

# Constitutive NF- $\kappa$ B activation, enhanced granulopoiesis, and neonatal lethality in I $\kappa$ B $\alpha$ -deficient mice

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Transcription factors belonging to the NF- $\kappa$ B family are controlled by inhibitory I $\kappa$ B proteins, mainly I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ . Apparently normal at birth, I $\kappa$ B $\alpha$ <sup>-/-</sup> mice exhibit severe runting, skin defects, and extensive granulopoiesis postnatally, typically dying by 8 days. Hematopoietic tissues from these mice display elevated levels of both nuclear NF- $\kappa$ B and mRNAs of some, but not all, genes thought to be regulated by NF- $\kappa$ B. NF- $\kappa$ B elevation results in these phenotypic abnormalities because mice lacking both I $\kappa$ B $\alpha$  and the p50 subunit of NF- $\kappa$ B show a dramatically delayed onset of abnormalities. In contrast to hematopoietic cells, I $\kappa$ B $\alpha$ <sup>-/-</sup> embryonic fibroblasts show minimal constitutive NF- $\kappa$ B, as well as normal signal-dependent NF- $\kappa$ B activation that is concomitant with I $\kappa$ B $\beta$  degradation. Our results indicate that I $\kappa$ B $\beta$ , but not I $\kappa$ B $\alpha$ , is required for the signal-dependent activation of NF- $\kappa$ B in fibroblasts. However, I $\kappa$ B $\alpha$  is required for the postinduction repression of NF- $\kappa$ B in fibroblasts. These results define distinct roles for the two forms of I $\kappa$ B and demonstrate the necessity for stringent control of NF- $\kappa$ B.

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Members of the NF- $\kappa$ B transcription factor family have been identified in various organisms, ranging from flies to mammals (for review, see Nolan and Baltimore 1992; Liou and Baltimore 1993; Baeuerle and Henkel 1994). In mammals, the most widely distributed  $\kappa$ B-binding activity is a heterodimer (called NF- $\kappa$ B) of a 50-kD (p50) and a 65-kD (p65 or RelA) protein (Baeuerle and Baltimore 1989). Members of this family, which also include the proto-oncogene c-Rel, RelB, and p100/p52, share a highly conserved amino-terminal sequence called the Rel-homology region (RHR) (Nolan and Baltimore 1992; Liou and Baltimore 1993; Baeuerle and Henkel 1994). This region is required for dimerization as well as DNA binding and its structure is known in detail for p50 homodimers (Ghosh et al. 1995; Muller et al. 1995). NF- $\kappa$ B complexes are localized typically to the cytoplasm bound to inhibitory cytoplasmic retention proteins called the I $\kappa$ Bs.

NF- $\kappa$ B can be activated by a wide variety of signals including cytokines, such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and interleukin-1 (IL-1), bacterial products, such as lipopolysaccharide (LPS), oxidative stress, viruses, and DNA-damaging agents (for review, see Grilli et al. 1993; Baeuerle and Henkel 1994). Stimulation of cells with these agents can lead to rapid translocation of NF- $\kappa$ B to

the nucleus where it can regulate transcriptionally various target genes. NF- $\kappa$ B responsive genes include those for various cytokines, adhesion molecules, cell surface receptors, and immune modulators (Grilli et al. 1993; Baeuerle and Henkel 1994). The recent generation of p105/p50 and RelA-deficient mice have revealed defects in immune responses and the inability to induce cytokine-dependent gene expression (Beg et al. 1995; Sha et al. 1995). In addition, studies carried out on RelA-deficient mice have demonstrated the importance of that protein for hepatocyte survival, whereas studies on RelB-deficient mice have revealed defects in the formation of thymic dendritic cells (Beg et al. 1995; Burkly et al. 1995; Weih et al. 1995). These studies underscore the critical role played by members of the NF- $\kappa$ B family in various developmental and functional processes.

The I $\kappa$ B proteins comprise a distinct family that is also highly conserved in disparate species (for review, see Haskill et al. 1991; Inoue et al. 1992; Beg and Baldwin 1993; Thompson et al. 1995). The region of homology is limited to the ankyrin repeats that have been shown to be required for interaction with the RHR of NF- $\kappa$ B proteins (Beg and Baldwin 1993). Although, several proteins with I $\kappa$ B-like activity have been identified, the I $\kappa$ B $\alpha$  (Haskill et al. 1991) and I $\kappa$ B $\beta$  (Thompson et al. 1995) proteins are the best studied. In addition, the carboxy-terminal regions of the p105 and p100 precursors of the p50 and p52 subunits of NF- $\kappa$ B also contain ankyrin re-

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peats and thus, can function as I $\kappa$ B proteins through an intramolecular interaction mechanism (Henkel et al. 1992; Rice et al. 1992; Mercurio et al. 1993; Scheinman et al. 1993). Recent studies have shown that stimulation of cells with NF- $\kappa$ B inducers, such as TNF $\alpha$  and IL-1, can lead to the rapid phosphorylation and degradation of I $\kappa$ B $\alpha$ , allowing NF- $\kappa$ B to translocate to the nucleus (Beg et al. 1993; Brown et al. 1993; Sun et al. 1993). Site-specific phosphorylation of I $\kappa$ B $\alpha$  (Brockman et al. 1995; Brown et al. 1995; Chen et al. 1995) does not cause dissociation from NF- $\kappa$ B but apparently renders it susceptible to degradation (Finco et al. 1994; Israel 1995). The I $\kappa$ B $\beta$  protein is proposed to be targeted by a subset of NF- $\kappa$ B inducers and may be responsible for persistent activation of NF- $\kappa$ B in response to these stimuli (Thompson et al. 1995).

