RESEARCH COMMUNICATION

Complete lack of NF- κ B activity in IKK1 and IKK2 double-deficient mice: additional defect in neurulation

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NF- κ B activity is induced by cytokines, stress, and pathogens. IKK1 and IKK2 are critical I κ B kinases in NF- κ B activation. In this study mice lacking IKK1 and IKK2 died at E12. Additional defect in neurulation associated with enhanced apoptosis in the neuroepithelium was also observed. MEF cells from *IKK1*^{-/-}/*IKK2*^{-/-} embryos did not respond to NF- κ B inducers. Upon crossing with κ B-*lacZ* transgenic mice, double-deficient embryos also lost *lacZ* transgene expression in vascular endothelial cells during development. Our data suggest that IKK1 and IKK2 are essential for NF- κ B activation in vivo and have an important role in protecting neurons against excessive apoptosis during development.

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NF- κ B activity is required for the induction of a large number of genes involved in cell growth, differentiation, and development. A wide variety of external stimuli including cytokines, pathogens, stress, and pharmacological agents can lead to the activation of the NF- κ B family of transcription factors (Baeuerle and Henkel 1994; Baeuerle and Baichwal 1997). These stimuli induce phosphorylation and subsequent degradation of I κ B inhibitory proteins, thereby releasing NF- κ B proteins for translocation to the nucleus to function as transcription factors (Verma et al. 1995). Phosphorylation of I κ B is mediated by IKK complexes containing two highly homologous I κ B kinases, IKK1 (IKK α) and IKK2 (IKK β) (DiDonato et al. 1997; Mercurio et al. 1997; Regnier et al. 1997; Woronicz et al. 1997; Zandi et al. 1997; Karin 1999). A scaffolding protein, NEMO (IKK γ), has also been implicated in NF- κ B activation (Rothwarf et al. 1998; Yamaoka et al. 1998; Mercurio et al. 1999). IKK1- and IKK2-deficient mice were generated by a gene targeting approach and displayed different spectra of defects (Hu et al. 1999; Li et al. 1999a, b, c; Takeda et al. 1999; Tanaka et al. 1999). IKK2-deficient mice died progressively from E12.5 to E14 because of enhanced apoptosis in liver that could be overcome by mating to *TNFR1*^{-/-} background (Li et al. 1999b). In contrast, IKK1-deficient mice died at

birth with multiple developmental defects in skin, limb, and skeleton (Li et al. 1999a). Because NF- κ B activation in mouse embryonic fibroblasts (MEFs) is blocked only partially in the absence of IKK1 or IKK2, it raises the question as to whether genetic redundancy of IKK1 and IKK2 can account for all of the NF- κ B activity in mice.

Results and Discussion

To study the redundant functions of IKK1 and IKK2, we generated compound homozygous mice carrying null alleles of both *IKK1* and *IKK2* genes. *IKK1*^{-/-}/*IKK2*^{-/-} mice were generated from intercrosses of *IKK1*^{+/-}/*IKK2*^{+/-} mice. *IKK1*^{-/-}/*IKK2*^{-/-} embryos were recovered with expected frequency at E11.5, but they died at E12. Nearly 70% of *IKK1*^{-/-}/*IKK2*^{-/-} embryos revealed a failure of neural tube closure in the hindbrain (Fig. 1) that was not observed in wild-type littermates. The telencephalic vesicle of *IKK1*^{-/-}/*IKK2*^{-/-} embryos was smaller than that of wild-type embryos (Fig. 1A). Neural folds at the hindbrain in E9.5 *IKK1*^{-/-}/*IKK2*^{-/-} mutants failed to elevate on either side of the midline and did not bend toward each other, whereas the remaining length of the neural tube other than the hindbrain was able to form a tube (Fig. 1B). Hindbrain defects in *IKK1*^{-/-}/*IKK2*^{-/-} embryos were also revealed by histological examination (Fig. 1C,D). To further define the neural tube defect (NTD) in double mutant embryos, we performed TUNEL assay on the transverse section through the hindbrain of E9.5 embryos. Increased apoptosis was detected in the neuronal epithelium at the hindbrain level (Fig. 1E,F). A massive increase of apoptosis in double mutant liver was detected at around E11.5–E12 (Fig. 1G,H), indicating that like the *IKK2*^{-/-} mutant, *IKK1*^{-/-}/*IKK2*^{-/-} embryos died from liver dysfunction. We also observed a twofold increase in apoptosis in the mutant spinal cord and dorsal root ganglia (not shown); however, no defects in neural differentiation were observed (not shown).

