IKK1-deficient mice exhibit abnormal development of skin and skeleton

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IKK1 and IKK2 are two putative IκBα kinases involved in NF-κB activation. To examine the in vivo functions of IKK1, we generated IKK1-deficient mice. The mutant mice are perinatally lethal and exhibit a wide range of developmental defects. Newborn mutant mice have shiny, taut, and sticky skin without whiskers. Histological analysis shows thicker epidermis, which is unable to differentiate. Limbs and tail are wrapped inside the skin and do not extend properly out of the body trunk. Skeleton staining reveals a cleft secondary palate, split sternebra 6, and deformed incisors. NF-κB activation mediated by TNFα and IL-1 is diminished in IKK1-deficient mouse embryonic fibroblast (MEF) cells. The IKK complex in the absence of IKK1 is capable of phosphorylating IκBα and IκBβ in vitro. Our results support a role for IKK1 in NF-κB activation and uncover its involvement in skin and skeleton development. We conclude further that the two related kinases IKK1 and IKK2 have distinct functions and can not be substituted for each other's functions.

[Key Words: IKK1, NF-κB, skin, skeleton, limb, mice]
Received March 9, 1999; accepted March 30, 1999.

NF-κB transcription factors are dimers composed of various combinations of structurally related proteins p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), c-Rel, and RelB (for review, see Verma et al. 1995; Baeuerle and Baichwal 1997). In resting cells, NF-κB complexes are retained in the cytoplasm in association with inhibitory proteins IκBα, IκBβ, IκBε. Upon stimulation by TNFα, IL-1α, UV, and γ-irradiation, or bacterial and viral infection, IκBs are phosphorylated at specific sites that lead to their ubiquitination, degradation by the proteosome, and release of NF-κB proteins for translocation to the nucleus where they regulate expression of target genes.

NF-κB proteins play a major role in many physiological and pathological processes. Analyses of mice deficient in different members of the NF-κB and IκB families have revealed essential roles for these transcription factors in lymphocyte development and immune responses (for review, see Attar et al. 1997), fetal liver development (Beg et al. 1995), and osteoclast maturation (Franzoso et al. 1997; Iotsova et al. 1997). Rel/NF-κB genes may also play a role in vertebrate limb development (Bushdid et al. 1998; Kanegae et al. 1998). Additionally, several groups have shown the involvement of NF-κB proteins in anti-apoptotic processes (Beg and Baltimore 1996; Van Antwerp et al. 1996; Wang et al. 1996). Lack of p65 (RelA) results in hepatocyte apoptosis and embryonic lethality at embryonic day 15 (E15), which may reflect its anti-apoptotic function in hepatocytes during development (Beg et al. 1995).

A central step to NF-κB activation is the induced phosphorylation of IκBs (Verma et al. 1995). Recently, the long-sought kinases for signal-induced phosphorylation of IκB have been identified by three independent groups (DiDonato et al. 1997; Mercurio 1997; Regnier et al. 1997; Woronicz et al. 1997; Zandi et al. 1997). Two highly homologous IκB kinases (IKKs), IKK1 [IKKα] and IKK2 [IKKβ], are present in a large 700–900 kD complex and can specifically phosphorylate IκBα and IκBβ in response to induction by TNFα and IL-1. The kinetics of induced complex activity matches with the kinetics of induced NF-κB activity. Overexpression of dominant-negative mutants of IKK1 or IKK2 can specifically inhibit the TNFα- and IL-1-induced NF-κB activity. Furthermore, in vitro kinase assays with the purified recombinant proteins, IKK1 and IKK2 site specifically phosphorylate all three known IκBs [IκBα at Ser-32 and Ser-36, IκBβ at Ser-19 and Ser-23, and IκBε at Ser-18 and Ser-22; Lee et al. 1998; Li et al. 1998; Zandi et al. 1998]. Thus, it appears that IKK1 and IKK2 are bona fide IκB kinases. However, the roles played by the individual kinases in NF-κB activation during development remain unknown.

