IKKi/IKKɛ Plays a Key Role in Integrating Signals Induced by Pro-inflammatory Stimuli

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We report that the product of the inducible gene encoding the kinase known as IKKi/IKKɛ (IKKi) is required for expression of a group of genes up-regulated by pro-inflammatory stimuli such as bacterial endotoxin (lipopolysaccharide (LPS)). Here, using murine embryonic fibroblasts obtained from mice bearing deletions in IKK2, p65, and IKKi genes, we provide evidence to support a link between signaling through the NF-κB and CCAAA/enhancer-binding protein (C/EBP) pathways. This link includes an NF-κB-dependent regulation of C/EBPβ and C/EBPδ gene transcription and IKKi-mediated activation of C/EBP. Disruption of the NF-κB pathway results in the blockade of the inducible up-regulation of C/EBPβ, C/EBPδ, and IKKi genes. Cells lacking IKKI are normal in activation of the canonical NF-κB pathway but fail to induce C/EBPδ activity and transcription of C/EBPβ and C/EBP-NF-κB target genes in response to LPS. In addition we show that, in response to LPS or tumor necrosis factor α, both β and δ subunits of C/EBP interact with IKKI promoter, suggesting a feedback mechanism in the regulation of IKKI-dependent cellular processes. These data are among the first to provide insights into the biological function of IKKI.

Gene expression during innate and adaptive immune responses involves the combined effects of multiple transcription factors. Among these are members of the activating protein 1, NF-κB, signal transducers and activators of transcription, and CCAAA/enhancer-binding proteins (C/EBP) families (1–3). Prototypic activators of innate immunity such as bacterial endotoxin (lipopolysaccharide (LPS)) are known to regulate both the NF-κB and the C/EBP pathways (4, 5). The C/EBP family is comprised of at least six proteins containing basic leucine zipper (bZIP) motifs (6, 7). The NF-κB family is made up of fewer members including p50, p52, p65/RelA, c-Rel, and RelB (8). These transcription factors regulate expression of distinct and overlapping subsets of genes encoding immune and pro-inflammatory modulators (3, 9). Whether or not NF-κB and C/EBP family members influence each other is not well understood at this time. Current paradigms suggest that the rapidly and transiently activated NF-κB pathway is central in a primary wave of gene induction followed by a second wave of gene transcription some hours later mediated by other transcription factors including members of the C/EBP family (3, 5).

Despite participating in regulation of overlapping sets of genes, the NF-κB and the C/EBP pathways are activated by distinct intracellular signaling mechanisms. Activation of the canonical NF-κB pathway depends on stability of the inhibitor known as IκBo. It stabilizes NF-κB complexes so that after its degradation the remaining subunits translocate from the cytoplasm to the nucleus. NF-κB-dependent transcription of IκBo gene also provides a feedback mechanism maintaining the balance between cytoplasmic and nuclear localization of the NF-κB subunits (8). Treatment of cells with specific inducers, such as TNF, IL-1, or LPS, results in the phosphorylation of IκBo at two serines (Ser-32 and Ser-36). This is a signal for its rapid ubiquitin-dependent proteolysis and translocation of free NF-κB to the nucleus. IκBo phosphorylation is catalyzed by the IκB kinase (IKK), a complex composed of three subunits, IKKa/IKKβ/IKKγ, and NEMO/IKKγ/IKKAP1/FIP3. IKK1 and IKK2 are the catalytic subunits, whereas NEMO serves a non-enzymatic, regulatory function. Biochemical and genetic analyses demonstrate that IKK2 is essential for NF-κB activation in response to TNF, IL-1, and LPS, whereas IKK1 is not required for such responses (10). Targeted deletion of p65 or IKK2 gene affects TNF-induced transcription of the IκBo gene (11, 12). Recently two additional IKK2-related kinases, IKKi/IKKɛ (13, 14) and TANK-binding kinase 1/NF-κB-activating kinase/T2K (15–17) have been identified. Whether these latter proteins play a role as enzymatic or non-enzymatic regulatory factors is not fully understood (4).