Because *IKK2* mutant embryos can be rescued from the embryonic lethal to the postnatal stage by inactivation of the *TNFR1* gene (Li et al. 1999b), we generated *IKK1*^{-/-}/*IKK2*^{-/-}/*TNFR1*^{-/-} triple mutant mice to assess if *IKK1*^{-/-}/*IKK2*^{-/-} mice can be rescued by blocking the TNFR1 signal transduction pathway. In *TNFR1*^{-/-} background, *IKK1*^{-/-}/*IKK2*^{-/-} embryos survived to around E16.5 and revealed a morphology similar to *IKK1*^{-/-} embryos, such as curled tail and dumpy limb buds (Fig. 1I,L). Interestingly, the NTD in *IKK1*^{-/-}/*IKK2*^{-/-} embryos cannot be rescued by loss of the *TNFR1* gene. In contrast to *IKK2*^{-/-} embryos, the phenotypes of *IKK1*^{-/-} mutants cannot be rescued either (Fig. 1I–K). Therefore, *IKK1*^{-/-}/*IKK2*^{-/-} mutants revealed combined phenotypes of *IKK1*^{-/-} and *IKK2*^{-/-} mutants, as well as additional defects in neurulation and neuronal survival, suggesting that their functions are distinctive and overlapping during development.

Degradation of I κ B α in response to a plethora of external stimulus is preceded by the phosphorylation at Ser-32

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Figure 1. Phenotypes of *IKK1*^{-/-}/*IKK2*^{-/-} embryos. Side (A) and views back (B) of wild type (left) and *IKK1*^{-/-}/*IKK2*^{-/-} embryos at E11.5 (A) and E9.5 (B). The neural tube in the hindbrain region failed to close (arrow). H&E-stained transverse sections of E9.5 wild type (C) and *IKK1*^{-/-}/*IKK2*^{-/-} mutant (D) at the hindbrain level showing that the roof of the hindbrain (arrow) is present in the wild type but absent in the mutant. TUNEL assay on transverse section of E9.5 wild type (E) and double mutant (F), revealing increased apoptosis (green) in the neuroepithelium of the hindbrain (arrow). Enhanced apoptosis (red arrowhead) was observed in an H&E-stained liver section from *IKK1*^{-/-}/*IKK2*^{-/-} at E11.5 (H) in comparison with that of a wild type littermate (G). (I–L) Whole-mount pictures of wild-type, *IKK2*^{-/-}, *IKK1*^{-/-}, and *IKK1*^{-/-}/*IKK2*^{-/-} embryos in *TNFR1*^{-/-} genetic background at E14.5. No morphologic differences were detected between *TNFR1*^{-/-} (I) and *TNFR1*^{-/-}/*IKK2*^{-/-} (J) embryos. Phenotypes of dumpy limb buds and curled tail were observed in both *TNFR1*^{-/-}/*IKK1*^{-/-} (K) and *TNFR1*^{-/-}/*IKK1*^{-/-}/*IKK2*^{-/-} (L) embryos. In addition, *TNFR1*^{-/-}/*IKK1*^{-/-}/*IKK2*^{-/-} embryos also had NTD (arrow in L). Scale bar, 1 mm in A, B, and I–L; 100 μ m in C–F; and 24 μ m in G and H.

