The IKKβ Subunit of IκB Kinase (IKK) is Essential for Nuclear Factor κB Activation and Prevention of Apoptosis

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Summary
The IκB kinase (IKK) complex is composed of three subunits, IKKα, IKKβ, and IKKγ (NEMO). While IKKα and IKKβ are highly similar catalytic subunits, both capable of IκB phosphorylation in vitro, IKKγ is a regulatory subunit. Previous biochemical and genetic analyses have indicated that despite their similar structures and in vitro kinase activities, IKKα and IKKβ have distinct functions. Surprisingly, disruption of the Iκκα locus did not abolish activation of IKK by proinflammatory stimuli and resulted in only a small decrease in nuclear factor (N F)-κB activation. Now we describe the pathophysiological consequence of disruption of the Iκκβ locus. IKKβ-deficient mice die at mid-gestation from uncontrolled liver apoptosis, a phenotype that is remarkably similar to that of mice deficient in both the R eElA (p65) and N F-κB1 (p50/p105) subunits of N F-κB. Accordingly, IKKβ-deficient cells are defective in activation of IKK and N F-κB in response to either tumor necrosis factor α or interleukin 1. Thus IKKβ, but not IKKα, plays the major role in IKK activation and induction of N F-κB activity. In the absence of IKKβ, IKKα is unresponsive to IKK activators.

Key words: inflammation • tumor necrosis factor α • interleukin 1 • knockout mice • signal transduction

The nuclear factor (N F)-κB is a transcription factor that plays a key role in activation of inflammatory and innate immune responses (1, 2). In nonstimulated cells, N F-κB dimers are kept as cytoplasmic latent complexes through binding of specific inhibitors, the IκBα, which mask their nuclear localization signal (NLS). Upon exposure to proinflammatory stimuli, such as bacterial LPS, TNF-α, or IL-1, the IκBα are rapidly phosphorylated at two conserved N H2-terminal serines, a posttranslational modification that is rapidly followed by their polyubiquitination and proteosomal degradation (3–6). This results in unmasking of the NLS of N F-κB dimers followed by their translocation to the nucleus, binding to specific DNA sites (κB sites), and target gene activation. N F-κB target genes include many of the cytokine and chemokine genes, as well as genes coding for adhesion molecules, cell surface receptors, and enzymes that produce secondary inflammatory mediators (7, 8).

The protein kinase that phosphorylates IκBα in response to proinflammatory stimuli has been identified biochemically and molecularly (9–11). Named IKK, this protein kinase is a complex composed of at least three subunits IKKα, IKKβ, and IKKγ (for a review, see reference 12). IKKα and IKKβ are highly similar protein kinases that act as the catalytic subunits of the complex (9, 11, 13, 14). In vitro, both IKKα and IKKβ form homo- and heterodimers that can phosphorylate IκB proteins at their N H2-terminal regulatory serines (15). In mammalian cells, IKKα and IKKβ form a stable heterodimer that is tightly associated with the IKKγ (NEMO) subunit (16, 17). As cell lines that fail to express IKKγ (NEMO) exhibit a major defect in IκB degradation and N F-κB activation in response to proinflammatory stimuli and double-stranded RNA, this regulatory subunit plays an essential function (at least in the examined cell lines) in IKK and N F-κB activation (17). The physiological function of the two catalytic subunits has been less clear. Initially, overexpression of catalytically inactive forms of IKKα and IKKβ that blocked IKK and N F-κB activation suggested that both subunits play similar and possibly redundant roles in IκB phosphorylation and N F-κB activation (13, 14). This hypothesis was fostered by finding that in vitro IKKα and

