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Embryonic Lethality, Liver Degeneration, and Impaired NF-κB Activation in IKK-β-Deficient Mice

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Summary

IκB kinase-α and -β (IKK-α and IKK-β), the catalytic subunits of the IKK complex, phosphorylate IκB proteins on specific serine residues, thus targeting IκB for degradation and activating the transcription factor NF-κB. To elucidate the in vivo function of IKK-β, we generated IKK-β-deficient mice. The homozygous mouse embryo dies at ~14.5 days of gestation due to liver degeneration and apoptosis. IKK-β-deficient embryonic fibroblasts have both reduced basal NF-κB activity and impaired cytokine-induced NF-κB activation. Similarly, basal and cytokine-inducible kinase activities of the IKK complex are greatly reduced in IKK-β-deficient cells. These results indicate that IKK-β is crucial for liver development and regulation of NF-κB activity and that IKK-α can only partially compensate for the loss of IKK-β.

Introduction

NF-κB is a transcription factor that regulates expression of many inflammatory response genes, including proinflammatory cytokines, chemokines, and adhesion molecules (Baeuerle and Henkel, 1994; Barnes and Karin, 1997). NF-κB is composed of homo- and heterodimers of members of the Rel protein family. In most cells, the heterodimer consisting of p50 and p65 (RelA) is the major species of NF-κB. This p50/p65 complex is maintained in the cytoplasm by binding to IκB inhibitory proteins (Baeuerle and Henkel, 1994; Thanos and Maniatis, 1995; Verma et al., 1995; Baeuerle and Baltimore, 1996). In response to cellular stimulation with proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) or with bacterial lipopolysaccharide (LPS), IκB proteins are phosphorylated on two conserved serine residues (Ser-32 and -36 on IκB-α and Ser-19 and -23 on IκB-β) and then ubiquitinated and degraded by the 26S proteosome pathway (Verma et al., 1995). This removal of IκB from NF-κB allows NF-κB to translocate into the nucleus where it transactivates numerous genes by binding to specific sequences in their promoters.

The kinase responsible for the signal-induced phosphorylation of IκB exists in a high molecular weight protein complex that can be activated in vitro by the kinase MEKK1 or by ubiquitination (Lee et al., 1997). The two kinases in this complex, IκB kinase-α (IKK-α) and -β (IKK-β), were identified by biochemical purification and interaction cloning methods (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). These kinases are highly related in sequence, are rapidly activated following treatment with TNF or IL-1, and specifically phosphorylate the two critical serines of IκB. In addition, catalytically inactive forms of IKK-α and IKK-β inhibit NF-κB activation mediated by TNF and IL-1, suggesting that IKK-α and -β are responsible for IκB phosphorylation and subsequent NF-κB activation. Recently, a third component of the IKK complex, designated NEMO/IKK-γ, was identified by complementation cloning in NF-κB-unresponsive cells (Yamaoka et al., 1998) and by affinity purification using antibodies to IKK-α (Rothwarf et al., 1998). NEMO is a 47 kDa protein that interacts with IKK-α and IKK-β. NEMO-deficient cells are unable to activate NF-κB in response to TNF, IL-1, or LPS. Furthermore, IKK complexes lacking NEMO cannot be activated to phosphorylate IκB, indicating that NEMO is an essential component of the IKK complex.

NF-κB-inducing kinase (NIK) and MEKK1 have been proposed to be upstream activators of IKKs (Malinin et al., 1997; Nemoto et al., 1998). NIK is a MEKK family member that binds to and activates both IKK-α and IKK-β when overexpressed (Regnier et al., 1997; Woronicz et al., 1997). In addition, NIK can phosphorylate Ser-176 of IKK-α, a modification that is required for the kinase activity of IKK-α (Ling et al., 1998). Since overexpressed NIK binds to TRAF2, TRAF5, and TRAF6, and since kinase inactive mutants of NIK are potent dominant-negative inhibitors of TNF and IL-1 mediated NF-κB activation (Malinin et al., 1997; Song et al., 1997), it is possible that TNF and IL-1 act through TRAF proteins to activate NIK, and that activated NIK may transduce the signal for NF-κB activation by phosphorylating IKKs. MEKK1, another member of the MEKK family, is also capable of activating the kinase activity of the IKK complex (Lee et al., 1998; Nemoto et al., 1998). However, whether inflammatory cytokine signaling pathways actually activate NIK and MEKK1 is still unknown.

It is well established that NF-κB activation inhibits cells from undergoing TNF-mediated apoptosis (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996). The binding of TNF to the type I TNF receptor (TNFR1) results in the generation of an apoptotic signal through a pathway involving TRADD and FADD (Hsu et al., 1995, 1996b; Yeh et al., 1998). However, most cells are resistant to TNF-induced apoptosis because TNFR1 simultaneously activates NF-κB through the TRADD-RIP pathway (Hsu et al., 1996a, 1996b; Kelliher et al., 1998). The mechanism by which

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NF-κB activation is antiapoptotic may include the transcriptional activation of genes encoding inhibitor of apoptosis proteins (IAPs) (Chu et al., 1997; Stehlik et al., 1998; Wang et al., 1998), two of which are also a part of the TNFR1 signaling complex (Rothe et al., 1995; Shu et al., 1996).

IKK-α and IKK-β contain leucine zipper and helix-loop-helix motifs and can form both homodimers and heterodimers. However, they exist preferentially as a heterodimer (Woronicz et al., 1997). The catalytically inactive form of each kinase inhibits TNF and IL-1-induced NF-κB activation (Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997), suggesting that both kinases may play an essential role in IKK activity. However, the actual physiological role of each individual kinase is still unknown.

To elucidate the in vivo function of IKK-β, we generated *IKK*-β knockout (KO) mice by gene targeting. Homozygous mice die at about embryonic day 14.5 (E14.5), apparently due to liver degeneration. TNF and IL-1-mediated activation of NF-κB is severely compromised in IKK-β-deficient embryonic fibroblasts (EFs). IKK kinase activity in these cells is also greatly reduced in comparison to wild-type (WT) cells. These results show that the related kinase IKK-α cannot compensate for the loss of IKK-β.