We have generated IKK1-deficient mice by a gene targeting approach. Mice lacking the IKK1 gene are perinatally lethal and reveal a remarkable abnormal appearance including shorter limbs, a fused tail, and a shiny skin. The undifferentiated epidermis in mutant skin lacks degradation of nuclei and keratinized stratified epider-
mis in their stratum corneum. IKK1-deficient mice have a cleft secondary palate, split sternebra 6, and abnormal incisors. Furthermore, TNFα- and IL-1-induced NF-κB activation is diminished in mouse embryonic fibroblasts (MEFs) isolated from IKK1-deficient mice.

Results

Generation of IKK1-deficient mice

To mutate the IKK1 gene, a neomycin-resistance gene was used to replace a 3-kb genomic DNA fragment containing exon 1 and the upstream promoter region [Fig. 1A]. Homologous recombinant ES cell clones were identified by Southern analysis [Fig. 1B] and injected into C57/BL6 blastocysts that, in turn, developed into chimeric mice. Heterozygous mice generated by germ-line transmission of the targeted ES clones into a C57/BL6 genetic background were normal and fertile. Although they could develop to term, the IKK1 homozygous mutant mice were stillborn or died shortly after birth. Examination of E18 and E19 embryos by Caesarean section revealed that all of the mutant embryos were alive in utero but died soon after section. To determine the expression of IKK1 protein in IKK1−/− mice, we performed Western blot analysis on primary MEF cell extracts [Fig. 1D]. Anti-IKK1 sera detected IKK1 protein in wild-type MEFs and approximately one-half of that amount in heterozygous MEFs but failed to detect any signal in IKK1−/− MEFs. The relative amount of IKK2, IkBa, and IkBβ proteins was not affected in IKK1−/− MEFs [Fig. 1D]. We conclude that homozygous mice are IKK1 null.

General phenotype of IKK1−/− mice

Newborn IKK1 homozygous mutant mice could easily be identified by the marked malformation of their body morphology [Fig. 2A] and their genotypes were confirmed by PCR analysis [Fig. 1C]. The mutant skin was shiny, translucent, sticky, and without whiskers [Fig. 2B,C]. All four limbs appeared shorter and, most remarkably, the hind limbs together with the curled tail were embedded in the thick skin. Mutant embryos could be distinguished from their normal littermates as early as E12.5 of gestation because of their short, dumpy limb buds and curled tail [Fig. 2D].

Gross examination of internal organs revealed alterations of the gastrointestinal tract. IKK1−/− mice had a much smaller stomach and a shorter and narrower intestine (data not shown). All the mutant newborn pups had an expanded bladder that was not observed in wild-type or heterozygous littermates. Delayed umbilical hernia withdrawal was also observed in mutant embryos at E18 (data not shown). No apparent histological abnormalities were noticed in other organs.

Figure 1. Targeting of the IKK1 gene in mice. (A) Strategy for targeting the IKK1 allele. Simplified restriction maps of the wild-type IKK1 allele, the targeting construct, and the mutated allele are shown. A 3-kb genomic fragment is deleted and replaced by a PGK-neo cassette in an antisense orientation. (B) Southern blot analysis of an ES clone showing the correct insertion of the targeting construct. DNA was digested with BamHI and hybridized to the probe shown in A. The wild-type allele yields an 8.9-kb fragment whereas the mutant allele yields a 4-kb fragment. (C) PCR detection of mouse genotypes. (D) Western blot analysis confirmed the absence of IKK1 in IKK1−/− MEFs, whereas the expression of IKK2, IkBa, and IkBβ was not changed. Forty micrograms of whole-cell protein lysates from IKK2+/+, IKK2+/−, and IKK2−/− MEFs were loaded and immunoblotted with IKK1, IKK2, IkBa, and IkBβ antibodies.
Whole-mount examination of *IKK1*−/− mice revealed shorter limb buds at mid-gestation and shorter limbs at birth (Fig. 2A). Surprisingly, cartilage and bone staining of newborn mice with alizarin red/alcian blue did not reveal any major changes in the pattern and size of the proximal limb elements (Fig. 2E). However, alterations in the curvature and bending of the most distal limb elements were observed, and the phalanges were retarded and deformed [data not shown].