Regulatory mechanisms for the C/EBP pathways differ markedly from those of NF-κB and include transcriptional and/or post-translational mechanisms as well as protein-protein interactions via dimerization through leucine-zipper domains (6, 7, 18, 19). Phosphorylation also regulates the C/EBP family by directing nuclear localization and transcription-activating potential (20–23). Some members of this family, specifically C/EBPβ and C/EBPδ, have been linked to gene expression in the acute phase response and during inflammation (24–26). Furthermore, up-regulation of C/EBPβ and C/EBPδ gene expression occurs after exposure to pro-inflammatory stimuli such as TNF, IL-1, IL-6, or LPS (3, 5).

Here we have performed experiments to identify possible regulatory links between the NF-κB and C/EBP pathways, with an emphasis on events related to innate immune re-
sponses. We have used murine embryonic fibroblasts (MEFs) obtained from various strains of mice bearing targeted gene deletions in components of the NF-κB pathway. We show that fibroblasts isolated from p65−/− or IKK2−/−, but not from control, mice fail to induce C/EBPβ and C/EBPδ transcripts in response to cell stimulation with LPS. Furthermore, we observed that both p65 and IKK2 are required for induction of IKKi mRNA. In turn, in primary fibroblasts lacking the IKKi gene LPS induced C/EBPβ and C/EBPδ mRNA and activation of NF-κB, as observed in control cells, but importantly, fail to induce C/EBPδ-specific DNA binding activity. Furthermore, we report that LPS treatment of IKK−/− MEFs reveals deficits in expressions of genes associated with immune and pro-inflammatory responses. In totality, these data support the contention that there is a link between the NF-κB and C/EBP pathways and that IKKi may be a key element in integration of signals from both pathways during inflammatory and immune responses.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Reagents—RelA/p65, IKK2, IKKi, or Egr1-deleted primary fibroblasts corresponding to the corresponding immortalized MEFs (immMEFs) were generated and maintained as described (11, 27, 28). Human umbilical vein endothelial cells were purchased from Clonetics Corp. and maintained in EGM (Cambrex, Gaithersburg, MD). The IKKK, IKK2, IKKI, NEMO, p65, p50, C-Rel, C/EBPδ, and C/EBPβ antibodies were purchased from Santa Cruz. LPS (Escherichia coli 011:B4) was purchased from List Biological Laboratories. Total RNA was prepared by using TRIzol reagent (Invitrogen). The NF-κB, Oct-1, and C/EBP gel shift oligonucleotides were from Santa Cruz. RNA oligonucleotides were purchased from Dharmacon Research. Double-stranded small interfering RNAs (siRNAs) (IKKδ, 5′-GUGAAGGUCUUCACAGCUACC-3′; 5′-UGAGUGGUGGAGGCUCACAG-3′; 5′-CAAGUCGAGGCAUCCAG-3′; 5′-UCCGAGAUCUCGCCGUGUG-3′) were prepared and used for transfection of human umbilical vein endothelial cells (5 × 10⁶ cells per transfection) by using electroporation performed as described (29).

Assays—Samples of total RNA (10 μg) were analyzed by Northern blot as described (13). A blot was hybridized with specific antisense oligonucleotide labeled by T4 polynucleotide kinase using 32P-γ-ATP. Nuclear extracts were prepared and used for electrophoretic mobility shift assays (EMSA) as described (30). Kinase activity of endogenous IKKK or IKKi were measured by immune-complex kinase assay with glutathione S-transferase-IκBα (1–46) as substrate (12, 13). The immune-complexes were also subjected to Western blot to estimate the amount of precipitated proteins. Chromatin immunoprecipitation (ChIP) were performed as described (31). The antibodies specific for C/EBPδ, C/EBPβ, or p65 were used for the ChIP assay. The levels of IKKK or IκBα promoter DNA were determined by PCR using oligonucleotides from the 5′-untranslated region of IKKK gene (5′-TCTGTTAGACGAATGAGCAAG-3′; 5′-AGGAACGCTGGACAGTGTGG-3′) or IκBα gene (5′-AGGGAGAAGGGCTTCTGC-3′; 5′-CTGACTGTGGTG-GGCTGTCG-3′).