Results

Generation of IKK-β-Deficient Mice

A single *IKK*-β allele in embryonic stem (ES) cells was disrupted by homologous recombination using an *IKK*-β targeting vector (Figure 1A). The vector was designed to disrupt the *IKK*-β gene by deletion of exon 2, which encodes the ATP binding site of the kinase domain of IKK-β (Woronicz et al., 1997). Heterozygous ES cell lines containing a mutant *IKK*-β allele were injected into C57Bl/6 blastocysts. Male chimeras with germline transmission were used to generate *IKK*-$\beta^{+/-}$ animals. Intercrossing of heterozygous animals generated only heterozygous and WT mice, suggesting that IKK-β deficiency results in embryonic lethality.

We next used timed matings of heterozygous animals to generate embryos at various stages of development. Thirty-five embryos obtained at 12.5 days of gestation (E12.5) were genotyped by Southern blotting (Figure 1B). The three genotypes were represented in expected Mendelian ratios ($8^{+/+}$, $19^{+/-}$, and $8^{-/-}$). To confirm that IKK-β-deficient embryos do not express IKK-β protein, we examined embryonic fibroblasts from E12.5 embryos. IKK-β is normally expressed in most tissues (Hu and Wang, 1998) in a complex with IKK-α (Mercurio et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). Therefore, we immunoprecipitated the IKK complex from lysates of embryonic fibroblasts with an anti-IKK-α antibody, followed by Western blotting with either anti-IKK-α or anti-IKK-β antibodies. IKK-β protein was coimmunoprecipitated with IKK-α in fibroblasts from both WT and heterozygous embryos. The amount of IKK-β protein coimmunoprecipitated with IKK-α was indistinguishable in heterozygous and WT cells. However, no IKK-β protein could be detected in homozygous$^{-/-}$ embryos (Figure 1C). The amount of IKK-α protein in homozygous deficient embryos was greater than in wild-type

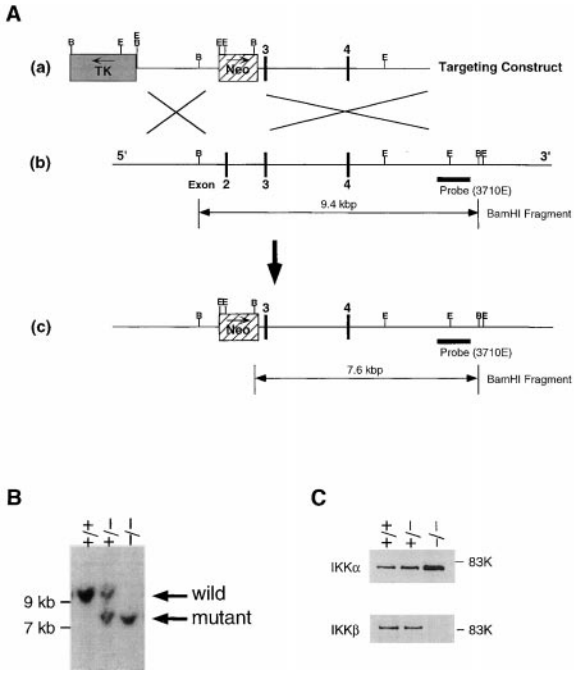

Figure 1. Targeted Disruption of the Mouse *IKK*-β Gene

(A) Schematic drawing of targeting procedure. (Aa) The targeting vector MTp038-2; (Ab) the genomic structure of the *IKK*-β locus with the region surrounding exons 2 through 4; (Ac) the disrupted *IKK*-β allele after homologous recombination. Exons 2 through 4 are indicated by solid bars. The TK cassette at the 5′ end of the targeting vector is indicated by a shaded box, and the Neo cassette is indicated by a hatched box. The probe (3710E) used for Southern blotting is indicated as a solid line together with the predicted hybridizing fragments.

(B) Southern blotting of genomic DNA from the embryos of a heterozygous cross. DNA extracted from embryos was digested with BamHI and hybridized to the radiolabeled probe (3710E). The bands predicted from wild-type and mutant alleles are indicated by arrows. Southern blotting gave a single 9.4 kb band for wild-type (+/+), a 7.6 kb band for homozygous mice (−/−), and both bands for heterozygous mice (+/−).

(C) Expression of IKK proteins in homozygous mutant embryonic fibroblasts. Since our anti-IKK-β antibody does not work in immunoprecipitations, cell lysates from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) embryonic fibroblasts were subjected to immunoprecipitation with anti-IKK-α antibody, followed by Western blotting with either anti-IKK-α antibody (top) or anti-IKK-β antibody (bottom). Position of an 83 kDa of molecular weight standard (NEB) is shown.

embryos, suggesting that IKK-α expression is upregulated in IKK-β-deficient cells.

Liver Degeneration in IKK-β-Deficient Embryos

To examine the time of lethality, further timed pregnancies were performed. At E13.5, 15 of the 45 embryos were *IKK*-$\beta^{-/-}$, and all of these appeared normal. However, at E14.5, only about 10% of the embryos were *IKK*-$\beta^{-/-}$. Histological examination of viable E14.5 WT and *IKK*-$\beta^{-/-}$ embryos indicated that massive liver degeneration had occurred in the homozygous embryos (Figure 2B). The hepatocytes showed pyknotic nuclei, suggesting that the cells were undergoing apoptosis. These results suggest that IKK-β-deficient embryos die