There were additional defects in skeletal development, including cleft palate [Fig. 2F,G] and unfused sternebra 6 [Fig. 2H,I]. In the mutant mice, the vomer and presphenoid were visible because of the cleft secondary palate [Fig. 2F,G]. Sternebra 6 was split and showed a reminiscence of its dual origin. Moreover, the sternal bands were shorter and broader and had a kinked shape instead of a straight one as a result of incomplete and asymmetric ossification of the sternum [Fig. 2H,I]. However, the sternal bands were well fused and functional. The gross appearance of incisors in newborn wild-type and *IKK1* mutant mice is shown in Figure 2 [J,K]. Incisors were present but deformed.

**Skin defects**

Histological examination of newborn (P0) mouse skin showed abnormalities in epidermal morphogenesis. The superficial keratinized squamous layer was missing, and the density of the suprabasal cells was much higher in *IKK1*−/− mutant skin (Fig. 3C), keratin 10-positive cells invaginated deeply into dermis in the mutant mice (Fig. 3A,B). Although anti-keratin 14 immunostaining showed no difference of the single basal layer in both wild-type and mutant skin [Fig. 3C,D], keratin 10-positive cells increased dramatically in the mutant suprabasal layer (SB) of skin compared with littermates [Fig. 3E,F]. The suprabasal cells failed to differentiate into granular cells and cornified cells as determined by the immunohistochemistry with antibodies to filaggrin [Fig. 3G,H] and loricrin [Fig. 3I, J, K]. Both filaggrin and loricrin are expressed in differentiated epidermal cells. They are present in the keratohyalin granules of the granular cells and the cornified layer in normal epidermis. The expression of both differentiation markers in *IKK1*-deficient epidermis was reduced considerably.

**Reduced NF-κB activity in *IKK1*−/− MEFs**

Because IKK1 functions as an IkB kinase, loss of IKK1 may impair signal-induced phosphorylation and degradation of IkBs and, therefore, block NF-κB nuclear translocation and subsequent DNA binding. To assess whether NF-κB activation in *IKK1*−/− MEFs was impaired, we performed a gel mobility shift analysis on nuclear extracts with a HIV-κB probe. A significant reduction of the NF-κB binding activity in *IKK1* mutant MEFs was observed upon induction with TNFα or IL-1 [Fig. 4A]. Next, we examined the significance of the reduced NF-κB DNA-
binding activity by measuring the expression of NF-κB-targeted genes such as those encoding IκBα, macrophage colony-stimulating factor (M-CSF), and IL-6. Consistent with the decreased NF-κB-binding activity, the IκBα mRNA level in IKK1−/− MEF was reduced (Fig. 4B). We measured the basal and induced expression of M-CSF and IL-6 in IKK1+/+ and IKK1−/− MEFs. The induced expression of M-CSF mRNA was reduced considerably in IKK1−/− MEFs, whereas its basal expression level was not affected (Fig. 4C). Similarly, the TNFα-induced IL-6 expression was also reduced (Fig. 4C). Together, these results support a role for IKK1 as an IκB kinase, although a considerable amount of NF-κB binding activity remains in the absence of IKK1.

In addition to IKK1 and IKK2, two other proteins, NEMO (IKKγ/IKKAP1) and IKAP, have been identified in the large IKK complex (Cohen et al. 1998; Rothwarf et al. 1998; Yamaoka et al. 1998; Mercurio et al. 1999). NEMO is not a kinase but is necessary for NF-κB activity induced by TNFα and other external stimuli (Yamaoka et al. 1998). To examine the activity of IKK complex in the absence of the IKK1, we performed an in vitro kinase assay with immunocomplexes precipitated with NEMO antibody (Fig. 4D). Interestingly, both IκBα and IκBβ, as well as p65 (RelA) were phosphorylated efficiently by NEMO immunocomplexes from both IKK1+/+ and IKK1−/− MEFs [Fig. 4D]. Thus, it appears that IKK1 may not be involved directly in IκBα or IκBβ phosphorylation but has another and yet unknown function for full NF-κB activation.

