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

By Zhi-Wei Li,* Wenming Chu,* Yinling Hu,* Mireille Delhase,* Tom Deerinck,§ Mark Ellisman,§ Randall Johnson,§ and Michael Karin*

From the *Department of Pharmacology, Laboratory of Gene Regulation and Signal Transduction, #Department of Biology, and §Department of Neuroscience, University of California, San Diego, La Jolla, California 92036-0636.

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 midgestation from uncontrolled liver apoptosis, a phenotype that is remarkably similar to that of mice deficient in both the RelA (p65) and NF-κB1 (p105) subunits of NF-κB. Accordingly, IKKβ-deficient cells are defective in activation of IKK and NF-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 NF-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 transcription factor plays a key role in activation of inflammatory and innate immune responses (1, 2). In nonstimulated cells, NF-κB dimers are kept as cytoplasmic latent complexes through binding of specific inhibitors, the IκBs, which mask their nuclear localization signal (NLS). Upon exposure to proinflammatory stimuli, such as bacterial LPS, TNF-α, or IL-1, the IκBs are rapidly phosphorylated at two conserved NH₂-terminal serines, a posttranslational modification that is rapidly followed by their polyubiquitination and proteasomal degradation (3–6). This results in unmasking of the NLS of NF-κB dimers followed by their translocation to the nucleus, binding to specific DNA sites (κB sites), and target gene activation. NF-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 IκBs 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 NH₂-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 NF-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 NF-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 NF-κB activation suggested that both subunits play similar and possibly redundant roles in IκB phosphorylation and NF-κB activation (13, 14). This hypothesis was fostered by finding that in vitro IKKα and

Abbreviations used in this paper: EF, embryonic fibroblast; EMSA, electrophoretic mobility shift assay; ES, embryonic stem; H&E, hematoxylin and eosin; IKK, IκB kinase; NF, nuclear factor; NIK, NκB 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 kinase, 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 excessive apoptosis of the developing liver. This phenotype is similar to that of mice deficient in the RelA (p65) subunit of NF-κB (21). It was recently shown that the lethality of RelA−/− 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 Ikkβ−/− mice. Using a 0.2-kb BseEII–Bsu36I restriction fragment from the 5′ end of human IKKβ cDNA as a probe, three murine IKKβ 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 IkkKO. A 1.4-kb SacI 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–XhoI restriction fragment containing part of the third intron. The two arms were inserted into the XmnI and Smal sites, respectively, of pGNA, which contains the G418 resistance gene (Neor) 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 DNA. Mouse genomic DNA was digested with EcoRI and probed with probe A (1.2-kb HindIII–PstI fragment of Ikkβ). After homologous recombination, the 9.7-kb EcoRI fragment of wild-type Ikkβ is replaced by a 7.2-kb EcoRI fragment, as indicated in panel A. (C) Western blot analysis of mouse proteins using antibody H470 specific for IKKβ. Location of the IKKβ band is indicated. The lower band is nonspecific (ns). The same blot was also probed with antibodies to IKKα, Ikkγ, p65 (RelA), and p50 (NF-κB1). The genotypes are as indicated.

Figure 1. Generation of Ikkβ−/− mice. (A) The mouse Ikkβ locus and the targeting vector. Map of the Ikkβ genomic fragment used for gene targeting is shown. The exons are indicated by solid black boxes, the introns are indicated by bold lines, and the selection markers, lengths of restriction fragments, restriction enzyme sites, the probes used for Southern analysis, and the location of primers used in PCR screening are also shown. RI, EcoRI; P, PstI; S, SacI; RV, EcoRV; Xh, XhoI. (B) Southern blot analysis of mouse genomic DNA. Mouse genomic DNA was digested with EcoRI and probed with probe A (1.2-kb HindIII–PstI fragment of Ikkβ). After homologous recombination, the 9.7-kb EcoRI fragment of wild-type Ikkβ is replaced by a 7.2-kb EcoRI fragment, as indicated in panel A. (C) Western blot analysis of mouse proteins using antibody H470 specific for IKKβ. Location of the IKKβ band is indicated. The lower band is nonspecific (ns). The same blot was also probed with antibodies to IKKα, Ikkγ, p65 (RelA), and p50 (NF-κB1). The genotypes are as indicated.
mouse tail DNA. Embryos from intercrosses of heterozygous (Ikk\(^{-/-}\)) 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\(^\circ\)C for 5 min, amplification for 35 cycles (94\(^\circ\)C for 30 s, 55\(^\circ\)C for 30 s, 65\(^\circ\)C for 2 min), and elongation at 72\(^\circ\)C for 10 min. Primers used were: P1 (5\(^\prime\)-AGTCCAACTGGCAGCAATA-3\(^\prime\)) located outside of the homology arm and P2 (5\(^\prime\)-CAA-CATTAAATGTGAGCGAG-3\(^\prime\)) 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. Ikk\(^{β-/-}\), Ikk\(^{β+/-}\), and Ikk\(^{β+/-}\) 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). Immunoblotting was performed as described (14, 16). Electrophoretic mobility shift assays (EMSA) using the consensus κB and NF-κB 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\(^\circ\)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, and then for 1 h at 4\(^\circ\)C. 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 keV with either a JEOL 100CX or 2000EX transmission electron microscope.