Metabolic Labeling—106 cells were plated per 60-mm dish. On the second day, the cells were washed 3 times with phosphate-free Dulbecco's modified Eagle's medium containing 5% of dialyzed fetal bovine serum and then incubated in the same medium containing 400 μCi/ml [32P]Pi PO4 for 4 h. The last 2 h some cells were incubated with 100 μCi/ml [32P]Pi PO4 for 4 h. The last 2 h some cells were incubated with 100 μg/ml LPS. The cells were then washed three times with cold phosphate-buffered saline and used for preparation of nuclear extract according to EMSA protocol (see above). The nuclear extracts were diluted by the addition of 10 volumes of standard radioimmunoprecipitation assay buffer, and C/EBPδ or p65 proteins were recovered by immunoprecipitation with specific antibodies as indicated in Fig. 5C. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

RESULTS

We first sought an experimental system to investigate relationships between the C/EBP and NF-κB pathways. The promoter region of C3 gene contains C/EBP sites (32); it is known that C3 expression is regulated by C/EBPδ (26). Despite the fact that there are no identifiable NF-κB sites in the C3 promoter, transcription of the C3 gene is induced by NF-κB activators including LPS (9, 33). Thus, we reasoned that measurements of LPS induction of C3 mRNA would be an appropriate marker for initial studies to investigate possible relationships between the NF-κB and C/EBP pathways. Here we have used MEFs from mice bearing targeted deletions of genes encoding IKKK, p65, and IKKi. We first examined induction of C3 mRNA in LPS-treated MEFs derived from IKK2−/− and control (IKK2+/+) embryos; the induction of C3 mRNA was observed in control cells but not in the IKK2-deficient cells (Fig. 1A). In contrast, LPS-mediated induction of c-Jun mRNA was nearly identical in both types of MEFs. Moreover, as shown here and as previously noted, IKK2−/−/− MEFs showed LPS-induced IκBα mRNA and NF-κB DNA binding activity that was partially reduced when IKK2−/− and IKK2+/− cells were compared (Ref. 12; Fig. 1, A and B). Similar findings were noted in experiments with spontaneously imMEFs derived from IKK2−/− and control cells (Fig. 1C). Thus, the absence of IKKK revealed a deficiency in LPS-induced C3 induction that is not likely to result from loss of LPS responsiveness. To further probe the role of the NF-κB pathway we also examined the effects of p65 deficiency on LPS induction of C3 and IκBα mRNA. LPS-mediated induction of both IκBα and C3 mRNAs was completely abolished in p65−/− imMEFs (Fig. 1D). These data suggest that regulation of C3 gene expression requires an intact NF-κB pathway. However, we hypothesize that the role of NF-κB involves indirect mechanisms and requires additional gene expression under the control of NF-κB. In fact it has been shown that protein synthesis is required for LPS-induced expression of C3 mRNA (34).

The observation that LPS induces IKKK mRNA (13) prompted us to evaluate the effects of IKKK deletion on C3 gene expression. Thus, we next measured LPS-induced C3, IκBα, and IKKI mRNA in IKK−/− and control MEFs as well as in IKK2−/− cells (Fig. 2A). As shown above, LPS-mediated induction of IκBα mRNA was reduced in cells lacking IKK1, whereas IKKK deficiency had no effect on LPS-induced expression of IκBα mRNA. In contrast, the induction of C3 mRNA was abolished in the IKK−/− cells as well as in the IKK2−/− cells. Furthermore, we failed to observe induction of IKKI mRNA in the IKK2−/− cells. Nearly identical results were also obtained in similar experiments with LPS-stimulated IKK1−/− and IKK1+/− imMEFs derived from the corresponding primary isolates of MEFs (Fig. 2B). As an additional specificity control, we also examined the LPS responses in MEFs derived from Egr1-deficient embryos (28). The LPS-mediated induction of C3 or IKKI mRNA was practically identical in Egr1−/− and Egr1+/+ cells (Fig. 2C), emphasizing the specificity of the effects observed here with IKK2−/−, p65−/−, and IKK1−/− MEFs. Thus, IKKK appears to be a key molecule for transcriptional induction of C3 gene in response to LPS, and its expression requires an intact NF-κB pathway.