1 Abbreviations used in this paper: EF, embryonic fibroblast; EMSA, electrophoretic mobility shift assay; ES, embryonic stem; H&H, hematoxylin and eosin; IKK, IκB kinase; N F, nuclear factor; NIK, N F-κB inducing kinase; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling.
IKKβ can directly phosphorylate IκBα and IκBβ at the serines that trigger their degradation in vivo (15). However, it was also suggested that IKKα rather than IKKβ is responsible for activation of the entire complex in response to certain stimuli, such as the NF-κB inducing kinases, NIK (18). Recently, we found that in addition to an IKKγ subunit with an intact COOH terminus (16), IKK activation requires the phosphorylation of IKKβ at two serines within its activation loop (19). Replacement of these serines, whose phosphorylation is stimulated by proinflammatory stimuli or NIK, with alanines abolishes IKK activation. Interestingly, although the entire activation loop is identical in sequence between IKKα and IKKβ, replacement of the same two serines in IKKα with alanines has no effect on IKK activation (19). These results were further substantiated by gene targeting (knockout) experiments. Cells and tissues from mice that no longer express IKKα (Ikkα−/− mice) exhibit normal IKK activation in response to TNF, IL-1, or LPS (20). Although NF-κB is fully inducible, for an unknown reason, IKKα-deficient fibroblasts exhibit approximately twofold reduction in both basal and induced NF-κB binding activity (20). Thus, IKKα may somehow stimulate NF-κB DNA binding despite not being required for IκB phosphorylation and degradation in most cell types. The gene targeting experiments reveal that, although not involved in activation of IKK by proinflammatory stimuli, IKKα plays an instrumental role in morphogenesis (20). The most important function of IKKα appears to be in the control of keratinocyte differentiation and formation of the epidermis (20). It is not yet clear whether these morphogenetic functions of IKKα are exerted through localized NF-κB activation in response to developmental cues.

To determine the physiological function(s) of IKKβ, we have used gene targeting to create Ikkβ knockout mice. We now show that the loss of IKKβ results in embryonic lethality at mid-gestation due to extensive apoptosis of the developing liver. This phenotype is similar to that of mice deficient in the R eRα (p65) subunit of N F-kB (21). It was recently shown that the lethality of R eRα−/− mice is completely suppressed by the loss of TNF-α (22). As NF-κB is required for protection of cells from TNF-α-induced apoptosis (23–25), the apoptotic phenotype of Ikkβ−/− mice strongly suggests that the absence of IKKβ results in a severe defect in NF-κB activation. Indeed, neither IKK nor NF-κB can be activated by TNF-α or IL-1 in IKKβ-deficient cells. Furthermore, we show that in the absence of IKKβ, the IKKα subunit is not responsive to NIK even though it can still associate with the IKKγ subunit.

Materials and Methods

Generation of IKK β-deficient Mice. Using a 0.2-kb BstEII-Bsu36I restriction fragment from the 5′ end of human IKK β cDNA as a probe, three murine IKK β genomic fragments were isolated from a 129/SvJ mouse genomic library (Stratagene, Inc.). One of the clones contained at least the first three coding exons and was used to construct the targeting vector Ikk KO. A 1.4-kb BclI restriction fragment harboring part of the second exon was used as the short homology arm, and the long arm was a 5.5-kb EcoRV-Xhol restriction fragment containing part of the third intron. The two arms were inserted into the XmnI and SmaI sites, respectively, of pGNA, which contains the G418 resistance gene (Neo) and LacZ (26). As a negative selection marker, a diphtheria toxin gene cassette (DT) was inserted into the KpnI site of pGNA. After cutting with Pmel, 20 μg of the linearized targeting vector was electroporated into 107 mouse embryonic stem (ES) cells (line GS from Genome Systems). After selection with G418 at 0.4 mg/ml, G418-resistant colonies were picked and screened by PCR. The genotype of the PCR-positive clones was confirmed by Southern blotting analysis. Homologous recombinants were karyotyped and analyzed for mycoplasma. Two homologous recombinant ES clones were injected into C57BL/6 blastocysts. Resulting male chimeras were crossed with C57BL/6 females, and germline transmission was scored by coat color. Heterozygous mice were identified by PCR and Southern analysis of...
mouse tail DNA. Embryos from intercrosses of heterozygous (Ikkβ−/−) mice, as well as mouse embryonic fibroblasts (EFs), were genotyped by PCR and Southern analysis using DNA isolated from a piece of each embryo or a cell pellet, respectively.