Despite recent advances in understanding the regulation of NF- $\kappa$ B and I $\kappa$ B proteins, major issues remain unresolved. How can so many seemingly diverse stimuli lead to NF- $\kappa$ B activation? How do NF- $\kappa$ B proteins specifically regulate subsets of target genes in response to particular stimuli? How are the optimal levels of NF- $\kappa$ B maintained in the cytoplasm and nucleus, both before and after stimulation? Is degradation of I $\kappa$ B proteins sufficient to allow nuclear translocation of NF- $\kappa$ B? Do different I $\kappa$ B forms perform distinct functions? The key to deciphering these issues is understanding the function and regulation of the I $\kappa$ B proteins.

Here we describe the generation of I $\kappa$ B $\alpha$ -deficient mice by gene targeting. I $\kappa$ B $\alpha$ <sup>-/-</sup> mice show significantly elevated nuclear NF- $\kappa$ B levels in thymocytes and splenocytes but only slightly elevated levels in brain and fibroblasts. NF- $\kappa$ B activation leads to up-regulation of certain target genes, including granulocyte colony stimulating factor (G-CSF). The appearance of phenotypic abnormalities in the I $\kappa$ B $\alpha$ <sup>-/-</sup> mice, which include elevated granulopoiesis and severe runting, are significantly delayed in the absence of p50. These results suggest strongly that constitutive nuclear NF- $\kappa$ B is the cause of abnormalities in I $\kappa$ B $\alpha$ <sup>-/-</sup> mice. I $\kappa$ B $\alpha$  is not necessary for the signal-dependent activation of NF- $\kappa$ B in embryonic fibroblasts but is required for the down-regulation of nuclear NF- $\kappa$ B after stimulation. Our results indicate that persistent NF- $\kappa$ B elevation can be lethal, and that I $\kappa$ B $\alpha$  plays the major role in hematopoietic cells by keeping NF- $\kappa$ B latent in the cytoplasm. I $\kappa$ B $\alpha$  also plays the more general role of rapidly repressing NF- $\kappa$ B once an inducing stimulus is terminated, assuring that NF- $\kappa$ B will only be present in the nucleus for a limited time.

## Results

### Generation of I $\kappa$ B $\alpha$ <sup>-/-</sup> mice

The murine I $\kappa$ B $\alpha$  gene was rendered dysfunctional in embryonic stem (ES) cells by homologous recombination with the gene-targeting vector pPNT-I $\kappa$ B $\alpha$ KO (Fig. 1A). The gene was replaced with the bacterial *lacZ* gene such that *lacZ* expression would be regulated by the I $\kappa$ B $\alpha$  promoter, although that capability is not studied here. ES

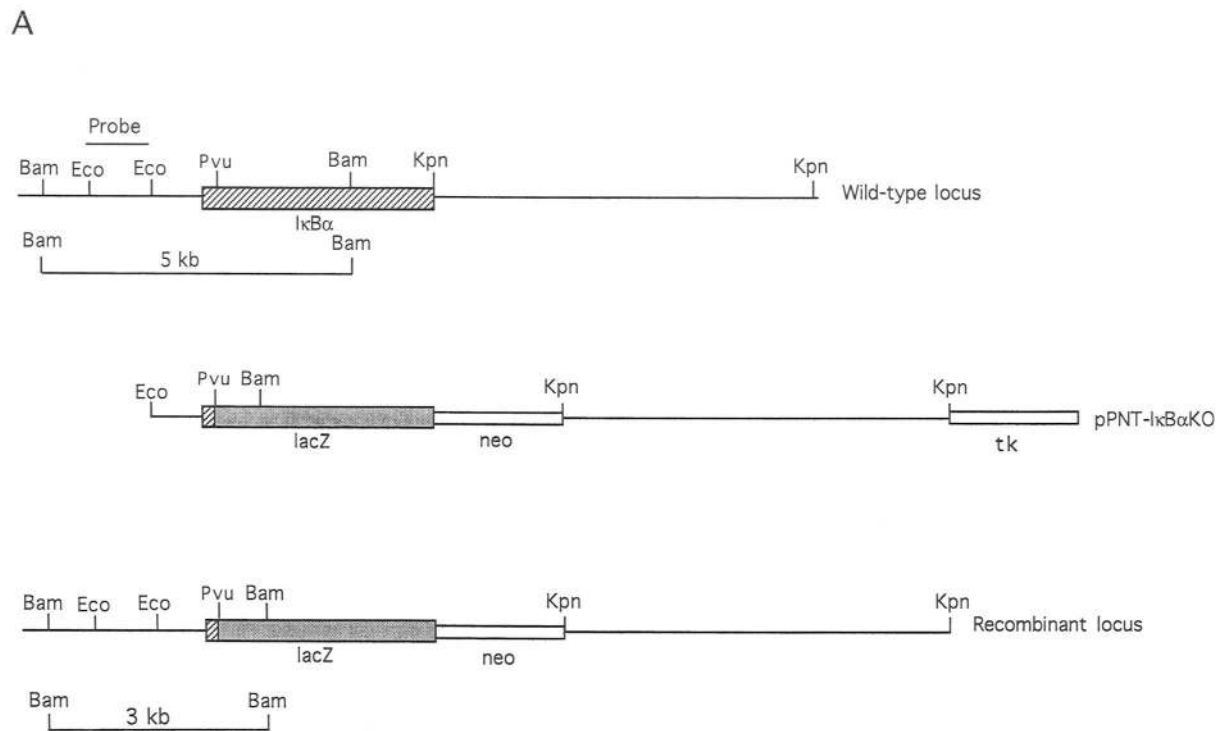
cell lines with a disrupted I $\kappa$ B $\alpha$  allele were injected into blastocysts to generate chimeric animals. Male mice from a clone that gave >70% chimerism transmitted the recombinant allele to their progeny. Heterozygous animals were indistinguishable from wild-type animals, and subsequently, were interbred to obtain homozygotes. A Southern blot analysis of the different genotypic categories is shown in Figure 1B.