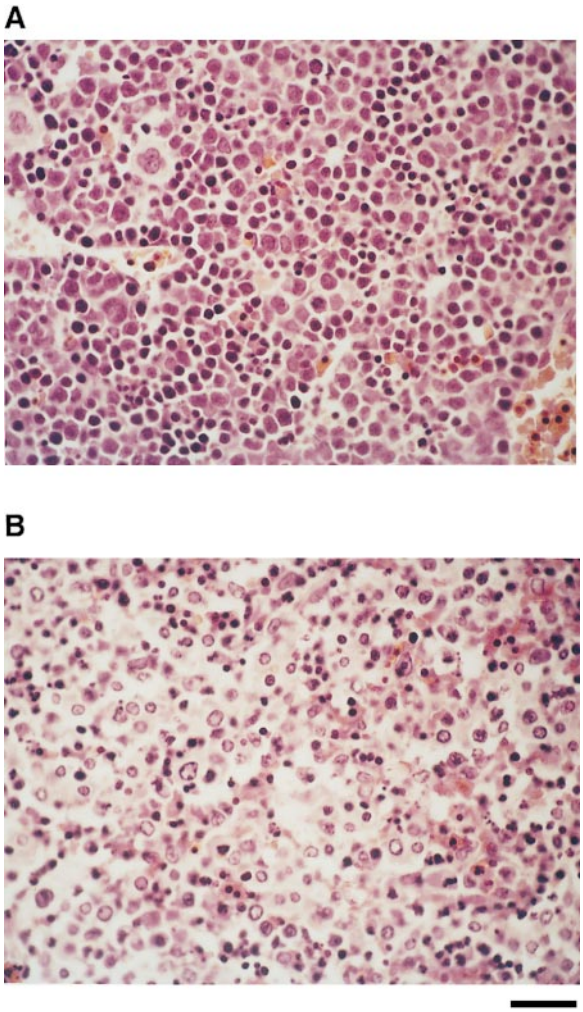

Figure 2. Histological Analysis of the Liver of IKK-β-Deficient Embryos

IKK-$\beta^{+/+}$ (A) and *IKK*-$\beta^{-/-}$ (B) embryos at E14.5 were fixed with 10% neutral buffered formalin and embedded in paraffin. Four micrometer sections were stained with hematoxylin and eosin. The scale bar indicates 10 μm.

at approximately E14.5 due to liver degeneration associated with hepatocyte apoptosis. Thus, the phenotype of IKK-$\beta^{-/-}$ mice is similar but slightly more severe than that of RelA-deficient mice that die between E15 and E16 (Beg et al., 1995).

Since liver degeneration was observed in IKK-β-deficient embryos, we assessed the status of fetal liver hematopoietic progenitor cells by performing in vitro colony-forming assays. Fetal liver cells from WT and IKK-β-deficient E13.5 embryos were assayed in the presence of appropriate cytokines for their ability to differentiate into either myeloid or erythroid colonies (Table 1). IKK-β-deficient hematopoietic progenitors were indistinguishable from WT controls in their ability to form mixed myeloid (CFU-Mix) colonies or late erythroid (CFU-E) colonies. Additionally, the distribution of specific colony types in the CFU-Mix assay was similar between WT and IKK-β-deficient fetal liver cells. These data indicate that IKK-β is not required for fetal liver hematopoiesis.

Impaired NF-κB Activation in IKK-β-Deficient Cells

IKK-α and IKK-β become rapidly activated by TNF or IL-1 treatment. Both IKKs specifically phosphorylate IκB proteins on two critical serine residues (Regnier et al., 1997; Woronicz et al., 1997), leading to the activation of NF-κB. To determine whether cytokine-inducible NF-κB activation requires IKK-β, WT and *IKK*-$\beta^{-/-}$ primary EF cells were stimulated with either cytokine, and NF-κB activation was examined by electrophoretic mobility shift assay. In WT cells, NF-κB activation occurred within 15 min of TNF stimulation (Figure 3A). However, in IKK-β-deficient cells, NF-κB activation occurred with delayed kinetics, and at 30 min this activation was 4-fold less than in WT cells. Similar delayed kinetics and reduced magnitude of NF-κB activation were observed in IKK-β-deficient cells following stimulation with IL-1 (Figure 3B).

IκB phosphorylation and degradation are required for NF-κB activation. Thus, we examined IκB protein levels after treatment with inflammatory cytokines. WT and IKK-β-deficient EF cells were stimulated with IL-1, and IκB-α levels were determined by immunoblotting. In WT cells, the amount of IκB-α was greatly reduced 15 min after stimulation but was restored to normal levels by 60 min. This restoration is the result of de novo production of IκB-α induced by NF-κB activation (Barnes and Karin, 1997). In contrast, in IKK-β-deficient cells, only a small amount of IκB-α was degraded 30 min after stimulation. These results indicate that NF-κB activation by TNF and IL-1 is significantly impaired, but not completely abolished, in IKK-β-deficient cells.

Decreased IL-6 Production in IKK-β-Deficient Cells

TNF and IL-1 transduce signals culminating in the expression of various inflammatory gene products (Baeuerle and Baltimore, 1996; Dinarello, 1996; Barnes and Karin, 1997). One such product is interleukin 6 (IL-6), a mediator of acute phase reactions that is induced by TNF and

Table 1. In Vitro Colony-Forming Assay of Fetal Liver Cells

	CFU-Mix						
Genotype	CFU-G	CFU-M	CFU-GM	BFU-E	CFU-Multi	Total	CFU-E
+/+ (n = 7)	23 ± 3.5	90 ± 10.8	120 ± 19.2	37 ± 6.5	23 ± 3.7	293 ± 37.7	1006 ± 152
−/− (n = 8)	33 ± 7.3	73 ± 15.7	101 ± 27.3	35 ± 9.5	16 ± 4.0	258 ± 60.4	845 ± 244

Fetal liver cells of E13.5 embryos were cultured in the conditions as described in the Experimental Procedures. Data are expressed as mean colony number ± SEM per 10^5 (CFU-Mix) or 5×10^4 (CFU-E) fetal liver cells. For the CFU-Mix, only colonies with more than 50 cells were counted.