PCR and Southern Blotting Analysis. PCR was performed in the presence of 10% DMSO with Taq DNA polymerase using a Perkin-Elmer 9600 thermocycler programmed for denaturation at 95°C for 5 min, amplification for 35 cycles (94°C for 30 s, 55°C for 30 s, 65°C for 2 min), and elongation at 72°C for 10 min. Primers used were: P1 (5'-AGTCACTGGCAGCGATA-3’) located outside of the homology arm and P2 (5’-CAACTTAAATGTGAGCGAG-3’) located within the LacZ gene. Southern blotting analysis was performed according to a standard protocol (27) except that hybridization was performed in phosphate-SDS buffer (28).

Kinase Assay, Immunoprecipitation, and Electrophoretic Mobility Shift Assays. Ikkβ−/−, Ikkβ+/−, and Ikkβ−/− ES and EF cells were treated with TNF-α or IL-1 at 20 ng/ml. Kinase assays and immunoprecipitations were performed as described (9). Immuno blotting was performed as described (14, 16). Electrophoretic mobility shift assays (EMSA) using the consensus κB and NF-1 sequences were performed as described (16, 29).

Histology, In Situ TUNEL Assay, and Transmission Electron Microscopy. Mouse embryos or embryo livers were fixed in 10% buffered formalin and embedded in paraffin. After routine processing, the sections (5-μm thick) were stained with hematoxylin and eosin (H&E) for histological analysis. In situ TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling) assay was done using the in situ cell death detection kit according to the manufacturer’s instructions (Boehringer Mannheim). For electron microscopy, embryonic day 13 (E13) embryos were removed and the livers were dissected out and fixed for 1 h in 2% formaldehyde and 2% glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.4) at 4°C. The remainder of the embryos were placed in PBS for subsequent PCR and Southern analysis. After washing in cacodylate buffer, the livers were postfixed in 1% osmium tetroxide in cacodylate buffer for an additional 1 h. After postfixation, the samples were rinsed in double distilled water, dehydrated in a graded ethanol series, and infiltrated and polymerized in Durcupan ACM resin (Electron Microscopy Sciences). Sections 80-nm thick were stained with Sato lead and examined at 80 kV with either a JEOL 100CX or 2000EX transmission electron microscope.

Results

Generation of Ikkβ Knockout Mice. To create a strain of IKKβ-deficient mice, we used gene targeting technology (30). Mouse genomic Ikkβ DNA was cloned from a 129 strain library and, after mapping and sequencing, was used

![Figure 2](image2.png)

**Figure 2.** Appearance of an Ikkβ−/− E13.5 embryo and a normal littermate. Wild-type (Ikkβ+/+, WT) and mutant (Ikkβ−/−, M) embryos were isolated at E13.5 and photographed. The genotypes of the embryos were later determined by PCR and Southern blot analysis.

![Figure 3](image3.png)

**Figure 3.** Analysis of wild-type (WT) and mutant (M) livers. E13.5 embryos were fixed and sectioned. Paraffin-embedded transverse sections at the area of the liver were subjected to H&E (top; original magnification: 400×) or TUNEL (bottom; original magnification: 600×) staining. The stained sections were photographed.
to construct the targeting vector (Fig. 1 A). To eliminate IKKβ kinase activity, part of the second and the entire third coding exon that specifies an essential part of the kinase domain were replaced with a DNA fragment encoding β-galactosidase (lacZ) and neomycin resistance (Neo+). Because the Neo+ gene contains transcription termination and polyadenylation signals, the COOH-terminal three quarters of IKKβ including its protein interaction motifs are unlikely to be expressed from the targeted allele.