and Ser-36 (Verma et al. 1995). To test if loss of IKK1 and IKK2 blocks phosphorylation, degradation of I κ B, and subsequent NF- κ B induction, we examined NF- κ B activation in MEF cells from *IKK1*^{-/-}/*IKK2*^{-/-} embryos. First, we examined NF- κ B binding activity by gel shift analysis using NF- κ B-responsive elements. We failed to detect induced NF- κ B binding activity in nuclear extracts from double-deficient MEFs treated with human (h)TNF α , IL-1 α , and LPS (Fig. 2A). No degradation of I κ B α and I κ B β in *IKK1*^{-/-}/*IKK2*^{-/-} MEFs was detected by the Western blot analysis, whereas I κ B α and I κ B β were degraded in response to induction in wild-type MEFs

(Fig. 2B). Furthermore, as expected, I κ B α was resynthesized rapidly in wild-type MEFs (Fig. 2B). Additionally, IKK complex immunoprecipitated from *IKK1*^{-/-}/*IKK2*^{-/-} MEFs by NEMO (IKK γ) antisera was unable to phosphorylate I κ B α , I κ B β , and p65 (Fig. 2C). To substantiate our observations further, we performed RNA analysis by Northern blot to examine NF- κ B target gene expression upon TNF α induction. I κ B α expression induced by TNF α was observed in wild-type MEFs but not in double mutant MEFs (Fig. 2D, E). In contrast, lack of either IKK1 or IKK2 individually resulted in only partial blocking of I κ B α induction (Fig. 2D, E). Consistent with the previous observation (Li et al. 1999a, b), NF- κ B activation is more attenuated in *IKK2*^{-/-} than in *IKK1*^{-/-} MEFs. We conclude that IKK1 and IKK2 are essential for NF- κ B activation in MEFs by hTNF α , hIL-1 α , and LPS.

To further explore the roles of IKK1 and IKK2 in NF- κ B activation in vivo, we introduced a κ B-*lacZ* transgene into the double homozygotes as a marker for NF- κ B activity. In mice containing the κ B-*lacZ* transgene, *lacZ* expression is driven by κ B sites, which mirrors the tran-

Figure 2. NF- κ B activation is blocked in *IKK1*^{-/-}/*IKK2*^{-/-} MEFs. (A) No NF- κ B DNA binding activity was detectable in *IKK1*^{-/-}/*IKK2*^{-/-} MEFs upon induction. Nuclear extracts (5 μ g) were used for electrophoretic mobility shift analysis with ³²P-end-labeled HIV- κ B oligonucleotide. (B) Induced I κ B degradation was blocked in the absence of IKK1 and IKK2. Cytoplasmic extract (40 μ g) was used for immunoblotting with I κ B α , I κ B β , IKK1, and IKK2 antisera (Santa Cruz Biotechnology). (C) TNF α -induced kinase activity of the IKK complex for I κ B and p65 was abolished in the absence of IKK1 and IKK2. Cells from three 15-cm plates, untreated or treated with TNF α for 7 min, were lysed and immunoprecipitated with anti-NEMO serum. IP complexes were eluted from the antibody by the synthetic peptide. Equal amounts of eluates were used for kinase assays using the substrate indicated. (D, E) I κ B α induction by TNF α was abolished in IKK1 and IKK2 deficient MEFs. RNA (10 μ g) from wild-type, *IKK1*^{-/-}, *IKK2*^{-/-}, and *IKK1*^{-/-}/*IKK2*^{-/-} MEFs, untreated or treated with 10 ng/ml hTNF α for 1 hr was subjected to Northern blot analysis with probe specific for I κ B α and GAPDH. Quantification and original data are shown in D and E, respectively.