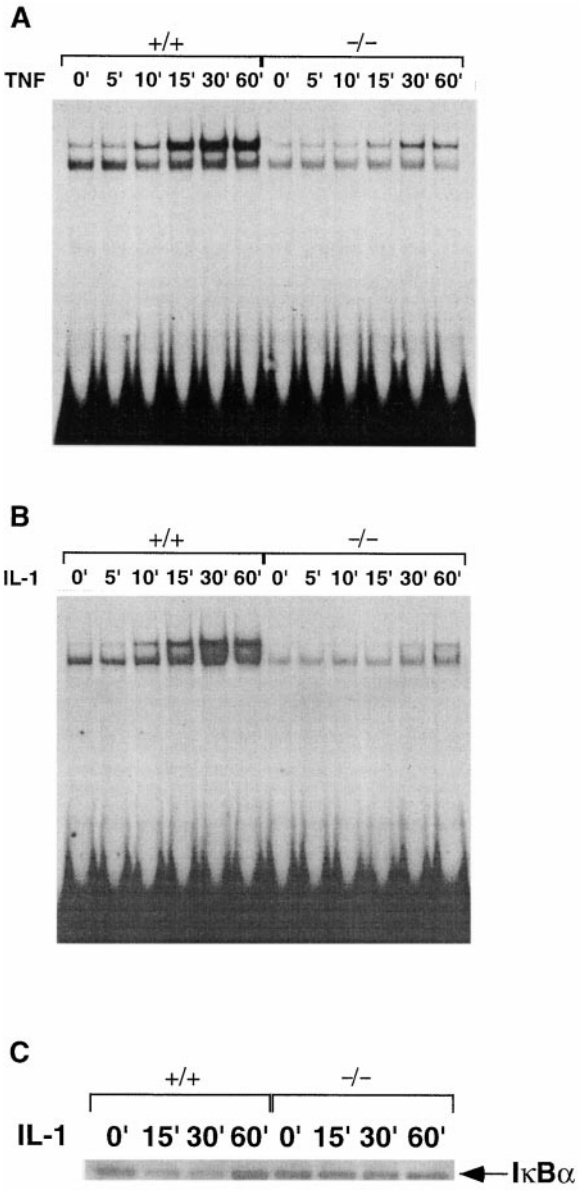

Figure 3. NF-κB Activation after Stimulation of IKK-β-Deficient Embryonic Fibroblasts

IKK-β-deficient EF cells (−/−) and wild-type EFs (+/+) were incubated with 10 ng/ml of TNF (A) or 10 ng/ml of IL-1β (B) for the indicated times. Total cell extracts were incubated with a radioactive probe containing an NF-κB binding site, and NF-κB activation was determined by gel shift assay as described in the Experimental Procedures. (C) Degradation of IκB-α protein. IKK-β-deficient embryonic fibroblasts (−/−) and wild-type controls (+/+) were incubated with 10 ng/ml of IL-1β for the indicated times. IκB-α protein was immunoprecipitated from the cell lysate with an anti-IκB-α antibody-conjugated agarose gel and was detected by Western blotting.

IL-1 through the coordinated action of NF-κB and NF-IL6 (Akira and Kishimoto, 1992; Bankers-Fulbright et al., 1996). We examined EF cells and found that IL-6 was secreted from WT EF cells upon stimulation with either TNF or IL-1 (Figure 4). In IKK-β-deficient cells, IL-6 induction by the cytokines was reduced by 2- and 4-fold following stimulation with TNF and IL-1, respectively.

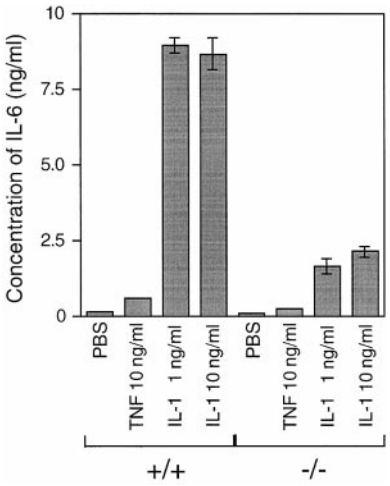

Figure 4. Cytokine-Induced IL-6 Production in IKK-β-Deficient Embryonic Fibroblasts

IKK-β-deficient embryonic fibroblasts (−/−) and wild-type control (+/+) were incubated with 10 ng/ml of TNF and 1 or 10 ng/ml of IL-1β for 16 hr. IL-6 in the culture supernatant was determined by ELISA. Data are shown as average values ± standard deviation.

These results are consistent with the reduced activation of NF-κB by TNF and IL-1 in IKK-β-deficient cells.

Reduced IκB Kinase Activity in the IKK-β-Deficient Cells

Endogenous IKK-α and IKK-β as well as overexpressed or recombinant IKKs can phosphorylate recombinant IκB protein in vitro (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997, 1998; Li et al., 1998; Yamaoka et al., 1998). The kinase activity of the endogenous IKK complex is enhanced by treatment with TNF or IL-1. We analyzed the kinase activity of IKKs in WT and *IKK*-$\beta^{-/-}$ EF cells by an in vitro kinase assay. For this analysis, we used an anti-NEMO antibody to immunoprecipitate the IKK complex. Western blotting with anti-IKK-α and anti-IKK-β revealed that both IKK proteins were coimmunoprecipitated with NEMO in WT cells (Figure 5A). However, only IKK-α protein was detected in immunoprecipitates from *IKK*-$\beta^{-/-}$ cells. This result demonstrates that IKK-α can bind to NEMO in the absence of IKK-β. Although the total amount of IKK-α in IKK-β-deficient cells is greater than in WT cells (Figure 1C), we observed no difference between WT and *IKK*-$\beta^{-/-}$ EF cells in the amount of IKK-α that coimmunoprecipitated with NEMO. This result suggests that NEMO may interact with or immunoprecipitate IKK-α less effectively in the absence of IKK-β.