Discussion

NF-κB/Rel signaling pathways have been studied extensively in the past decade. Knockout mice for individual members of the NF-κB family have been generated and showed no major developmental defects except liver degeneration in p65-deficient mice (Beg et al. 1995; Attar et
Figure 4. NF-κB activity in IKK1−/− MEFs. (A) NF-κB binding activity is reduced in IKK1−/− MEF. Gel mobility shift analysis was conducted on 5 µg of nuclear extract from IKK1+/+ and IKK1−/− MEFs with or without 10 ng/ml TNFα induction for the indicated time. (B) TNFα-induced IκBα RNA synthesis is reduced in MEFs lacking IKK1. Northern blot analysis was performed on 10 µg of total RNA from each sample, using probe from a full-length IκBα cDNA. (C) RNase protection shows that TNFα-induced mRNA expression of M-CSF and IL-6 are diminished in IKK1−/− MEF. (D) Phosphorylation of IκBα, IκBβ, and p65 by NEMO immunocomplexes from IKK1−/− MEFs is not affected. Cells from three 15-cm plates were lysed and immunoprecipitated with anti-NEMO serum. Equal amounts of NEMO immunoprecipitates were incubated with the substrates indicated for the in vitro kinase assay. NEMO immunoprecipitates were loaded on a 10% SDS–polyacrylamide gel for anti-IKK1 and anti-IKK2 immunoblotting (D, bottom).
Twenty-five micrograms of Not-I-linearized targeting construct was electroporated into J1 ES cells, and the targeted clones were selected with G418 (0.2 mg/ml, active form) and FIAU (200 μM) in DMEM medium. The resistant colonies were screened for homologous recombination by Southern blot analysis with a 240-bp 5'-external PstI fragment as a probe. A 4-kb BamHI fragment from the targeted allele was distinguished from a 8.9-kb wild-type BamHI fragment.

Two independent targeted ES cell clones [5B5 and 2D1] with only a single insertion were identified and injected into C57BL/6 blastocysts. Heterozygous mutant mice were generated from one line (5B5). Crossing of the chimeras with C57BL/6 blastocysts. Heterozygous mutant mice were generated as described (Martin et al. 1995).

Histology analysis, skeleton staining, and in situ immunostaining

Newborn mice or embryos at E12.5, E13.5, E18, and E19 were harvested and fixed in 4% paraformaldehyde (PFA) at 4°C for 24 hr. Genotyping was performed on genomic DNAs from yac sacs or tails. Embryos were grossly examined and photographed before and after fixation. Fixed embryos were then dehydrated, paraffin-embedded, and serially sectioned at 7 μm. The selected sections were stained with hematoxylin and eosin for routine histologic examination. Cartilage and bone staining was performed as described [Martin et al. 1995].

Immunohistochemical staining for keratin 14 [Noco Castra], keratin 10 [BAHCO Berkeley Antibody Company], filaggrin [BAHCO Berkeley Antibody Company], and loricrin [BAHCO Berkeley Antibody Company] was performed on 4% PFA-fixed cryosections as recommended by the manufacturer. Briefly, fresh frozen slides were fixed in 4% PFA for 10 min, washed with PBS, and blocked for 15 min. Then, slides were incubated with primary antibodies for 1 hr at room temperature, washed three times with PBS buffer, and probed with FITC-conjugated goat anti-mouse (for keratin 14) or Cy3-conjugated goat anti-rabbit secondary antibody (for keratin 10, filaggrin, and loricrin).

RNA isolation, Northern blot analysis, and RNase protection

Cultured MEFs from Ikk2+/−, Ikk2+/−, and Ikk2−/− embryos were stimulated with or without 10 ng/ml of human TNFα for 30 and 60 min. Cells were lysed in 2 ml of Rnase B buffer [Tel-Test, Inc., Friendswood, TX]. Total RNA was prepared from MEFs following the manufacturer’s instruction [Tel-Test, Inc.]. Ten micrograms of total RNA was fractionated on formaldehyde–agarose gels, blotted onto GeneScreen Plus membrane (Biotechnology Systems), and hybridized with a 32P-labeled probe from full-length IkkBα cDNA. Quick Hyb (Stratagene, San Diego, CA) was used for Northern analysis. Prehybridization, hybridization, and washes were performed as recommended by the manufacturer (Stratagene).