scription activity of endogenous NF- κ B (Schmidt-Ullrich et al. 1996). To examine NF- κ B activity during early mouse development, we first carried out whole-mount X-gal staining on wild-type transgenic embryos. Extensive *lacZ* expression was observed at E9.5 and E10.5 (Fig. 3A,B) and was detected as early as E8.5 (not shown). Detailed studies on parasagittal sections of whole-mount X-gal-stained embryos revealed that *lacZ* expression was located at the blood vessel walls, such as intersomitic vasculature (ISV) and dorsal aorta (DA) (Fig. 3C). To further characterize β -gal-positive cells, we double-labeled the sections immunohistologically using β -gal antibody and an antibody specific for an endothelial cell marker, PECAM-1. β -Gal-positive cells in all tissues including neuroepithelium were also PECAM-1 positive, suggesting that they are endothelial cells (Fig. 3D–F). However, not all of the PECAM-1-positive cells, for example, endocardium of heart, express *lacZ* (Fig. 3G). We conclude that NF- κ B activity is present in vascular endothelial cells during early development.

We examined κ B-*lacZ* transgene expression further in *IKK1*^{−/−}, *IKK2*^{−/−}, and *IKK1*^{−/−}/*IKK2*^{−/−} mutants. In comparison with control embryos, *lacZ* expression was weaker in *IKK1*^{−/−} and *IKK2*^{−/−} mutants and almost un-

Figure 3. Endothelial cell-specific expression of *lacZ* in κ B-*lacZ* transgenic mice during early development. Whole-mount X-gal staining of κ B-*lacZ* transgenic embryos at E9.5 (A) and E10.5 (B). (C) Sagittal sections of an X-gal-stained embryo in B counterstained with nuclear fast red. *lacZ* expression was readily detected everywhere, especially in vascular vessels such as the DA and ISV. (End) Endocardium; (HB) hindbrain; (NE) neuroepithelium. (D–G) β -Gal (green) and PECAM-1 (red) double staining on the sagittal sections of an E11.5 transgenic embryo. The transgene, NLS-containing β -gal, is expressed in the nuclei of PECAM-1-positive cells in blood vessels (D, E) and primary capillary plexus at the neuroepithelium (F) but not in PECAM-1-positive endocardium (G). Scale bar, 1 mm in A–C and 24 μ m in D–G.

Figure 4. IKK1 and IKK2 are required for NF- κ B activity in vascular endothelial cells. Whole-mount X-gal staining showing different expression levels of transgenes in different *IKK* mutants at E11.5 (A) and E10.5 (C). (B) Sagittal section of embryos in A. X-gal expression in *IKK1*^{−/−}/*IKK2*^{−/−} embryos is lost almost completely; its expression in *IKK1*^{−/−} and *IKK2*^{−/−} embryos is also attenuated in comparison with wild-type controls. (See text and legend to Fig. 3 for abbreviations.) Whole-mount PECAM-1 staining of an *IKK1*^{−/−}/*IKK2*^{−/−} mutant (E) and a control littermate (D) at E10.5 revealed normal vasculogenesis in the absence of IKK1 and IKK2 kinases. Scale bar, 1 mm in A–E.

detectable in *IKK1*^{−/−}/*IKK2*^{−/−} embryos (Fig. 4A–C). This result demonstrates that IKK1 and IKK2 are essential for NF- κ B activity in vascular endothelial cells during development. We also evaluated vasculogenesis in *IKK1*^{−/−}/*IKK2*^{−/−} embryos by whole-mount PECAM-1 staining. The overall pattern of PECAM-1 staining was similar in both *IKK1*^{−/−}/*IKK2*^{−/−} mutants and in wild-type controls, suggesting normal vasculogenesis in the absence of IKK1 and IKK2 (Fig. 4D,E).