Anti-NEMO immunoprecipitates were incubated with γ^{32}P-ATP and a recombinant IκB protein comprising amino acids 1–250 of IκB-α [IκB-α(1–250)]. The IKK complex from WT fibroblasts phosphorylated IκB-α(1–250) in vitro, and this kinase activity was stimulated approximately 4-fold by either TNF or IL-1 treatment (Figure 5A). As expected, these same IKK complexes did not phosphorylate recombinant IκB-α(1–250) in which both Ser-32 and Ser-36 were replaced by alanine (data not

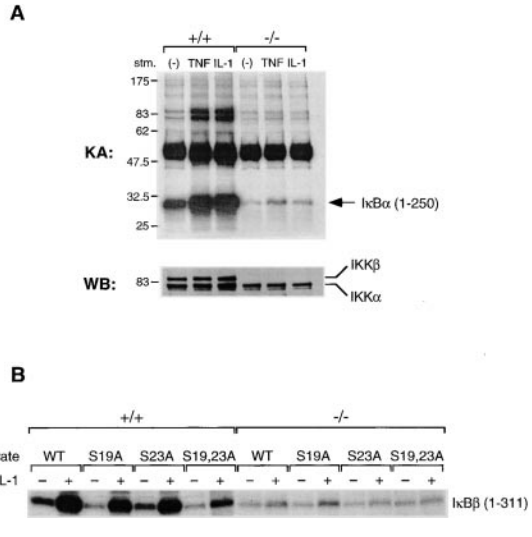

Figure 5. The In Vitro Kinase Activity of the IKK Complex in IKK-β-Deficient Fibroblasts

(A) In vitro kinase assay of IKK complex with IκB-α. IKK-β-deficient embryonic fibroblasts (−/−) and wild-type controls (+/+) were incubated with or without 10 ng/ml of TNF or 10 ng/ml of IL-1β for 10 min. The IKK complexes were immunoprecipitated from the cell lysates with anti-NEMO antibody, and kinase activity was assayed using a recombinant IκB-α(1–250) protein as a substrate, as described in the Experimental Procedures. The IKK-α and IKK-β proteins in the immuoprecipitates were detected by Western blotting with a mixture of anti-IKK-α and anti-IKK-β antibodies. Molecular weight standards (NEB) are shown in kilodaltons.

(B) The kinase activity of the IKK complex for IκB-β. IKK-β-deficient embryonic fibroblasts (−/−) and wild-type controls (+/+) were incubated with or without 10 ng/ml of IL-1β for 10 min. The cell lysates were immunoprecipitated with anti-NEMO antibody, and immunoprecipitates were subjected to in vitro kinase assay with either wild-type (WT) recombinant IκB-β (1–311) or mutant versions of recombinant IκB-β (1–311), in which either Ser-19 (S19A) or Ser-23 (S23A) was mutated to alanine or in which both Ser-19 and 23 were replaced with alanine (S19, 23A) as described in the Experimental Procedures.

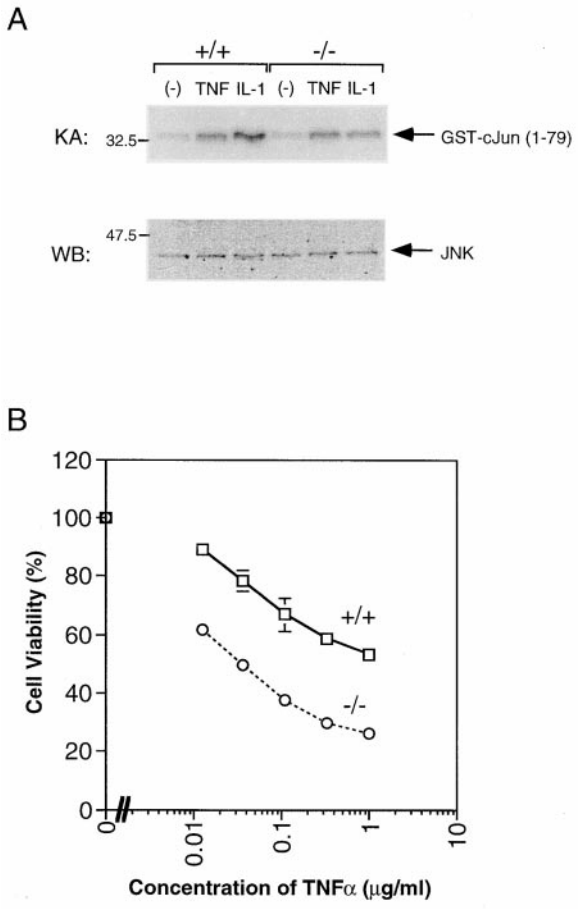

Figure 6. JNK Activation and TNF Cytotoxicity in IKK-β-Deficient Fibroblasts

(A) Cytokine-induced JNK activation. IKK-β-deficient embryonic fibroblasts (−/−) and wild-type controls (+/+) were incubated with or without 10 ng/ml of TNF or 10 ng/ml of IL-1β for 10 min. The cell lysates were immunoprecipitated with anti-JNK antibody, and the kinase activity was assayed with recombinant c-Jun (1–79) protein as substrate. To determine JNK protein levels, immunoblotting was performed with an anti-JNK antibody.

(B) Cytotoxicity of TNF plus cyclohexamide. Fibroblasts from IKK-β-KO (−/−) and wild-type (+/+) embryos were incubated with various amounts of TNF for 24 hr in the presence of 5 μg/ml of cycloheximide. Cell viability was determined by WST-1 assay as described in the Experimental Procedures. Data are shown as average values ± standard deviation for triplicate determinations.

shown). Both IKK-α and IKK-β from WT cells also became phosphorylated in this assay. In contrast, the basal level of kinase activity for IκB-α was greatly reduced in IKK-β-deficient cells (16-fold less) compared with the basal levels in WT cells. Although the kinase activity for IκB-α was slightly increased by TNF and IL-1 stimulation in IKK-β-deficient cells, these levels were 19-fold less (TNF) and 40-fold less (IL-1) than those seen in WT cells. Furthermore, the phosphorylation of IKK-α in this assay was barely detectable in IKK-β-deficient cells.

