are Both Required for PI3K- and AKT-mediated Activation of NF-κB in Response to IL-1 and TNF—We have investigated the roles of IKKα and IKKβ in the activation of NF-κB through the PI3K/AKT pathway in response to IL-1 and TNF. IKKα is required for the PI3K/AKT pathway to phosphorylate and transactivate the p65 subunit of NF-κB. In addition to the role of IKKβ as the IκBα kinase, it also plays a role in the PI3K/AKT-mediated phosphorylation and activation of p65. Our previous findings indicated that activation of the PI3K/AKT pathway is not sufficient to activate NF-κB-dependent transcription, but is necessary for IL-1 and TNF to activate NF-κB together with the pathway that leads to IκBα degradation and nuclear translocation of NF-κB (13). Our present results (Figs. 4B, lower panel; and 6) agree with these findings and with the studies of others, indicating that the IL-1- and TNF-stimulated PI3K/AKT pathway functions to stimulate the transactivation potential of NF-κB and does not participate in the pathway leading to IκBα phosphorylation, IκBα degradation, and NF-κB liberation (13, 16, 34). We also demonstrated that the endogenous antagonist of the PI3K/AKT pathway, the tumor suppressor PTEN, inhibits the IL-1- and TNF-stimulated activation of NF-κB in a manner that depends on its lipid phosphatase function (Fig. 6 and data not shown). Our finding that wild-type PTEN is able to inhibit NF-κB-dependent transcriptional activation agrees with two previous reports. In one, PTEN suppressed both the activation of IKK and the ability of NF-κB to bind to DNA (35), whereas in the second, PTEN affected the ability of NF-κB to bind to DNA by inhibiting the phosphorylation of the p50 subunit of NF-κB independently of IκBα degradation (18). We found no substantial difference in IL-1- and TNF-stimulated NF-κB DNA binding between wild-type and IKKα-null MEFs (Fig. 3B). However, the total levels of induced NF-κB DNA binding in IKKα-null MEFs are ~2-fold lower than those in wild-type MEFs (Fig. 3B). This may be due to reduced phosphorylation of p50 in these cells and remains to be investigated. Neither constitutively activated PI3K nor AKT is able to enhance the activation of NF-κB by IL-1 or TNF in either IKKα- or IKKβ-null MEFs (Fig. 5). These data indicate that both IKKα and IKKβ are required for the PI3K/AKT pathway to activate NF-κB.

IKKα, Unlike IKKβ, Is Dispensable for NF-κB Liberation, but Both Are Required for the PI3K/AKT-mediated Phosphorylation of p65—Targeted gene disruption studies have demonstrated that IKKβ (but not IKKα) is largely responsible for cytokine-induced IκBα degradation and translocation of NF-κB to the nucleus in response to IL-1 and TNF. In this respect, the failure of PI3K and AKT to activate NF-κB in IKKβ-null MEFs may be due in part to the defective liberation of NF-κB in these
cells (Fig. 3). However, the diminished capacity of overexpressed p65 to drive both basal and cytokine-induced NF-κB promoter activation in IKKβ-null MEFs also indicates a role of IKKβ in the phosphorylation and activation of NF-κB p65 (Fig. 5). Both IKKα and IKKβ are required for IL-1 and TNF to activate NF-κB-dependent transcription (Fig. 1). However, the IKK complex from IKKα-null MEFs is not deficient in IκBα phosphorylation (Fig. 4B, lower panel), IκBα degradation, or NF-κB liberation, but is deficient in activating NF-κB-dependent transcription in response to IL-1 and TNF (Figs. 1 and 3, A and B). There are no defects in the IL-1- or TNF-dependent phosphorylation and activation of Akt, ERK, JNK, p90RSK, or p38 in either IKKα- or IKKβ-null MEFs (Fig. 2). Our demonstration that the phosphorylation and activation of p38 in response to IL-1 are intact in IKKα-null MEFs differs from a recent report indicating that IL-1 activates NF-κB by inducing p38 activity in a manner dependent on AKT and IKKα (34). We have no explanation for this discrepancy other than that the IKKα-null MEFs utilized in the study of Madrid et al. (34) were obtained from a different source (19). However, our demonstration that the absence of IKKα (Fig. 2) or inhibition of P38K (data not shown) does not affect p38 phosphorylation and activation in response to IL-1 and TNF, but does inhibit Akt activation and NF-κB-dependent transcription (Fig. 6), indicates that cytokine-mediated p38 activation is independent of the IKKα and P38/akt pathways. However, we cannot exclude a separate role of p38 in the activation of NF-κB as suggested by Madrid et al. (34) and corroborated by previous reports of NF-κB modulation by p38 (36, 37). The sustained activation of p38 and the other kinases observed in IKKα- and IKKβ-null MEFs compared with wild-type MEFs is an interesting observation (Fig. 2). A recent report suggests that both IKKβ- and p65-null MEFs demonstrate a sustained JNK activation in response to TNF compared with wild-type MEFs, contributing to TNF-induced apoptosis, which can be reversed by overexpression of NF-κB-induced X-IAP (X-chromosome-linked inhibitor of apoptosis) (38). A second report demonstrated that inhibition of NF-κB activation by the super-repressor IκBα (A32/36) mutant elicits sustained JNK activation without inhibiting MAPK phosphatase-1, a JNK phosphatase (39). These reports suggest that NF-κB target genes may downregulate JNK activation. Our results indicate that inhibition of NF-κB and NF-κB target genes may have a broader impact on the down-regulation of the IL-1- and TNF-stimulated MAPKs and remain to be further investigated. Our studies demonstrate a novel role of IKKα in the phosphorylation and activation of p65 by the P38K/AKT pathway. We have demonstrated that the IKK complex from IKKα-null MEFs is unable to phosphorylate the transactivation domain of p65 efficiently (Fig. 4B, upper panel). The phosphorylation of the p65 transactivation domain by the IKK complex depends on the activation of the P38K/AKT pathway by IL-1 and TNF, as preincubation with LY 294002 almost completely blocked this phosphorylation in wild-type MEFs (Fig. 4B, upper panel). Our study demonstrates that the IKK complex from IKKβ-null MEFs is also deficient in phosphorylating the transactivation domain of p65 (Fig. 4B, upper panel). The deficiency of IKKβ-null MEFs in phosphorylating p65 is in agreement with the study of Madrid (34) and corroborated by previous reports of NF-κB modulation by p38 (36, 37).
et al. (34), demonstrating that AKT targets the transactivation function of p65 in a manner that is dependent on IKKaβ, and studies demonstrating the direct phosphorylation of p65 by IKKaβ (12). Therefore, both IKKa and IKKaβ are required for the IL-1- and TNF-stimulated PI3K/AKT pathway to induce the phosphorylation and transactivation potential of p65. However, the inability of IKKaβ to substitute for IKKa in restoring NF-κB activation to IKKa-null MEFs (data not shown) indicates an essential function of IKKa separate from that of IKKaβ in mediating the cytokine-stimulated activation of p65.