After selection and screening by Southern blotting, six ES cell clones with homologous integration of the targeting vector into the Ikkβ locus were isolated, and two of them were used to generate chimeric mice. Chimeric mice derived from these clones transmitted the targeted Ikkβ allele to their progeny (Fig. 1 B). Although Ikkβ+/− male and female mice appeared normal and were fertile, upon intercrossing they did not give rise to live Ikkβ−/− progeny. Analysis of protein extracts of Ikkβ+/+, Ikkβ+/−, and Ikkβ−/− cells revealed that, as expected, no IKKβ protein was expressed from the targeted allele (Fig. 1 C). In addition, Ikkβ−/− cells expressed approximately half the dose of IKKβ present in wild-type cells. No compensatory increases in IKKα, IKKγ, p65(RelA), or p50(NF-κB1) expression were observed.

Phenotype of Ikkβ−/− Mice. Given the expected importance of IKKβ for NF-κB activation and the embryonic lethality of Relα−/− mice (21), we suspected that the loss of IKKβ would result in a similar phenotype. Therefore, we analyzed embryos from timed pregnancies of Ikkβ−/− intercrosses. Although Ikkβ−/− embryos isolated at E11.5 were alive and had perfectly normal appearance (data not shown), Ikkβ−/− embryos isolated at E13.5 were no longer alive and were rather anemic in appearance (Fig. 2). Even external examination suggested that the liver of E13.5 Ikkβ−/− embryos had degenerated. Notably, however, the limbs and head of Ikkβ−/− embryos were normally developed, unlike those of Ikkα−/− E13.5 embryos (20). Histochemical examination of transverse sections of normal and mutant E13.5 mouse embryos stained with H&E revealed massive cell death in livers of Ikkβ−/− embryos (Fig. 3 A). Essentially, no viable hepatocytes could be detected, and the numbers of dead cells with highly condensed and fragmented nuclei were markedly increased. However, hematopoietic precursors retained their normal appearance in Ikkβ−/− livers. TUNEL staining revealed that the observed cell death is most likely due to apoptosis, whose rate was increased manyfold (Fig. 3 B). Examination of E13 Ikkβ−/− embryos revealed close to normal external appearance (data not shown), but electronmicroscopic examination of ultrathin sections from their livers revealed massive numbers of dead hepatocytes with highly condensed nuclei characteristic of apoptotic cell death (Fig. 4). The livers of Ikkβ+/+ or Ikkβ+/− littermates had perfectly normal appearance.

Defective NF-κB Activation in Ikkβ−/− Cells. We used two different approaches to determine the consequences of the loss of IKKβ expression on IKK and NF-κB activation. First, we prepared Ikkβ−/− ES cell lines by subjecting Ikkβ+/− ES cells to selection at higher G418 concentration. One Ikkβ−/− cell line was identified. As shown in Fig. 5 A, stimulation of these cells with either TNF-α or IL-1 did not result in IKK activation, whereas a normal activation response was observed in Ikkβ+/+ cells. Note, however, that Ikkβ−/− cells had ~50% of the IKK activity of wild-type Ikkβ+/+ ES cells, consistent with the reduced amount of IKKβ protein (data not shown). In addition to the defect in IKK activation, hardly any induction of NF-κB DNA binding activity was observed in Ikkβ−/− cells after stimulation with either IL-1 or TNF-α (Fig. 5 B). Even the basal level of NF-κB DNA binding activity was considerably reduced in Ikkβ−/− cells, despite no detectable changes in p65(RelA) or p50(NF-κB1) abundance (data not shown). The second approach to evaluate the function of IKKβ was to prepare cultures of EFs from E11.5 mouse embryos of all three genotypes. As shown in Fig. 6, essentially no induc-
expression vector into be activated by either TNF-α. This point, we cotransfected an HA epitope–tagged IKK (KA), and (B) NF-κB DNA binding activity. Whole cell extracts were prepared and used to measure (A) IKK activity (KA), and (B) NF-κB DNA binding activity. Immunoblotting. (in min) were incubated with 32P-labeled IκB (1-54) as a substrate. The kinase assay products were separated by SDS-PAGE, transferred to nitrocellulose membrane, and autoradiographed. The membrane was reprobed with antibody M280 (IB: IKKα) for loading control. (B) NF-κB binding activity. Nuclease extracts of Iκkβ+/− and Iκkβ−/− cells stimulated with IL-1 or TNF-α for the indicated times (in min) were incubated with 32P-labeled κB oligonucleotide probe and subjected to EMSA. Binding to an NF-1 probe was used to control the quality and amount of nuclear protein extracts.