Results

Generation of Ikkβ Knockout Mice. To create a strain of IKK-β-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
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(R elA) 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-

Figure 4. Electron microscopic analysis of livers from E13 Ikkβ+/+, Ikkβ−/−, and Ikkβ−/− embryos. Both E13 Ikkβ+/+ (A) and Ikkβ−/− (B) livers exhibited normal morphology. The Ikkβ−/− liver (C) exhibited varying degrees of apoptosis characterized by collapsed and condensed nuclei and general cellular degeneration. Bars = 5 μm.

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tion of IKK or NF-κB activity could be detected in Ikkβ−/−
EF cells treated with either IL-1 or TNF-α. Interestingly,
Ikkβ−/− EF cells exhibited an ~50% reduction in IKK activity
(consistent with the reduction in Ikkβ expression) but a
much larger decrease in NF-κB DNA binding activity.

IKKα cannot be activated by NIK in the absence of
IKKβ. The results described above indicate that IKKα,
which is expressed in normal levels in Ikkβ−/− cells, cannot
be activated by either TNF-α or IL-1. To further examine
this point, we cotransfected an HA epitope-tagged IKKα
expression vector into Ikkβ−/− ES cells in the absence or
presence of an NIK expression vector. NIK is the most potent
IKK activator identified to date (31) and was suggested to be
a direct IKKα kinase (18). Recently, however, we obtained
results that suggested that NIK-induced IKKα phosphoryla-
tion is not direct and is likely to be dependent on Ikkβ (19).
Consistent with this hypothesis, we found no increase in IKK
activity towards IκBα(1-54) substrate upon coexpression of
HA-IKKα with NIK in Ikkβ−/− cells (Fig. 7 A). Yet, when
an Ikkβ expression vector was included in these transfections,
NIK elicited a clear increase in IKK activity. As shown previ-
ously, NIK coexpression efficiently stimulates IKKα-associated
IKK activity in Ikkβ−expressing cells (19).

One reason for the inability of IKKα to respond to
proinflammatory stimuli or NIK in the absence of Ikkβ
could be its inability to directly associate with IKKγ, the
regulatory subunit of the IKK complex. Previous experi-
ments indicate that IKKγ is essential for recruitment of up-
stream activators to IKK (16). In addition, using recombi-
nant proteins, it was found that IKKβ directly interacts
with IKKγ much more efficiently than does IKKα (16, 17).
Having available IKKβ-deficient cells, we reexamined
the ability of IKKα to interact with IKKγ. In contrast to
the results obtained with recombinant proteins, very effi-
cient coprecipitation of IKKα by anti-IKKγ antibodies was
observed using lysates of Ikkβ−/− cells as a starting material
(Fig. 7 B). Therefore, the refractoriness of IKKα to IKK
activators in Ikkβ−deficient cells is not due to its inability
to associate with IKKγ.

**Discussion**

The enzymatic activity of the IKK complex, composed
of two catalytic subunits, IKKα and IKKβ, and one regula-

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**Figure 5.** Defective IKK and NF-κB activation in Ikkβ−
deficient ES cells. (A) IKK activity. Lysates of TNF-α or
IL-1–treated Ikkβ−/− and Ikkβ−/− 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 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 M 280 (IB: IKKα) for loading control. (B) NF-κB binding activity. Nuclear extracts of Ikkβ−/− and Ikkβ−/− 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 Ikkβ−/−, Ikkβ−/−, and Ikkβ−/− 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.

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**Figure 7.** Refractoriness of IKKα to IKKγ in Ikkβ−deficient ES cells. Immunoprecipitation was performed with antibodies to IKKα and
IKKβ, followed by Western blotting with antibodies to IKKα or IKKβ.
A: Detection of IKKγ and IKKβ association. B: Detection of IKKα,
IKKβ, and IKKγ association.
IKKα is refractory to activation in Ikkα−/− cells despite its association with IKKγ. (A) Ikkα−/− ES cells were transiently transfected by electroporation with an HA-IKKα expression vector alone or together with XpressIK or HA-IKKβ and XpressIK expression vectors 24 hours 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 Ikkα−/−, Ikkβ−/−, and Ikkβ−/− cells were immunoprecipitated (IP) with either anti-IKKα or anti-IKKβ 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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