Is the IκB Kinase the PI3K- and AKT-activated p65 Kinase?—Our data that the IKK complex from wild-type MEFs expressing activated AKT displays significantly increased kinase activity for the p65 TAD (Fig. 4C) indicate that the IKK complex phosphorylates the p65 TAD in response to activated AKT. We plan to investigate whether IKKa and IKKaβ require their kinase activities to stimulate the pathway leading to p65 activation. Reconstitution of IKKα/IKKβ double knockout MEFs with combinations of wild-type and kinase-dead mutants of IKKα and IKKβ will help to answer this interesting question. This line of experimentation may also lead to resolution of the controversy over the order of IKK activation and the possible interdependence of these two highly homologous kinases (40–42). In conclusion, IL-1 and TNF activate two separate but interrelated signal transduction pathways, and both are necessary for the full activation of NF-κB. One set of signals activates the IKK complex, primarily through IKKβ, to phosphorylate IκBα, inducing IκBα degradation and liberating NF-κB, which can then enter the nucleus and bind to DNA. The second set of signals utilizes PI3K and AKT to activate the IKK complex, requiring both IKKa and IKKaβ to phosphorylate and activate the transactivation domain of the p65 subunit of NF-κB.

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REFERENCES

1. Liu, H. C., and Baltimore, D. (1993) *Curr. Opin. Cell Biol.* 5, 477–487
2. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) *Genes Dev.* 9, 2723–2735
3. Basuuerle, P. A., and Henkel, T. (1994) *Annu. Rev. Immunol.* 12, 141–179
4. Siebenlist, U., Franzeno, G., and Brown, K. (1994) *Annu. Rev. Cell Biol.* 10, 405–455
5. Thanos, D., and Maniatis, T. (1995) *Cell* 80, 529–532
6. Anrather, J., Czismadia, V., Soares, M. P., and Winkler, H. (1999) *J. Biol. Chem.* 274, 13594–13603
7. Bird, T. A., Schooley, K., Dower, S. K., Hagen, H., and Virea, G. D. (1997) *J. Biol. Chem.* 272, 32606–32612
8. Diehl, J. A., Tong, W., Sun, G., and Hannink, M. (1995) *J. Biol. Chem.* 270, 2703–2707
9. Martin, A. G., and Fresno, M. (2000) *J. Biol. Chem.* 275, 24383–24391
10. Martin, A. G., San Antonio, B., and Fresno, M. (2001) *J. Biol. Chem.* 276, 15840–15849
11. Nannou, M., and Scheidereit, C. (1994) *EMBO J.* 13, 4957–4967
12. Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T., and Toriumi, W. (1999) *J. Biol. Chem.* 274, 30353–30356
13. Sironi, N., Leung, S., and Stark, G. R. (1999) *Mol. Cell. Biol.* 19, 4798–4805
14. Wang, D., and Baldwin, A. S. (1998) *J. Biol. Chem.* 273, 29411–29416
15. Zhong, H., Voll, R. E., and Ghosh, S. (1998) *Mol. Cell.* 2, 661–671
16. Madrid, L. V., Wang, C. Y., Guitridge, D. C., Schottelius, A. J., Baldwin, A. S., Jr., and Mayo, M. W. (2000) *Mol. Cell. Biol.* 20, 1626–1638
17. Tang, Y., Zhou, H., Chen, A., Pittman, R. N., and Field, J. (2000) *J. Biol. Chem.* 275, 9106–9109