To further define how IKKK participates in gene regulation we performed the following experiments. Extracts from IKK2−/−, IKK2+/+, IKK1−/− (as negative control), or IKK1+/+ (as positive control) were immunoprecipitated to enrich the samples for IKKI, and the resultant immunoprecipitants were electrophoresed and then subjected to Western blot analysis (Fig. 3A). As was expected, expression of IKKI protein was not detected in IKK1−/− cells. In contrast, IKK1+/+ or IKK2+/+ cells showed detectable IKKI protein expression that was increased after LPS addition. Compared with IKK2+/+ or IKK1+/+ cells, the basal level of IKKI protein was significantly reduced in IKK2-deficient cells. Importantly, LPS addition failed to up-regulate IKKI expression in this cell type. These data together with Northern blot studies support the contention that IKK2 is required for the inducible expression of IKKI mRNA and protein. These data prompted us to address the
question of how IKKi regulates LPS-induced C3 gene expression. Specifically, we asked whether the failure to induce C3 results from the absence of IKKi protein or whether essential signaling including expression and/or activation of IKK2 is also affected by IKKi deficiency. To address these issues, we used Western blot analysis to compare the levels of IKK2 protein in extracts from IKKi−/−, IKKi+/+, IKK2−/− (as negative control), or IKK2+/+ (as positive control) cells. The results showed that IKKi−/−, IKKi+/+, and IKK2+/+ cells express nearly identical levels of IKK2 protein (Fig. 3B). Expression of IKK1 and NEMO subunits of the IKK complex was also unchanged in IKKi−/− cells (Fig. 3B). Thus, the absence of IKKi does not reduce the protein expression of other key members of the IKK complex. Furthermore, it is unlikely that IKKi is required for activation of IKK complex because LPS treatment up-regulated the kinase activity of the IKK similarly in IKKi−/− and
IKK+/- cells (Fig. 3C). Thus, the absence of IKKi does not alter signaling that is directly related to NF-kB activation. The same extracts were subjected in parallel to an immunoprecipitation/kinase assay for IKKi. In addition LPS treatment did not alter IKKi kinase activity but, rather, up-regulate its expression (13). Thus, the absence of IKKi does not alter signaling that is directly related to NF-kB activation. In contrast IKKi deficiency resulted in the reduction of C/EBP DNA binding activity induced by LPS and affected the induction of DNA binding complexes containing essentially the same proteins including p65 (Fig. 4E). Phosphorylation of p65 subunit increases its ability to activate transcription of NF-kB target genes (8, 35). Although our data do not exclude a role for IKKi in this process, we believe that to be unlikely because LPS-induced expression of IkBa gene, a classical NF-kB target gene (8, 11), is not affected by IKKi deficiency (see Fig. 2, A and B). Finally we examined LPS-induced activation of IRF-3 in IKK+/− MEFs since the promoter region of IP-10 gene contains interferon stimuluss-responsive element, a binding site for transcription factor IRF-3 (36, 37). The results showed that LPS treatment induces essentially the same levels of interferon stimulus-responsive element binding activity in IKK+/− and IKK+/+ MEFs (Fig. 4F), suggesting that IKKi is not involved in regulation of IRF-3.

Others show that C/EBPβ is involved in IL-1-induced regulation of the C3 gene (26). Moreover, C/EBPβ appears to synergize with C/EBPβ, another member of the C/EBP family, in transcriptional regulation of the IL-6 gene (24, 25, 38). Consistent with previous reports (24, 25), we also observed that LPS induces C/EBPβ and C/EBPβ proteins in IKKi−/− and IKK+/+ MEFs (see Fig. 4A). As both nuclear localization and transcriptional activating potential of C/EBP family members may be regulated by phosphorylation (20, 21, 23, 39), we also investigated whether IKKi-deficiency affects the function of C/EBP genes in IKKi-deficient MEFs since the promoter region of IP-10 gene contains interferon stimuluss-responsive element, a binding site for transcription factor IRF-3 (36, 37). The results showed that LPS treatment induces essentially the same levels of interferon stimulus-responsive element binding activity in IKK+/− and IKK+/+ MEFs (Fig. 4F), suggesting that IKKi is not involved in regulation of IRF-3.