Overexpressed IKK-α preferentially phosphorylates Ser-23 over Ser-19 in IκB-β, while both serine residues are equally phosphorylated by overexpressed IKK-β (Regnier et al., 1997; Woronicz et al., 1997). Therefore, we examined the kinase activity of the IKK complex in WT and *IKK*-$\beta^{-/-}$ EF cells toward recombinant IκB-β. The WT IKK complex phosphorylated recombinant IκB-β (1–311), and the kinase activity was increased 8-fold upon IL-1 stimulation (Figure 5B). This WT IKK complex phosphorylated versions of IκB-β (1–311) in which either Ser-19 (S19A) or Ser-23 (S23A) alone was mutated to alanine with equal efficiency. The IKK complex from IKK-β-deficient cells had much weaker basal kinase activity for IκB-β than did the IKK complex from WT cells. IL-1-induced activation of the IKK complex in IKK-β-deficient cells was still seen when using wild-type IκB-β or IκB-β (S19A) mutant, but this activity was very weak. There was little or no inducible phosphorylation of IκB-β (S23A), results that are consistent with earlier observations using overexpressed IKK-α (Regnier et al., 1997).

JNK Activation in IKK-β Null Cells

In addition to activating NF-κB, TNF and IL-1 treatment are effective activators of the c-Jun N terminal kinase (JNK). To determine if TNF- or IL-1-induced JNK activation is altered in *IKK*-$\beta^{-/-}$ cells, JNK was immunoprecipitated from EF cells and its kinase activity measured in

an in vitro kinase assay using c-Jun (1–79) as a substrate. JNK was activated equally well in both WT and IKK-β-deficient cells following TNF or IL-1 treatment (Figure 6A). This result is consistent with earlier studies showing that JNK and IKK pathways diverge upstream of NIK in the TNF and IL-1 signaling cascades (Song et al., 1997).

IKK-β-Deficient Cells Have Increased Sensitivity to TNF

TNFR1 transduces signals for NF-κB activation through TRADD-RIP (Hsu et al., 1995, 1996a, 1996b; Kelliher et al., 1998) and signals for apoptosis through TRADD-FADD (Hsu et al., 1995, 1996b; Yeh et al., 1998). *RelA*$^{-/-}$ cells, which are defective in TNF-induced NF-κB activation (Beg et al., 1995), display increased sensitivity to TNF (Beg and Baltimore, 1996), indicating that NF-κB activation provides protection against TNF's apoptotic pathway. We examined the susceptibility of WT and IKK-β-deficient cells to TNF in the presence or absence of cycloheximide (CHX). After 24 hr treatment with 1 μg/ml TNF, cell viability in the absence of CHX was unaffected (data not shown). In the presence of CHX, IKK-β-deficient cells were approximately 30-fold more sensitive than WT cells to TNF (Figure 6B).

Discussion

The mammalian NF-κB/Rel family consists of five members that can form homo- and heterodimers: RelA/p65, RelB, c-Rel, p50, and p52. It is likely that each dimer plays a distinct role and activates a different set of target genes, as knockout mice for each Rel family member exhibit a different phenotype. RelB-deficient mice show multiorgan inflammation due to infiltration of mixed inflammatory cells, splenomegaly, and myeloid hyperplasia in bone marrow (Weih et al., 1995). They also show impaired T cell–mediated immune function including DTH reaction. In *c-Rel* KO mice, the proliferation responses of T and B cell to various stimuli are impaired (Kontgen et al., 1995), whereas p50- and p52-deficient mice have distinct B cell abnormalities (Sha et al., 1995; Caamano et al., 1998). None of these KO mice exhibit developmental abnormalities. On the other hand, RelA/p65 deficiency results in embryonic lethality caused by massive liver degeneration with hepatocyte apoptosis (Beg et al., 1995).

Here, we describe the generation and characterization of *IKK*-β KO mice. IKK-β deficiency leads to embryonic lethality at E14.5 due to liver degeneration, a phenotype similar to that observed for RelA-deficient mice. These results suggest that IKK-β is essential for the activation of RelA in hepatocytes. Since activation of NF-κB is required for the prevention of TNF-induced apoptosis, one possibility is that liver apoptosis in both RelA- and IKK-β-deficient embryos is caused by TNF signaling in the absence of NF-κB activation. This possibility is consistent with RelA- (Beg and Baltimore, 1996) and IKK-β-deficient EF cells displaying increased sensitivity to the apoptosis-inducing activity of TNF. In addition, colony-forming assay data suggest that fetal liver hematopoietic cells are also more sensitive to TNF (data not shown). However, mice lacking RIP, which is a component of the TNFR1 signaling complex (Hsu et al., 1996a), do not have this embryonic lethal phenotype and liver degeneration, even though *RIP*$^{-/-}$ cells are defective in TNF-induced NF-κB activation and have increased sensitivity to TNF (Kelliher et al., 1998). These observations strongly suggest that RelA and IKK-β protect against apoptosis caused by additional, perhaps unknown, apoptosis-inducing factors.

The kinase activity of the IKK complex is greatly decreased in IKK-β-deficient cells in comparison to WT cells. This result is consistent with impairment of cytokine-induced NF-κB activation in IKK-β-deficient cells and indicates that IKK-β is a crucial component for the kinase activity of IKK complex and subsequent NF-κB activation. The IKK complex includes IKK-α and IKK-β, which exist as a heterodimer in most cells (Woronicz et al., 1997), and NEMO, a protein that lacks intrinsic kinase activity but is required for the activity of the IKK complex (Rothwarf et al., 1998; Yamaoka et al., 1998). Since IKK-α can homodimerize in the absence of IKK-β (Woronicz et al., 1997), it is likely to form a homodimer that binds to NEMO in IKK-β-deficient cells and provide partial compensation for the loss of the IKK-α/β heterodimer. Overall, IKK-α and IKK-β are 52% identical in amino acid sequence with the highest homology occurring in their N-terminal kinase domains (Mercurio et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). Although both kinases phosphorylate IκB at the appropriate serine residues (Li et al., 1998; Zandi et al., 1998), IKK-β has considerably greater kinase activity than IKK-α when the proteins are overexpressed at similar levels (Woronicz et al., 1997; Li et al., 1998). This quantitative difference in kinase activity between IKK-α and IKK-β probably explains the reduction of IKK kinase activity in IKK-β-deficient cells.