### General phenotype

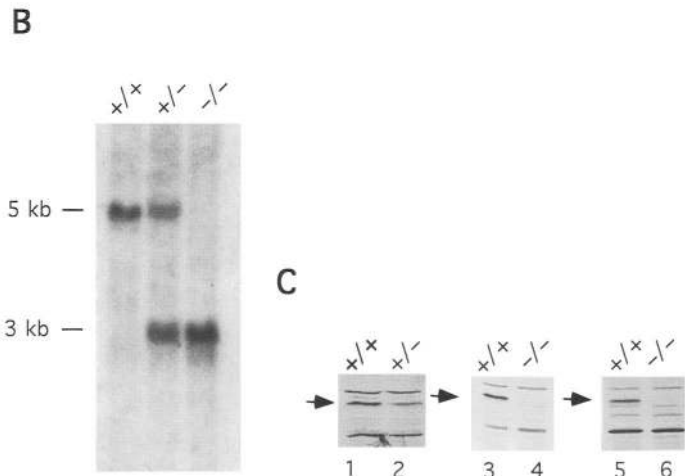
I $\kappa$ B $\alpha$ <sup>-/-</sup> mice were indistinguishable from wild-type or heterozygous mice at birth. In addition, they were present in expected Mendelian ratios suggesting no in utero lethality. Three to 4 days after birth, 25% of the pups appeared smaller than their littermates. By 7 days these pups were severely runted, typically being one-third normal weight, and died shortly thereafter. Genotyping of the runted and dead pups showed that they were I $\kappa$ B $\alpha$ <sup>-/-</sup>. The newborn I $\kappa$ B $\alpha$ <sup>-/-</sup> mice had normally formed internal organs. Thymocytes and splenocyte extracts obtained from these animals were analyzed by Western blotting using anti-I $\kappa$ B $\alpha$  serum. Heterozygous animals showed a reduction of I $\kappa$ B $\alpha$ , whereas homozygous animals showed the absence of I $\kappa$ B $\alpha$  (Fig. 1C). As early as 2 days after birth, the spleens of I $\kappa$ B $\alpha$ <sup>-/-</sup> mice appeared smaller than those of wild-type animals with a further reduction in spleen size until the time of death. Thymic atrophy, a well-documented response to stress, was also pronounced in I $\kappa$ B $\alpha$ <sup>-/-</sup> animals older than 6 days. At this stage, both the spleen and liver also appeared somewhat anemic.

Histologic analysis revealed no significant differences in internal organs between newborn I $\kappa$ B $\alpha$ <sup>-/-</sup> and control littermates. However, analysis of I $\kappa$ B $\alpha$ <sup>-/-</sup> pups older than 2 or 3 days revealed various abnormalities that became progressively more pronounced. Bone marrow and spleen sections displayed significantly elevated numbers of granulocytic precursors (Fig. 2A,B). Peripheral blood had a threefold rise in white blood cells (95% of which were neutrophils) and a significant reduction in the number of reticulocytes. However, red cell counts and hematocrits were not reduced in I $\kappa$ B $\alpha$ <sup>-/-</sup> mice. The skin of the I $\kappa$ B $\alpha$ <sup>-/-</sup> mice appeared scaly with significant sloughing and a poorly defined basal cell layer (Fig. 2C,D). Elevated numbers of granulocytes were present in skin sections and may contribute to the skin abnormalities (see Discussion). All other organs and tissues in the these animals were histologically unremarkable.

To analyze in more detail the myeloid and lymphoid lineages in the I $\kappa$ B $\alpha$ <sup>-/-</sup> animals, we carried out flow cytometric analysis on thymocyte and splenocyte populations (data not shown). At 3 days, a normal distribution of CD4/CD8 double-negative, double-positive, and single-positive cells was observed. Thy-1 and T-cell receptor (TCR) expression on thymocytes was also normal. However, a reduction in the proportion of double-positive cells was found in mice that were  $\geq$ 6 days, a likely consequence of depletion of the thymic cortex. Spleens had B220 and IgM-positive cells with both  $\kappa$  and  $\lambda$  light



**Figure 1.** Generation of  $I\kappa B\alpha^{-/-}$  mice. (A) Targeting of the  $I\kappa B\alpha$  gene. A map of the targeting vector is shown along with the wild-type and mutant locus. Restriction enzyme sites shown are abbreviated as Bam (*Bam*HI), Eco (*Eco*RI), Pvu (*Pvu*II), and Kpn (*Kpn*I). Homologous recombination of wild-type DNA with pPNT-IκBαKO results in the replacement of the  $I\kappa B\alpha$  gene with the bacterial *lacZ* gene and insertion of the PGK-*neo* gene 3' to *lacZ*. Southern blot analysis of *Bam*HI-digested DNA using the probe depicted above the wild-type locus was expected to yield either a 5-kb fragment for the wild-type locus or a 3-kb fragment for the targeted locus. (B) Southern blot analysis of tail DNA preparations from offspring of heterozygous matings. The three genotypic categories are indicated as wild type (+/+), heterozygote (+/-), and homozygote (-/-). The lengths of the *Bam*HI restriction fragments are also shown. (C) Western blot analysis of thymocyte and splenocyte extracts from mice of the three genotypic categories with anti-IκBα sera. (Lanes 1–4) Thymocytes; (lanes 5,6) splenocytes. The mobility of the IκBα protein is indicated by arrows.