Comparative analysis of the promoters of the group of IKKi-modulated genes depicted in Fig. 4A identified the presence of binding sites for multiple transcription factors including NF-kB, interferon regulatory factor-3 (IRF-3), and C/EBP. Although NF-kB is known to be involved in the regulation all of these genes (8, 9), a number of observations suggest that the absence of IKKi has no effect on activation of the canonical NF-kB pathway. First, we have shown that LPS-mediated activation of IKK complex activity (see Fig. 3C) and translocation of NF-kB were indistinguishable when IKK+/+ and IKK−/− MEFs were compared (Fig. 4D). Second, analysis of the subunit composition of the NF-kB binding activity with antibodies specific for NF-kB-related proteins revealed that treatment of IKK−/− and IKK+/+ cells with LPS induces DNA binding complexes containing essentially the same proteins including p65 (Fig. 4E). Phosphorylation of p65 subunit increases its ability to activate transcription of NF-kB target genes (8, 35). Although our data do not exclude a role for IKKi in this process, we believe that to be unlikely because LPS-induced expression of IkBa gene, a classical NF-kB target gene (8, 11), is not affected by IKKi deficiency (see Fig. 2, A and B). Finally we examined LPS-induced activation of IRF-3 in IKK+/− MEFs since the promoter region of IP-10 gene contains interferon stimuluss-responsive element, a binding site for transcription factor IRF-3 (36, 37). The results showed that LPS treatment induces essentially the same levels of interferon stimulus-responsive element binding activity in IKK+/− and IKK+/+ MEFs (Fig. 4F), suggesting that IKKi is not involved in regulation of IRF-3.

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revealed that IKKi-deficiency results in a significant reduction of a phosphoprotein precipitated by antibodies specific for C/EBPβ. In contrast the absence of IKKi did not effect p65/RelA phosphorylation (Fig. 5C). These results support the contention that IKKi is involved in the regulation of C/EBPβ activity.

To confirm and extend the findings obtained using MEFs we...
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Fig. 5. IKKi is required for post-transcriptional regulation of C/EBPβ.

A, EMSA of nuclear extracts from IKKi−/− and IKKi+/+ MEFs before and after 4 h of treatment with LPS. The nuclear extracts were incubated in the presence of specific antibody (Ab) to C/EBPβ (β) or C/EBPδ (δ), as indicated on the top of each lane. The DNA binding activity of C/EBP or Oct-1 (as a control) transcription factor is shown. B, Western blot (WB) analysis for C/EBPβ and actin (as a loading control) protein expression in IKKi+/+ and IKKi−/− MEFs before and after 4 h of treatment with LPS (100 ng/ml). C, analysis of [32P]orthophosphate for 2 h and LPS treatment, the nuclear extracts were prepared, immunoprecipitated (IP) by anti-C/EBPβ or anti-p65, and consequently subjected to SDS-PAGE and autoradiography. The phosphorylated products (C/EBPβ and p65) are shown on the left. The positions of size markers are shown on the right. D, Northern blot analysis of steady-state IKKi mRNA levels in untreated or treated with LPS human umbilical vein endothelial cells; cells were pre-transfected with siRNA as indicated. ns, nonspecific control.

Our findings support the contention that IKKi acting through C/EBPβ links the NF-κB and C/EBP pathways. It appears that control of IKKi gene expression plays an important role in this process. Studies with IKK2−/− MEFs indicate that activation of NF-κB is required for induction of IKKi and C3 mRNA. We further addressed this possibility using p65−/− and IKK2−/− cells. Northern blot analysis revealed that when compared with p65+/+ imMEFs, the p65−/− cells fail to induce IKKi, C/EBPβ and C/EBPδ mRNAs in response to LPS. In contrast basal levels of these transcripts were normal in p65−/− cells (Fig. 6A). Similar experiments using IKK2−/− cells also revealed a blockade in induction of C/EBPβ and C/EBPδ mRNA (Fig. 6B). Thus, these data support the conclusion that a functional NF-κB pathway is required for regulation of C/EBPβ and C/EBPδ genes. The data also raise the question of how IKKi is regulated - primarily through NF-κB or through C/EBP or through combined effects of both pathways.