It is also possible that IKK-α and IKK-β play different roles in the IKK complex. The protein kinase NIK, a potential upstream activator of the IKK complex in TNF and IL-1 signaling, can phosphorylate Ser-176 of IKK-α (Ling et al., 1998). Mutation of this serine, which is located in the activation loop between subdomains VII and VIII, abolishes the kinase activity of IKK-α for IκB-α. IKK-α (S176A) also acts as a dominant-negative inhibitor of NF-κB activation induced by TNF and IL-1 (Ling et al., 1998). These results suggest that phosphorylation of Ser-176 of IKK-α is essential not only for the activation of IKK-α itself but may also be required for the activation of the IKK complex and subsequent NF-κB activation. Conversely, IKK-β does not appear to be phosphorylated by NIK (Ling et al., 1998), and NIK coexpression enhances the kinase activity of overexpressed IKK-β much less than it does IKK-α (Regnier et al., 1997; Woronicz et al., 1997). These observations are consistent with a model in which IKK-α plays an initiating role in the activation of the whole IKK complex by accepting a signal from NIK, and with IKK-β subsequently being activated by IKK-α. However, other activation models not dependent on NIK or IKK-α are certainly possible.

The IKK complex in IKK-β-deficient cells is likely to consist of an IKK-α homodimer in association with NEMO. This complex is probably not unique to *IKK*-β KO mice since certain cell types may contain significantly less IKK-β than IKK-α. In these instances, IKK-α homodimers would exist, and basal and cytokine-induced

levels of NF-κB would be considerably reduced compared to cells containing only the IKK-α/β heterodimer in the IKK complex. Likewise, the formation of an IKK-β homodimer may also control the signal strength for NF-κB activation in some situations. For example, Mercurio et al. (1999) recently showed that HeLa cells contain both IKK-α/IKK-β heterodimers and IKK-β homodimers and that the TNF-induced kinase activity of the heterodimer is significantly greater than that of the IKK-β homodimer. Mechanisms may also exist to assemble IKK complexes that preferentially contain homodimers. In any event, IKK-α-deficient cells as well as IKK-α/IKK-β double knockout cells will provide additional information on the functional differences between IKK-α and IKK-β.

Experimental Procedures

Preparation of the IKK-β Targeting Construct

A 129/sv/J mouse genomic library was screened using a probe derived from the 5′ end of human *IKK*-β cDNA (Woronicz et al., 1997). Two overlapping genomic DNA fragments, an 8.4 kbp EcoRI fragment and a 9.4 kbp BamHI fragment, both of which include exons 2 through 4, were subcloned into pBluescript. The targeting vector was constructed by replacing a 1 kbp fragment containing exon 2 (95 bp) with a 1.1 kbp *neomycin* resistance gene cassette in the same orientation as the endogenous *IKK*-β (Figure 1A). A 2.2 kbp fragment containing the herpes simplex virus *thymidine* kinase gene driven by the MCI promoter was inserted in the opposite orientation for the negative selection. The final targeting construct (MTp038-2) was linearized with KpnI for transfection.

ES Cell Transfection

All experiments were carried out on the CJ7 cell line kindly provided by Dr. Tom Gridley (Swiatek and Gridley, 1993). Culture conditions were as described (Wurst and Joyner, 1993), except that 1000 U/ml of LIF (GIBCO-BRL) was used. Cells were cultured at all times on mouse embryonic fibroblasts (Genome System), including the selection step. Cells were harvested from nearly confluent plates for electroporation. Linearized DNA (100 μg) was used for 2×10^7 cells. Selection medium including 350 μg/ml G418 and 2 μM gancyclovir was added to the cells 24 hr after electroporation. G418-, gancyclovir-double resistant clones were selected and screened for homologous recombination by Southern blotting with a 1257 bp flanking probe located downstream of the construct (Figure 1A). In the positive ES clones, insertion of a single neo cassette was confirmed by Southern blotting.

Generation and Breeding of Chimeric Mice

Chimeric mice were generated by injection of the ES positive clones into C57Bl/6 blastocysts and implantation into pseudopregnant females. Male chimeric mice were bred to C57Bl/6 females. Germline transmission in F1 *IKK*-$\beta^{+/-}$ mice was verified by Southern blotting. Heterozygous mice were interbred to obtain *IKK*-β KO mice. The genotype of F2 mice or embryos was determined by PCR on tail or on embryo tissue genomic DNA and confirmed by Southern blotting. The PCR primers for wild-type *IKK*-β alleles were 5′-AGCGCTGGAT GGCTTAACCT-3′ (BG021: located in intron 1) and 5′-GATGGACAAC CAGACCTATGACCAC-3′ (BSA03: located in intron 1 and deleted in the targeting construct). The primer pairs for the KO allele were BG021 and 5′-GTGCAATCCATCTTGTTCAATGGCC-3′ (BSA01: located in the Neo gene).

Generation of *IKK*-$\beta^{-/-}$ Embryonic Fibroblasts

E12.5 embryos were harvested, and the head and blood-filled organs were removed for DNA preparation and genotyping. The remainder of the embryos were cut in small pieces and trypsinized. Supernatants containing individual cells were centrifuged and plated on 6-well plates. Embryonic fibroblasts were expanded twice and frozen.