18. Kula, D., Yao, Y., Abbruzzese, J. L., Yung, W. K., and Reddy, S. A. (2001) *J. Biol. Chem.* 276, 11402–11408
19. Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999) *Science* 284, 316–329
20. Li, Q., Estape, G., Memet, S., Israel, A., and Verma, I. M. (2000) *Genes Dev.* 14, 1729–1733
21. Li, Q., Lu, Q., Hwang, J. Y., Buscher, D., Lee, K.-F., Ipsioua-Belmonte, J. C., and Verma, I. M. (1999) *Science* 284, 321–325
22. Li, Q., Van Antwerp, D., Mercurio, F., Lee, K.-F., and Verma, I. M. (1999) *Science* 284, 321–325
23. Takeuchi, K., Takeuchi, O., Tsujimura, T., Itami, S., Adachi, O., Kawai, T., Sanjo, H., Yoshikawa, K., Terada, N., and Akira, S. (1999) *Science* 284, 313–316
24. Tanaka, M., Fuentes, M. E., Yamaguchi, K., Durnin, M. H., Dalrymple, S. A., Hardy, R. L., and Goeddel, D. V. (1999) *Immunity* 10, 421–429
25. Ozso, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) *Nature* 401, 82–85
26. Taka, K., Gu, J., Matsumoto, K., Aota, S., Parsons, R., and Yamada, K. M. (1998) *Science* 280, 1614–1617
27. Reif, K., Burgering, B. M., and Cantrell, D. A. (1997) *J. Biol. Chem.* 272, 14426–14433
28. Burgering, B. M., and Coffey, P. J. (1995) *Nature* 376, 599–602
29. Brockman, J. A., Scherer, D. C., McKinsey, T. A., Hall, S. M., Qi, X., Lee, W. Y., and Ballard, D. W. (1995) *Mol. Cell. Biol.* 15, 2809–2818
30. Ling, L., Cao, Z., and Goeddel, D. V. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 3792–3797
31. Bag, A. A., Sinner, P. V., and Baldwin, A. S., Jr. (1993) *Mol. Cell. Biol.* 13, 3291–3300
32. Welch, P. F., and Wang, J. Y. J. (1993) *Cell* 75, 779–790
33. Kaelin, W. G., Jr., Pallas, D. C., DeCaprio, J. A., Kaye, F. J., and Livingston, D. D. (1991) *Cell* 64, 521–532
34. Madrid, L. V., Mayo, M. W., Reutter, J. B., and Baldwin, A. S., Jr. (2001) *J. Biol. Chem.* 276, 18934–18940
35. Gustin, J. A., Machama, T., Dixon, J. E., and Donner, D. B. (2001) *J. Biol. Chem.* 276, 17247–17254
36. Bergmann, M., Hart, L., Lindsay, M., Barnes, P. J., and Newton, R. (1998) *J. Biol. Chem.* 273, 6607–6610
37. Wesselsloog, S., Bauer, M. K., Vogt, M., Schmitz, M. L., and Schulze-Osthoff, K. (1997) *J. Biol. Chem.* 272, 12422–12429
38. Thang, G., Minemoto, Y., Dibbing, B., Purell, N. H., Li, Z., Karin, M., and Lin, A. (2001) *Nature* 414, 313–317
39. Javelaud, D., and Besancon, F. (2001) *Oncogene* 20, 4365–4372
40. McKenzie, F. R., Connelly, M. A., Balzarano, D., Muller, J. R., Geleziunas, R., and Marcu, K. B. (2000) *Mol. Cell. Biol.* 20, 2635–2649
41. O'Mahony, A., Lin, X., Geleziunas, R., and Greene, W. C. (2000) *Mol. Cell. Biol.* 20, 1170–1178
42. Yamamoto, Y., Yin, M. J., and Gaynor, R. B. (2000) *Mol. Cell. Biol.* 20, 3655–3666
Distinct Roles of the IκB Kinase α and β Subunits in Liberating Nuclear Factor κB (NF-κB) from IκB and in Phosphorylating the p65 Subunit of NF-κB

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