To address questions of IKKi regulation we analyzed the 5′-untranslated regions of human and mouse DNA in the vicinity of the IKKi gene. Interestingly our analysis did not reveal the presence of NF-κB sites but rather indicated that both the murine and human genes have identical sequences containing a C/EBP-like DNA binding site (Fig. 6C). By using electrophoretic gel shift assay carried out on a nuclear extract prepared from LPS-treated cells we found that this sequence has the C/EBP-specific binding activity in vitro (Fig. 6D). The interaction of C/EBP with IKKi promoter was also demonstrated via chromatin immunoprecipitation (ChIP) assay. The results of ChIP assay performed on chromatin samples from untreated or LPS-treated imMEFs confirmed that both C/EBPβ and C/EBPδ are able to bind with the promoter region of IKKi gene in vivo (Fig. 6E). Similar results were obtained on chromatin samples from TNF-treated cells (Fig. 6E). The binding of p65 subunit of NF-κB with the IκBα promoter was used as positive control, whereas the chromatin samples from IKK2−/− imMEFs were tested as negative control for C/EBP-specific binding with IKKi promoter. Therefore our data provide support for the contention that up-regulation of the IKKi promoter by pro-inflammatory mediators such as LPS and TNF is likely to involve members of the C/EBP family.

DISCUSSION

The members of the C/EBP and NF-κB families are known to regulate overlapping cellular processes, including the control of inflammation and immunity (2, 3, 5, 8). C/EBP and NF-κB DNA binding motifs have been identified in the promoter regions of genes encoding immune and pro-inflammatory modulators, including IL-6 (24, 40), IL-1β (41), IP-10 (36), TNF (42), RANTES (43) and COX-2 (44). Previous studies have also shown that the p50 subunit of NF-κB associates with the C/EBPβ/NF-IL6, suggesting cross-talk between NF-κB and
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C/EBP in regulation of immune and acute-phase responses (45). The nature of the mechanisms responsible for coordination of C/EBP and NF-κB pathways has been unclear.

Here we provide experimental data to support the contention that the IKK2-related kinase IKKα is a key molecule for functional coordination between these two pathways. The IKKα-directed functional link between NF-κB and C/EBP seems to be a “nonlinear” mechanism that includes a NF-κB-dependent regulation of C/EBPβ and C/EBPδ gene transcription and IKKα-mediated activation of C/EBP-specific DNA binding activity. Consistent with this mechanism, our experiments carried out on MEFs derived from mice with targeted deletion of IKKα gene showed that IKKα is required for LPS induction of genes encoding IL-1β, IL-6, IP-10, TNF, RANTES and COX-2. Further support for the role of IKKα was obtained in the experiments showing that siRNA-directed reduction of IKKα expression in human umbilical vein endothelial cells results in significant inhibition of LPS-mediated up-regulation of the C/EBP-NF-κB-regulated target gene for IL-6.

Previous work by others demonstrated that the promoter of the C3 gene contains C/EBP binding sites (32) and is trans-activated by C/EBPδ (26). Our findings extend these observations by showing that IKKα is required for LPS-induced activation of a C/EBP-specific DNA binding complex containing C/EBPδ and driving expression of C3. Moreover, we found that IKKα-deficiency results in a significant reduction of LPS-induced phosphorylation of a protein precipitated by antibodies specific for C/EBPδ. Although further work is needed to characterize the nature of this phosphoprotein, these results are consistent with the assumption that IKKα is involved in the regulation of C/EBPδ activity induced by LPS. In contrast to the effect on C/EBP we did not find evidence for linkage of IKKα deficiency to other pathways initiated by LPS.

Previous studies have demonstrated that activators of the NF-κB pathway, such as LPS, TNF and IL-1 (9), induce up-regulation of C/EBPβ, C/EBPδ and IKKα mRNA (24, 25, 13). However the exact sequence of events in this process has not been defined. Here we show that LPS-mediated activation of the genes for both C/EBPβ and C/EBPδ requires intact NF-κB pathway, since neither gene is induced by LPS in IKK2- or p65-deficient MEFs. Further examination of the C/EBPβ and C/EBPδ promoters revealed the presence of κB-like sites, suggesting that NF-κB directly involves in the LPS-mediated regulation of C/EBPβ and C/EBPδ genes. However additional studies are needed to more completely understand the role of these sites. In contrast, the analysis of the promoter sequences in both human and mouse IKKα genes failed to identify a κB-like site. Surprisingly we also noted that IKKα promoter contains a C/EBP-like site with identical sequences in human and mouse genes. Our analysis revealed that both β and δ

![Image](http://www.jbc.org/)