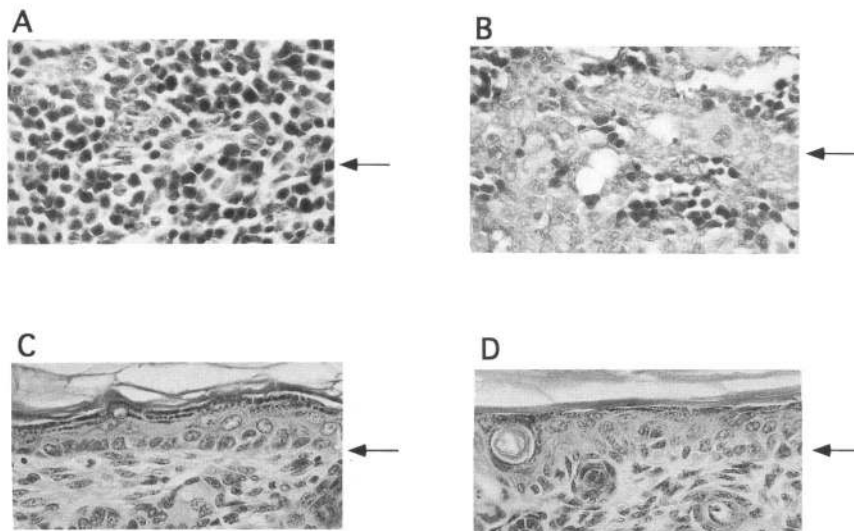


chains. Staining of splenocytes with the granulocyte-specific Gr-1 marker or the macrophage-specific Mac-1 marker showed that populations of both cell types were elevated, consistent with previous histological analysis. These results also suggest that the decrease in spleen size is caused by depletion of erythroid and/or lymphoid lineages but not of myeloid lineages. In any case, our results indicate that major hematopoietic lineages can form in the absence of  $I\kappa B\alpha$ .

#### Constitutive NF-κB activation in the absence of $I\kappa B\alpha$

Association with  $I\kappa B\alpha$  allows cytoplasmic retention of members of the NF-κB family leading to the expectation

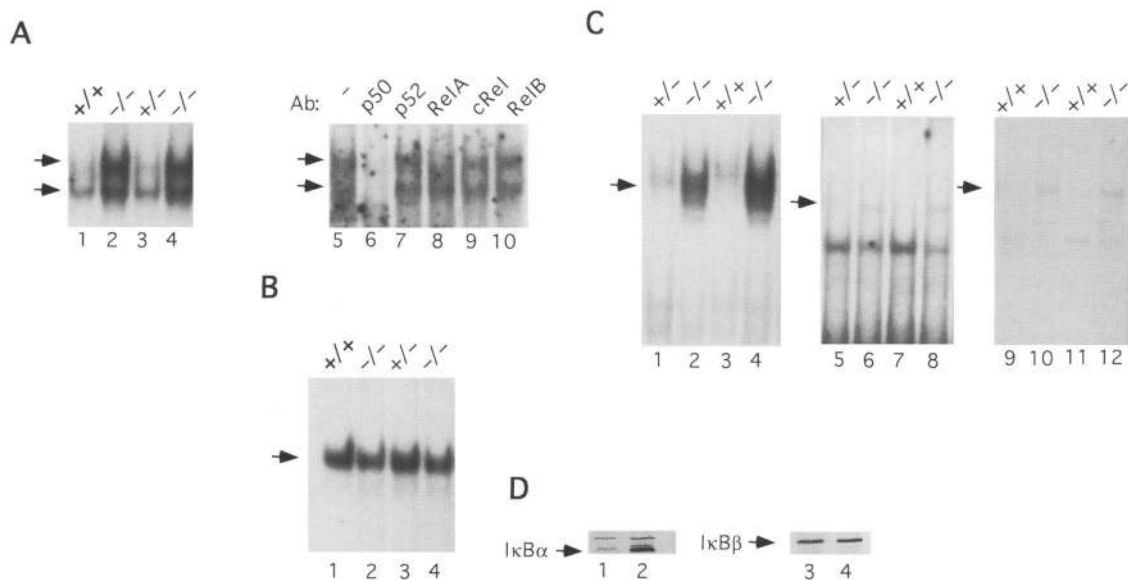
that constitutive nuclear NF-κB would be present in  $I\kappa B\alpha^{-/-}$  cells. Nuclear extracts obtained from 3-day-old thymocytes were analyzed by the electrophoretic mobility shift assay (EMSA) using an NF-κB-specific major histocompatibility complex (MHC) class I DNA probe. A dramatic increase in protein complexes with this DNA was evident in the nuclear extracts from  $I\kappa B\alpha^{-/-}$  cells compared to those from  $I\kappa B\alpha^{+/+}$  or  $I\kappa B\alpha^{+/-}$  cells (Fig. 3A, lanes 1–4). The slower migrating complex was reactive to both anti-p50 and anti-RelA sera, whereas the faster migrating complex was only reactive to anti-p50 sera (Fig. 3A, lanes 5–10). Thus, the elevated binding activity in  $I\kappa B\alpha^{-/-}$  thymocytes primarily consisted of