Immunoprecipitation and Western Blot Analysis

Primary cultured fibroblasts from *IKK*-$\beta^{+/+}$, *IKK*-$\beta^{+/-}$, and *IKK*-$\beta^{-/-}$ embryos were lysed with the lysis buffer containing 50 mM Hepes (pH 7.6), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X, protease inhibitor cocktail (Boehringer), 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 5 mM p-nitrophenyl phosphate. After 30 min incubation on ice, centrifugation was performed at 14 K for 20 min, and supernatants were collected. IKK-α and IKK-β proteins were immunoprecipitated from the cell lysates with anti-IKK-α antibody (Santa Cruz) or anti-NEMO antiserum (Yamaoka et al., 1998) and Protein G Sepharose (Pharmacia). The immunoprecipitates were fractionated by 10% SDS-PAGE and blotted onto nitrocellulose membrane. After the membrane was incubated with the blocking buffer containing 1× PBS, 5% skim milk, 3% goat serum, and 0.2% Tween 20 at room temperature for 1 hr, immunoblotting was performed with anti-IKK-α and anti-IKK-β polyclonal antibodies (Santa Cruz). After incubation with HRP-conjugated Protein A (BioRad), the blot was developed using an enhanced chemiluminescence system according to the manufacturer's instructions (Amersham). To detect JNK protein, the cell lysate was incubated with anti-JNK monoclonal antibody (Pharmingen) and Protein G Sepharose for immunoprecipitation, and Western blotting was performed with anti-JNK goat polyclonal antibody (Santa Cruz) and HRP-conjugated Protein G (BioRad). For detection of IκB-α protein, anti-IκB-α antibody-conjugated agarose beads (Santa Cruz) were used for immunoprecipitation, and anti-IκB-α antiserum (Pharmingen) was used for Western blotting.

In Vitro Kinase Assay

Primary cultured fibroblasts (5×10^6 cells) were either left untreated or treated with human TNF (Genentech) or human IL-1β (Biosources) for 10 min in the absence of serum. After stimulation, cells were immediately rinsed with ice-cold PBS and lysed with the lysis buffer described above. To measure the kinase activity of the IKK complex, the lysates were immunoprecipitated with the anti-NEMO antiserum. The immunoprecipitates were washed with the lysis buffer extensively, followed by three washes with the kinase buffer, which contains 20 mM Hepes (pH 7.9), 10 mM $MgCl_2$, 100 mM NaCl, EDTA free proteinase inhibitor cocktail (Boehringer), 20 mM β-glycerophosphate, 1 mM Sodium orthovanadate, 1 mM p-nitrophenyl phosphate, and 2 mM DTT. The immunoprecipitates were incubated with FLAG-tagged recombinant IκB-α(1–250) or IκB-β (1–311) (Regnier et al., 1997) and 20 nM γ^{32}P-ATP at 30°C for 30 min. After adding 1/5 volume of 5× Laemmli's sample buffer containing β-mercaptoethanol, the proteins were fractionated by 10% SDS-PAGE, and phosphorylated proteins were detected by exposing to X-ray film. For the JNK kinase assay, immunoprecipitation was performed with anti-JNK antibody, and the kinase activity was detected using recombinant GST-c-Jun (1–79) protein (Santa Cruz).

Gel Mobility Assay

EFs (2×10^5) were cultured with DMEM supplemented with 10% FCS in a 6-well dish for 24 hr. 1 hr prior to stimulation, culture medium was changed to serum-free DMEM. The cells, untreated or treated with TNFα or IL-1β for various times, were washed with ice-cold PBS and were lysed with total lysis buffer (20 mM Hepes [pH 7.9], 350 mM NaCl, 20% glycerol, 1% NP-40, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, and proteinase inhibitor cocktail [Boehringer]) to obtain the total cell lysate. The total cell lysate (5 μg protein) was incubated with an end-labeled, double-stranded, oligonucleotide derived from an NF-κB binding sequence in the E-selectin promoter (5′-GATGCCATTGGGGATTTCCTCTTTACTG-3′ and 5′-CAGTAAAG AGGAAATCCCCAATGGCATC-3′) (Schindler and Baichwal, 1994). The binding reaction was carried out in 20 mM Hepes (pH 7.5), 0.5 mM EDTA, 5 mM $MgCl_2$, 50 μg/ml bovine serum albumin, 0.05% NP-40, 60 mM KCl, 10 mM DTT, 10% glycerol, and 1.5 μg of dI/dC in a total volume of 20 μl. The reaction mixture was incubated at room temperature for 30 min and then was fractionated by 5% acrylamide gel. The DNA–protein complex was detected by exposing to X-ray film.

Measurement of IL-6 Production

EFs (3×10^4) were seeded in 24-well plates. After culturing for 24 hr, the cells were left untreated or treated with TNF or IL-1β for 16

hr. IL-6 concentration of the culture supernatant was determined by ELISA (Endogen).

EF Cell Death Assay

The cell killing assay was performed essentially as described previously (Tanaka et al., 1997). In brief, 1×10^4 cells were cultured with 10% FCS DMEM in a 96-well plate for 24 hr. Then, the cells were incubated with various amounts of TNF in the presence or absence of 5 μg/ml cycloheximide (Sigma) for 24 hr. Cell viability was determined using WST-1 (Boehringer).

Colony-Forming Assay

Individual fetal livers were isolated from E13.5 embryos and disrupted mechanically into single cell suspensions in Iscove's MDM plus 2% fetal bovine serum 1 + 2 (StemCell Technologies). Fetal liver cell suspensions were counted on a Coulter counter. Assays were plated in 35 mm culture dishes in duplicate. For the CFU-Mix assay, cells were mixed with MethoCult M3434 (StemCell Technologies) according to the manufacturer's instructions, giving final concentrations of 5×10^4 cells/dish, 0.9% methylcellulose in Iscove's MDM, 15% fetal bovine serum, 1% bovine serum albumin, 10 μg/ml bovine pancreatic insulin, 200 μg/ml human transferrin, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 10 ng/ml rmIL-3, 10 ng/ml rhIL-6, 50 ng/ml rmSCF, and 3 units/ml rh erythropoietin. Colonies were enumerated differentially on day 8. For the CFU-E assay, cells were mixed with MethoCult M3334 giving final concentrations as above except that erythropoietin was the only cytokine present. CFU-E were scored on day 3. Colonies were enumerated blindly with respect to genotype.

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