**FIG. 6.** IKKα couples the C/EBP and NF-κB pathways. A, Northern blot analysis of IKKα, C/EBPβ, C/EBPδ, and GAPDH steady-state mRNA levels in p65−/− and p65+/+ imMEFs treated with LPS (100 ng/ml) for the indicated times. B, Northern blot analysis of C/EBPβ, C/EBPδ, and GAPDH steady-state mRNA levels in IKK2−/− and IKK2+/+ imMEFs treated with LPS (100 ng/ml) for the indicated times. C, the 36-nucleotide sequence of mouse or human (shown in bold) chromosome 1 identified 480 bp upstream of the translation initiation site of IKKα gene. A sequence of C/EBP binding site is underlined. D, EMSA of nuclear extract from normal imMEFs treated with LPS for 2 h. The nuclear extracts were incubated with a 32P-labeled 36-bp DNA fragment (see a sequence of the upper strand on the panel C) in the absence or in the presence of competitive (Comp) unlabeled oligonucleotide (2 pmoI) containing the wild type 36-bp fragment (W), the C/EBP consensus sequence (C), or the wild type 36-bp fragment containing the mutation of a C/EBP binding motif (M). Ab, antibody. In addition, some samples were incubated in the presence of normal rabbit IgG (N) or in the presence of specific antibodies (2 μg per reaction) against C/EBPα, β, or δ as indicated. E, ChIP assays were carried out on chromatin samples from IKK2−/− (negative control), IKKα+/+, and IKKα−/− (positive control) imMEFs untreated or treated with LPS or TNF for 2 h. The chromatin was immunoprecipitated with antibodies to C/EBP (β or δ) or p65 as an additional control. Shown is an IKKα or an Iкκα (positive control) promoter fragment amplified by PCR from the ChIP samples.
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subunits of C/EBP interact with an IKKi fragment protein containing this sequence. This observation thus supports the conclusion that IKKi gene expression is regulated by C/EBP. After the submission of the present work, Sharma et al. (46) and Fitzgerald et al. (47) reported on experiments with human cell lines showing a link between TANK-binding kinase 1 and IKKi/IKKε and phosphorylation of endogenous IRF-3 (46) and reporter gene expression regulated by interferon regulatory factor-3 (IRF-3) and IRF-7 in response to virus infection. Here we have not examined effect of IKKi-deficiency on virus induced responses in MEFs so it is difficult to compare our studies with those recently published (46, 47). However we believe each report provides new evidence linking IKKi to innate immune responses to both viral and bacterial infection. Here we showed that LPS-mediated activation of IRF-3 DNA-binding activity was normal in IKKi−/− cells, whereas the activation of IRF family members target genes for IP-10 and RANTES was blocked in IKKi-deficient cells. This result may reflect differences in signaling via TRB4- and TRB3-dependent pathways. IKKi-deficiency also resulted in the reduction of LPS-mediated expression of TNF, IL-6, IL-1 and COX-2 genes; in none of these cases does the promoter appear to contain IRF sites. In contrast a common characteristic of the promoters for this group of IKKi-affected genes, including IP-10 and RANTES, is the presence of binding sites for C/EBP and NF-κB. Here we provide clear evidence that IKKi is not involved in the activation of NF-κB but significantly affects the activation of C/EBP. It has been shown that similar to IRF-3 IKKi couples the C/EBP. It has been shown that similar to IRF-3 signaling molecule required for LPS-inducible transcriptional activation of C/EBP. It has been shown that similar to IRF-3 signaling molecule required for LPS-inducible transcriptional activation of C/EBP. It has been shown that similar to IRF-3 signaling molecule required for LPS-inducible transcriptional activation of C/EBP. It has been shown that similar to IRF-3 signaling molecule required for LPS-inducible transcriptional activation of C/EBP. It has been shown that similar to IRF-3 signaling molecule required for LPS-inducible transcriptional activation of C/EBP. It has been shown that similar to IRF-3 signaling molecule required for LPS-inducible transcriptional activation of C/EBP. It has been shown that similar to IRF-3 signaling molecule required for LPS-inducible transcriptional activation of C/EBP. It has been shown that similar to IRF-3 signaling molecule required for LPS-inducible transcriptional activation of C/EBP. It has been shown that similar to IRF-3 signaling molecule required for LPS-inducible transcriptional activation of C/EBP. It has been shown that similar to IRF-3 signaling molecule required for LPS-inducible transcriptional activation of C/EBP.
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