RNase protection was performed by use of mCK-4 RiboQuant Multi-Probe Template Set [Pharmingen, San Diego, CA], containing multiple probes including IL-3, IL-11, IL-7, GM-CSF, M-CSF, C-CSF, LIF, IL-6, SCF, L32, and GAPDH. Ten micrograms of total RNAs from Ikk1+/+ and Ikk1−/− MEFs treated with or without 10 ng/ml human TNFα for 1 hr were used. The RNase protection assay was conducted following the manufacturer’s protocol.

Western blot analysis, gel mobility shift assays, immunoprecipitation, and kinase assay

Forty micrograms of each extract was separated on a 10% SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane, blocked in 1× PBST [PBST plus 0.2% Tween] and 5% milk powder for 15–30 min, washed twice in PBST, and probed further with primary antibodies to Ikk1, or Ikk2, or Ikkβ, or IκBα (Santa Cruz Biotechnology) for 1–2 hr at room temperature. After several washes in PBST, the filters were incubated with a horseradish peroxidase-conjugated secondary antibody for 30–60 min in PBST, washed several times in PBST, and eventually developed with ECL kit (Amersham).

To assess effects of Ikk1 inactivation on the NF-κB activation pathway, we subjected MEFs from all three genotypes to a variety of stimuli. MEFs at ~90% confluence on a 10-cm plate were either untreated or treated with 10 ng/ml human TNFα [Calbiochem] or 2 ng/ml human IL-1α at the indicated times. After treatment, cells were washed with cold PBS, the cytoplasmic and nuclear extracts were prepared, and binding assays were performed as described [Miyamoto et al. 1994; Van Antwerp et al. 1996].

Whole-cell extracts from >95% confluent MEFs in 10-cm plates were used for immunoprecipitation with anti-NEMO. The NEMO antibody [anti-C-IKKAP1] raised against the synthetic peptide from the carboxyl terminus of human NEMO was a gift from Signal Pharmaceutical Inc. [San Diego, CA, Mercurio et al. 1999]. Immunocomplexes precipitated from cell lysates with anti-NEMO antibody and then were eluted off the antibody by the synthetic peptide. NEMO immunocomplexes were subjected to an in vitro kinase assay as described [Mercurio et al. 1997]. Equal amounts of NEMO immunocomplex from different MEFs were used for kinase assays with 3 μg of GST–IkkBα[1–54] [amino acids 1–54], 1.5 μg of GST–IkkBα[1–54]/S [amino acids 1–54, Ser-32 → Thr-32 and Ser-36 → Thr-36], 2.5 μg of GST–IkkBβ[1–44] [amino acids 1–44], 2.5 μg of GST–IkkBβ[1–44]/A [amino acids 1–44, Ser-19 → Ala-19 and Ser-23 → Ala-23], 1 μg GST–IkkBα[1–317] (Santa Cruz Biotechnology), or 1 μg of p65/RelA as a substrate. Equal amounts of eluates were analyzed, and the amount of IKK1 and IKK2 proteins was determined by Western blot analysis with anti-IKK1 and anti-IKK2 antibodies.

Acknowledgments

We thank Yelena Marchuk and Bertha Dominguez for their excellent technical assistance, Frank Mercurio for Ikk1 cDNA, GST–IkkBα, GST–IkkBβ and NEMO antibody, Steven Crone for helpful advice in ES technology, Daniel Van Antwerp and Wen Xie for discussion, Conchi Rodriguez Esteban for helping with skeleton staining, and Beth Coyne for her help with this manuscript. Q.L. is supported by a training grant from the National Institutes of Health [NIH]; D.B. is supported by a fellowship from Deutsche Forschungsgemeinschaft, K.-F.L is a Pew Scholar and is supported by the NIH and the March of Dimes Foundation, J.C.I.B. is supported by NIH and Mathers Foundation grants, and I.M.V. is an American Cancer Society Professor of Molecular Biology and is supported by the Valley Foundation and grants from the NIH.
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References