Our results show that IKK1 and IKK2 have a redundant role during neural development. They are integral in preventing excessive apoptosis in liver and neural tissue during development. It is well established that NF- κ B activity is required for protecting the liver from TNF α -induced apoptosis (Doi et al. 1999; Li et al. 1999b). The involvement of NF- κ B in NGF-mediated neuronal survival and in protecting neurons from injury-induced apoptosis has also been suggested recently (Hamanoue et al. 1999; Mattson et al. 2000). Because misregulation of apoptosis has been implicated in NTD (Lill et al. 1997; Yao et al. 1998), it is possible that the NTD in *IKK1*^{−/−}/*IKK2*^{−/−} mutants may be a secondary effect of dysregulation of apoptosis. Alternatively, NF- κ B may regulate the expression of adhesion molecules important for neu-

ral tube folding. Increased apoptosis in neural tissue and development of NTD in the absence of NF- κ B activity during mouse embryonic development have not been reported to date. Recently NEMO/IKK γ -deficient mice were generated, which display a phenotype similar to IKK1 and IKK2 double mutants in terms of liver apoptosis and lack of NF- κ B activation (Rudolph et al. 2000). However, no NTD or other developmental phenotypes were reported in NEMO-deficient embryos. Thus it would appear that NTD in IKK1 and IKK2 double mutants may be caused at least partly by a NF- κ B-independent mechanism. NF- κ B-independent functions of IKK1 during development are already hinted at from the genetic analysis of skin phenotype (Li et al. 1999a). Identification of IKK1 downstream targets during development will be required to better understand the cause of the phenotypes. NF- κ B activity has been observed previously in blood vessels of adult mice (Schmidt-Ullrich et al. 1996), and it is thought to be important for leukocyte trafficking and regulation of inflammatory responses. Identification of strong constitutive NF- κ B activity in endothelial cells during early vasculogenesis is an intriguing observation. It will be of obvious interest to identify NF- κ B induced genes involved in endothelial cell development and function. Finally it is important to point out that our data with κ B-*lacZ* transgenic mice do not have the sensitivity to exclude other cell types, which may not require IKK1 or IKK2 for NF- κ B activation.

Materials and methods

Generation of IKK1^{-/-}/IKK2^{-/-} and IKK1^{-/-}/IKK2^{-/-} mice in TNFR1^{-/-} background or containing the κ B-*lacZ* transgene
IKK1^{-/-}/IKK2^{-/-} mice were generated from intercrosses of IKK1^{+/-}/IKK2^{+/-} mice. IKK1^{+/-}/IKK2^{+/-} mice were generated from the mating of IKK1^{+/-} and IKK2^{+/-} mice (Li et al. 1999a, b). TNFR1^{-/-} mice were obtained from The Jackson Laboratory (Pfeffer et al. 1993). κ B-*lacZ* transgenic line 252 was used in this study (Schmidt-Ullrich et al. 1996).

Histology analysis and TUNEL assay

Embryos were harvested and fixed in 4% paraformaldehyde (PFA) at 4°C overnight. Genotyping was performed on genomic DNAs from yolk sacs. Embryos were grossly examined and photographed after fixation. Fixed embryos were then dehydrated, paraffin embedded, and serially sectioned at 7 μ m. The selected sections were stained with hematoxylin and eosin (H&E) for routine histologic examination.

TUNEL assay was performed on E9.5 transverse frozen sections using an in situ cell death detection kit (Boehringer Mannheim) and counterstained with DAPI (Vector).

Whole-mount X-gal staining

Whole-mount X-gal staining was performed as described (Hogan et al. 1994). Embryos were dissected from uteri, and yolk sacs were saved for PCR genotyping. Embryos were fixed in 0.2% glutaraldehyde solution [0.1 M PBS (pH 7.3), 5 mM EGTA, 2 mM MgCl₂] for 15–30 min. The embryos were washed three times with rinsing buffer (0.1 M PBS at pH 7.3, 2 mM MgCl₂, 0.01 sodium deoxycholate, 0.02% NP-40). Embryos were stained with 1 mg/ml X-gal solution (0.1 M PBS at pH 7.3, 2 mM MgCl₂, 0.01 sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferri-cyanide, 5 mM potassium ferrocyanide) for 3–6 hr at 37°C. After staining, the embryos were postfixed with PBS/4% PFA in PBS for 2 hr.