**Figure 2.** Histological analysis of  $\text{I}\kappa\text{B}\alpha^{-/-}$  mice. (A,B) Bone marrow sections through the sternum of 6-day-old (A) wild-type and (B)  $\text{I}\kappa\text{B}\alpha^{-/-}$  mice, stained with hematoxylin–eosin. Erythroid or lymphoid precursors are indicated by an arrow in A and granulocyte precursors are indicated by an arrow in B. (C,D) Skin sections of 6-day-old (C) wild-type and (D)  $\text{I}\kappa\text{B}\alpha^{-/-}$  mice. The basal cell layers are indicated by arrows.

p50 homodimers and p50/RelA heterodimers (NF- $\kappa$ B). As a control, no elevation of binding activity was observed when the nuclear extracts from  $\text{I}\kappa\text{B}\alpha^{-/-}$  mice were tested with an octamer-specific probe (Fig. 3B). Nuclear extracts from splenocytes also showed elevated  $\kappa$ B-binding activity (Fig. 3C, lanes 1–4) that was reactive to

sera specific for p50 and RelA. Some component of nuclear NF- $\kappa$ B in these splenocyte extracts may be contributed by the elevated proportion of macrophage and granulocyte precursors present in  $\text{I}\kappa\text{B}\alpha^{-/-}$  mice. In contrast to the hematopoietic organs, brain (Fig. 3C, lanes 5–8) and embryonic fibroblasts (Fig. 3C, lanes 9–12) had only



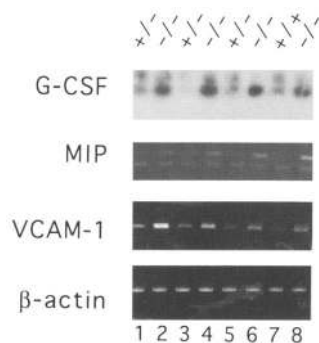
**Figure 3.** NF- $\kappa$ B levels in  $\text{I}\kappa\text{B}\alpha^{-/-}$  mice. (A) EMSA of thymocyte nuclear extracts obtained from 3-day-old mice. A radiolabeled MHC class I  $\kappa$ B hairpin oligonucleotide was used as a binding probe. The two major DNA–protein complexes are indicated by arrows. The genotypes of the mice from which the thymocytes were derived are indicated above lanes 1–4. For lanes 5–10, antibodies specific for various subunits of the NF- $\kappa$ B family were added to thymocyte extracts derived from  $\text{I}\kappa\text{B}\alpha^{-/-}$  mice. (B) EMSA of thymocyte nuclear extracts used in A with an octamer-specific probe. The genotypes of mice from which the thymocytes were derived are indicated above the lanes. (C) EMSA of nuclear extracts from splenocytes (lanes 1–4), brain (lanes 5–8), and embryonic fibroblasts with an MHC class I  $\kappa$ B probe (lanes 9–12). The mobilities of the inducible  $\kappa$ B-binding activities are indicated by arrows. The genotypes of the mice from which the extracts were derived are indicated above the lanes. (D) Western blot analysis of  $\text{I}\kappa\text{B}\alpha$  and  $\text{I}\kappa\text{B}\beta$  proteins. (Lanes 1,3) Embryonic fibroblast; (lanes 2,4) thymocyte cytoplasmic extracts from wild-type mice were analyzed using antisera against  $\text{I}\kappa\text{B}\alpha$  (lanes 1,2) or  $\text{I}\kappa\text{B}\beta$  (lanes 3,4). The mobilities of the two proteins are indicated.

slightly elevated constitutive NF- $\kappa$ B levels. These results demonstrate that the loss of I $\kappa$ B $\alpha$  causes constitutive NF- $\kappa$ B to be present in the nuclei of many cell types with a predominant effect in hematopoietic cells.

The difference between constitutive NF- $\kappa$ B levels in hematopoietic cells compared to others suggested that I $\kappa$ B $\alpha$  may play a variably important role in repressing NF- $\kappa$ B present in different cell types. To examine this issue, we determined the levels of I $\kappa$ B $\alpha$  in wild-type thymocyte and embryonic fibroblast cytoplasmic extracts. The same amount of extracted protein contained significantly lower levels of I $\kappa$ B $\alpha$  in embryonic fibroblasts than in thymocytes (Fig. 3D, lanes 1,2). In contrast, similar levels of I $\kappa$ B $\beta$  were present in both thymocyte and embryonic fibroblast cytoplasmic extracts (Fig. 3D, lanes 3,4). These results suggest that the contribution of I $\kappa$ B $\alpha$  to the regulation of NF- $\kappa$ B is cell-type dependent, I $\kappa$ B $\alpha$  being quantitatively more significant for the regulation of NF- $\kappa$ B in hematopoietic cells compared to nonhematopoietic cells.