Figure 5. Defective IKK and NF-κB activation in IKKβ-deficient ES cells. (A) IKK activity. Lysates of TNF-α or IL-1–treated Iκkβ+/− and Iκkβ−/− cells were prepared at the indicated time points (in min) after stimulation and immunoprecipitated with antibody M 280 to IKKα. IKK activity (KA) was measured by an immunocomplex kinase assay using GST-IκBα(1-54) as a substrate. The kinase assay products were separated by SDS–PAGE, transferred to nitrocellulose membrane, and autoradiographed. The membrane was reprobed with antibody M280 (IB: IKKα) for loading control. (B) NF-κB binding activity. Nuclease extracts of Iκkβ+/− and Iκkβ−/− cells stimulated with IL-1 or TNF-α for the indicated times (in min) were incubated with 32P-labeled κB oligonucleotide probe and subjected to EMSA. Binding to an NF-1 probe was used to control the quality and amount of nuclear protein extracts.

Figure 6. Defective IKK and NF-κB activation in IKKβ-deficient EF cells. Second passage EFs from E11.5 Iκkβ+/−, Iκkβ+/−, and Iκkβ−/− embryos were stimulated with TNF-α or IL-1. At the indicated times, whole cell extracts were prepared and used to measure (A) IKK activity (KA), and (B) NF-κB DNA binding activity. IB, immunoblotting.

Discussion

The enzymatic activity of the IKK complex, composed of two catalytic subunits, IKKα and IKKβ, and one regulatory subunit, IKKγ, is rapidly stimulated by proinflammasome
transiently transfected with an HA-IKK vector alone or together with XpressIK or HA-IKKα and XpressIK expression vectors 24 h after transfection. HA-IKK proteins were immunoprecipitated (IP) with anti-HA antibody and their associated IKK activity (KA) was determined using GST-IκBα(1-54) as a substrate. Protein expression levels were determined by immunoblotting (IB) with anti-HA. (B) Lysates of iκκα/−, iκκβ/−, and iκκβ/− cells were immunoprecipitated (IP) with either anti-IKKα or anti-IKKγ antibodies as indicated. The immunocomplexes were dissolved in SDS loading buffer and separated by SDS-PAGE. After transfer to an Immobilon membrane, the proteins were analyzed by immunoblotting (IB) with anti-IKKα antibody. A lysate of 3T3 cells was used as a control (Cont).

Figure 7. IKKα is refractory to activation in iκκβ−/− cells despite its association with IKKγ. (A) iκκβ−/− ES cells were transiently transfected by electroporation with an HA-IKKα expression vector alone or together with XpressIK or HA-IKKα and XpressIK expression vectors 24 h after transfection. HA-IKK proteins were immunoprecipitated (IP) with anti-HA antibody and their associated IKK activity (KA) was determined using GST-IκBα(1-54) as a substrate. Protein expression levels were determined by immunoblotting (IB) with anti-HA. (B) Lysates of iκκα/−, iκκβ/−, and iκκβ/− cells were immunoprecipitated (IP) with either anti-IKKα or anti-IKKγ antibodies as indicated. The immunocomplexes were dissolved in SDS loading buffer and separated by SDS-PAGE. After transfer to an Immobilon membrane, the proteins were analyzed by immunoblotting (IB) with anti-IKKα antibody. A lysate of 3T3 cells was used as a control (Cont).

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