Immunohistochemical staining for β -gal and PECAM-1

Cryosections from 4% PFA/PBS-fixed embryos were postfixed in 4% PFA/PBS for 10 min, washed with PBS, and blocked with PBS-blocking

buffer (0.1% BSA, 0.2% powdered skim milk, 0.3% Triton X-100) for 15 min. The slides were incubated with primary antibodies specific for β -gal (Promega) and PECAM-1 (PharMingen) for 1 hr at room temperature, washed three times with PBS buffer, and probed with FITC-conjugated donkey anti-mouse (for β -gal) or Cy3-conjugated donkey anti-rat secondary antibody (for PECAM-1).

Whole-mount PECAM-1 staining

PECAM-1 staining was carried out using anti-mouse PECAM-1 mAb MEC13.3 (PharMingen). Embryos were fixed in 4% PFA/PBS at 4°C for overnight, washed three times with PBS, dehydrated in MeOH, bleached with 5% H₂O₂ in MeOH for 60 min at room temperature, washed three times with PBST (0.1% Tween 20)/1% DMSO, blocked with blocking buffer (0.1% BSA, 0.2% powdered skim milk, 0.3% Triton X-100) for overnight, and incubated overnight with anti-PECAM-1 antibody (PharMingen, 1:500 dilution in blocking buffer) at room temperature. Following three washes with PBST/1% DMSO, embryos were incubated with HRP-donkey anti-rat antibody for overnight, and washed with PBST/1% DMSO and PBST. Color was developed using a DAB kit (Vector Laboratory Inc.).

RNA isolation and Northern blot analysis

Primary MEFs from wild-type, IKK1^{-/-}, IKK2^{-/-}, and IKK1^{-/-}/IKK2^{-/-} embryos were treated or untreated with 10 ng/ml hTNF α for 60 min. Cells were lysed in RNazol B buffer (Tel-Test, Inc.). Total RNA was prepared from MEFs according to the manufacturer's instructions (Tel-Test, Inc.). Ten micrograms of total RNA was fractionated on formaldehyde agarose gels, blotted onto GeneScreen Plus membrane (Biotechnology Systems), and hybridized with ³²P-labeled probe from full-length I κ B α cDNA. Quick Hyb (Stratagene) was used for Northern analysis. The same membrane was stripped and reprobed with GAPDH as an RNA loading control. The intensities of the hybridization signals were quantitated using a storage phosphorimaging system (Molecular Dynamics).

Western blot analysis and gel shift mobility assays

MEFs with ~90% confluence on a 10-cm plate were either untreated or treated with 10 ng/ml hTNF α (Calbiochem), 2 ng/ml hIL-1 α (Calbiochem), or 10 μ g/ml LPS (Sigma) at the indicated times. After treatment, cells were washed with cold PBS, cytoplasmic and nuclear extracts prepared, and Western blot analysis and gel shift binding assays performed as described previously (Miyamoto et al. 1994; Li et al. 1999a).

Immunoprecipitation and kinase assay

Three 15-cm plates of MEFs from wild-type and IKK1^{-/-}/IKK2^{-/-} embryos were untreated or treated with 10 ng/ml hTNF α for 7 min. Whole-cell lysates from each 15-cm plate were prepared and immunoprecipitated with 10 μ l of anti-NEMO antibody (Mercurio et al. 1999) in 1 ml of immunoprecipitation (IP) buffer (Mercurio et al. 1997). Twenty microliters of protein A was added and samples were rotated for 2 hr at 4°C. The immunoprecipitates were then washed three times with IP buffer. Samples from all three 15-cm plates were pooled into one tube and washed once with kinase assay (KA) buffer (Mercurio et al. 1997). Sixty micrograms of synthetic peptide in 140 μ l of KA buffer was added to the protein A beads, and samples were rotated for 6 hr at 4°C. After a brief spin, the eluates were transferred to new tubes. Twenty microliters of eluates was used for each kinase assay reaction or Western blot analysis.

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