#### Up-regulation of NF- $\kappa$ B responsive genes in I $\kappa$ B $\alpha$ <sup>-/-</sup> mice

A wide variety of genes have been proposed to be regulated by members of the NF- $\kappa$ B transcription factor family (for review, see Grilli et al. 1993; Baeuerle and Henkel 1994). Elevated NF- $\kappa$ B levels in cells from I $\kappa$ B $\alpha$ <sup>-/-</sup> animals suggested that NF- $\kappa$ B-responsive genes might also be up-regulated. We were interested particularly in genes that may cause phenotypic abnormalities in the I $\kappa$ B $\alpha$ <sup>-/-</sup> mice. Among the various genes that have been proposed to be regulated by NF- $\kappa$ B is that for G-CSF (Tsuchiya et al. 1986; Nishizawa and Nagata 1990), a key cytokine that stimulates the production of granulocytes. To determine mRNA levels of G-CSF, we used RNA isolated from thymocytes because they display high levels of nuclear NF- $\kappa$ B. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed significantly elevated levels of G-CSF mRNA in thymocytes from I $\kappa$ B $\alpha$ <sup>-/-</sup> animals (Fig. 4). A similar elevation



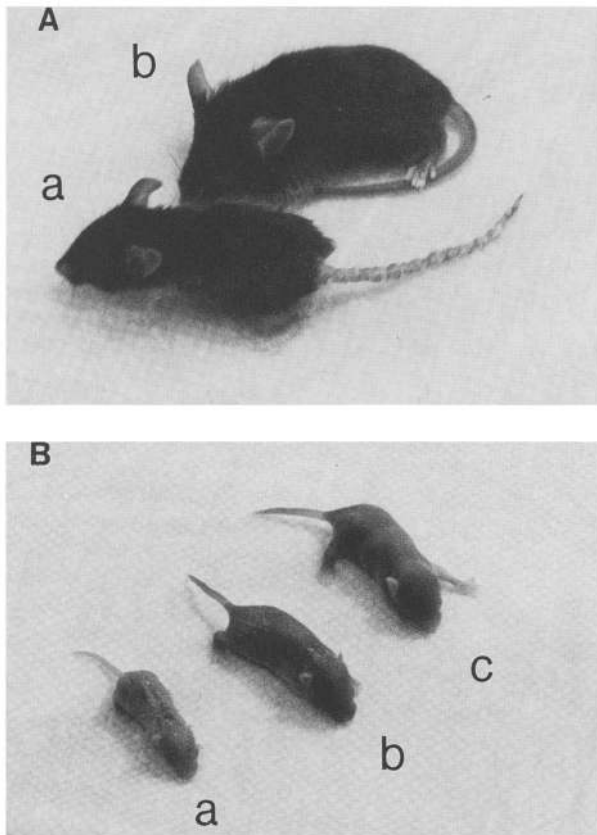
**Figure 4.** Analysis of mRNA levels of NF- $\kappa$ B responsive genes. RNA was isolated from 3-day-old (lanes 1–4) or 6-day-old (lanes 5–8) mice of different genotypes as indicated above the lanes. The RNA was analyzed by RT-PCR using primers specific for various genes, as indicated at left.

was seen in thymocytes from 3- or 6-day-old mice. Thus, elevated production of this cytokine may be responsible for the extensive granulopoiesis seen in the I $\kappa$ B $\alpha$ <sup>-/-</sup> animals.

The mRNA levels of other genes involved in the recruitment or adherence of granulocytes were examined using the PCR to amplify regions of the transcripts. The mRNA levels for the chemo-attractant murine macrophage inflammatory protein 2 (MIP-2) (Tekamp-Olson et al. 1990) (a mouse IL-8 homolog) and the adhesion molecule vascular cell adhesion molecule-1 (VCAM-1) (Araki et al. 1993) were clearly elevated (Fig. 4). Genes for both of these proteins contain NF- $\kappa$ B sites in their promoter regions and have been postulated to be regulated by NF- $\kappa$ B (Lademarco et al. 1992; Widmer et al. 1993). In contrast, the mRNAs of other genes thought to be regulated by NF- $\kappa$ B, including GM-CSF, IL-2, IL-2R $\alpha$ , IL-6, and *c-myc* (Baeuerle and Henkel 1994), were not detectably elevated in cells from I $\kappa$ B $\alpha$ <sup>-/-</sup> animals (data not shown). Thus, the presence of nuclear NF- $\kappa$ B is sufficient to up-regulate some but not all NF- $\kappa$ B responsive genes, underscoring the importance of other transcription factors in activating many NF- $\kappa$ B target genes.

#### Absence of p50 leads to prolonged survival of I $\kappa$ B $\alpha$ <sup>-/-</sup> mice

To examine the role of constitutive NF- $\kappa$ B in the neonatal lethality displayed by I $\kappa$ B $\alpha$ <sup>-/-</sup> mice, we initiated crosses with p50<sup>-/-</sup> mice to generate mice lacking both I $\kappa$ B $\alpha$  and the p50 subunit of NF- $\kappa$ B. Although p50<sup>-/-</sup> mice exhibit multifocal defects in immune responses, they develop normally (Sha et al. 1995), allowing us to assess the effects of the absence of p50 on the I $\kappa$ B $\alpha$  phenotype. In initial crosses between p50<sup>+/-</sup>I $\kappa$ B $\alpha$ <sup>+/-</sup> mice, 68 offspring were genotyped at weaning, at least 2 weeks after all I $\kappa$ B $\alpha$ <sup>-/-</sup> mice with p50 had died. Interestingly, 5 of the 68 offspring (7.4%) were phenotypically unusual, and were the only 5 offspring genotyped as p50<sup>-/-</sup>I $\kappa$ B $\alpha$ <sup>-/-</sup>. This result corresponds to the expected percentage of p50<sup>-/-</sup>I $\kappa$ B $\alpha$ <sup>-/-</sup> mice (1 of 13) if the absence of p50 was significantly prolonging the survival of I $\kappa$ B $\alpha$ <sup>-/-</sup> mice. Subsequent breeding experiments indicated that p50<sup>-/-</sup>I $\kappa$ B $\alpha$ <sup>-/-</sup> mice typically survived 2 to 4 weeks, with the majority dying shortly after weaning at 3 weeks. At the time of death, p50<sup>-/-</sup>I $\kappa$ B $\alpha$ <sup>-/-</sup> mice (Fig. 5A) exhibited many of the phenotypes that characterize the neonatal lethality of I $\kappa$ B $\alpha$ <sup>-/-</sup> mice. In addition to severe runting, bone marrow from p50<sup>-/-</sup>I $\kappa$ B $\alpha$ <sup>-/-</sup> mice displayed significantly elevated numbers of granulocyte precursors. Pronounced, but variable, sloughing and scaling of skin and fur was evident, with tail skin demonstrating marked hyperkeratinization (Fig. 5Aa). Spleen and thymus were also atrophied. These abnormalities were not evident in younger animals and became progressively more pronounced as p50<sup>-/-</sup>I $\kappa$ B $\alpha$ <sup>-/-</sup> mice aged. Between days 6 and 8 (Fig. 5B), when I $\kappa$ B $\alpha$ <sup>-/-</sup> mice typically died, p50<sup>-/-</sup>I $\kappa$ B $\alpha$ <sup>-/-</sup> mice were virtually indistinguishable from control littermates. Bone marrow and skin sections



**Figure 5.** Increased survival of  $p50^{-/-}I\kappa B\alpha^{-/-}$  mice. (A) Representative 25-day-old littermates whose genotypes were (a)  $p50^{-/-}I\kappa B\alpha^{-/-}$  and (b)  $p50^{-/-}I\kappa B\alpha^{+/-}$ . (B) Representative 6-day-old littermates whose genotypes were (a)  $p50^{+/+}I\kappa B\alpha^{-/-}$ , (b)  $p50^{-/-}I\kappa B\alpha^{-/-}$ , and (c)  $p50^{+/+}I\kappa B\alpha^{+/-}$ .

taken from  $p50^{-/-}I\kappa B\alpha^{-/-}$  mice during this early time period appeared histologically normal. These results indicate that absence of the p50 subunit of NF- $\kappa$ B appears to suppress, but not completely eliminate, the onset of the  $I\kappa B\alpha^{-/-}$  phenotype.

#### Absence of p50 significantly reduces constitutive NF- $\kappa$ B activity

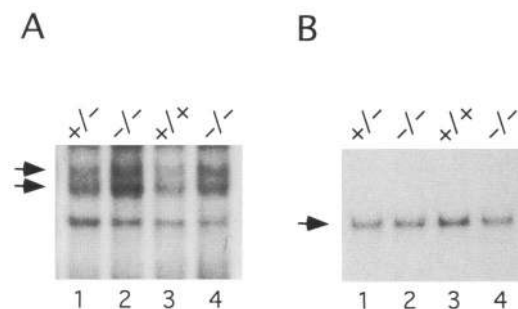
The markedly increased survival of  $p50^{-/-}I\kappa B\alpha^{-/-}$  mice suggested that constitutive activation of NF- $\kappa$ B could be the primary cause of the neonatal lethal phenotype, and that absence of p50-containing complexes lead to a reduction in constitutive NF- $\kappa$ B activity. To test this hypothesis, we determined the nuclear levels of NF- $\kappa$ B in cells from  $I\kappa B\alpha^{+/+}$ ,  $I\kappa B\alpha^{+/-}$ , or  $I\kappa B\alpha^{-/-}$  littermates lacking p50. In contrast to the results observed with  $p50^{+/+}I\kappa B\alpha^{-/-}$  mice (see Fig. 3A), only a minor increase in nuclear NF- $\kappa$ B from extracts prepared from 3-day-old thymocytes was seen in  $p50^{-/-}I\kappa B\alpha^{-/-}$  mice (Fig. 6A). Again, equivalent binding activity was observed with a control octamer-specific probe (Fig. 6B). The nuclear NF- $\kappa$ B complexes from  $p50^{-/-}I\kappa B\alpha^{-/-}$

mice were reactive primarily to anti-p52 and anti-RelB, but not to anti-RelA sera (data not shown). The lack of reactivity to anti-RelA sera may be because RelA is associated predominantly with p50 and thus, in the absence of p50, RelA DNA binding is also not detected. In any case, our results demonstrate that constitutive nuclear  $\kappa$ B-binding activity is greatly reduced in  $p50^{-/-}I\kappa B\alpha^{-/-}$  mice and implicate constitutive NF- $\kappa$ B as the primary cause of neonatal lethality in  $I\kappa B\alpha^{-/-}$  mice.

#### Signal-dependent NF- $\kappa$ B activation in $I\kappa B\alpha^{-/-}$ fibroblasts

Several studies have identified  $I\kappa B\alpha$  as a key target molecule that is phosphorylated and degraded rapidly after stimulation of cells with NF- $\kappa$ B inducers (for review, see Beg and Baldwin 1993). Free NF- $\kappa$ B translocates to the nucleus after  $I\kappa B\alpha$  loss and regulates various target genes. Furthermore,  $I\kappa B\beta$  has been described as not being degraded after exposure to certain inducers (Thompson et al. 1995). To determine whether NF- $\kappa$ B could be activated in the absence of  $I\kappa B\alpha$ , embryonic fibroblasts were treated with TNF $\alpha$  for different periods. A Western blot analysis showed that  $I\kappa B\alpha$  was degraded after TNF $\alpha$  stimulation in wild-type embryonic fibroblasts, consistent with previous studies on other cell types (data not shown). However, NF- $\kappa$ B was activated by TNF $\alpha$  in  $I\kappa B\alpha^{-/-}$  embryonic fibroblasts to levels similar to wild-type embryonic fibroblasts (Fig. 7A). Thus, degradation of  $I\kappa B\alpha$  after TNF $\alpha$  treatment cannot be solely responsible for NF- $\kappa$ B activation in embryonic fibroblasts. Stimulation of  $I\kappa B\alpha^{-/-}$  embryonic fibroblasts with phorbol, 12-myristate, 13-acetate (PMA) or LPS also showed NF- $\kappa$ B activation (Fig. 7B,C).

To determine the mechanism of NF- $\kappa$ B activation in the absence of  $I\kappa B\alpha$ , we examined the two other proteins



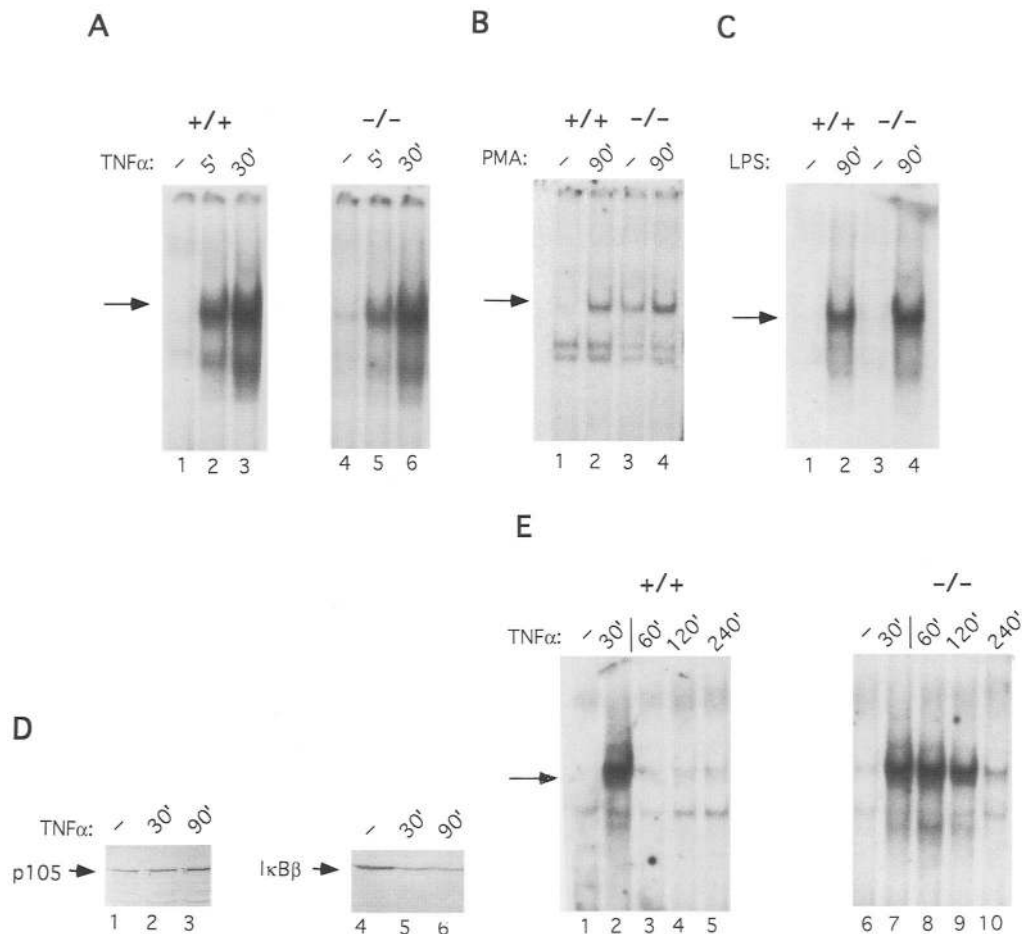
**Figure 6.** NF- $\kappa$ B levels in  $p50^{-/-}I\kappa B\alpha^{-/-}$  mice. (A) EMSA of thymocyte nuclear protein extracts obtained from 3-day-old mice. The  $I\kappa B\alpha$  genotypes of thymocytes from  $p50^{-/-}$  littermates derived from matings of  $p50^{-/-}I\kappa B\alpha^{+/-}$  mice are indicated above the lanes. A radiolabeled MHC class I  $\kappa$ B hairpin DNA oligonucleotide was used as binding probe. The major DNA-protein complexes supershifted with sera against NF- $\kappa$ B/Rel family members (data not shown) are indicated by arrows. (B) EMSA of same thymocyte nuclear extracts (lanes 1–4) used in A with an octamer-specific probe.

that might be responsive to activation:  $\text{I}\kappa\text{B}\beta$  and the p105 precursor of p50 (Fan and Maniatis 1991; Mercurio et al. 1993; Palombella et al. 1994; Thompson et al. 1995). A Western blot analysis of  $\text{I}\kappa\text{B}\alpha^{-/-}$  embryonic fibroblast extracts with anti-p105 sera showed no significant change in p105 levels after treatment of embryonic fibroblasts with  $\text{TNF}\alpha$  (Fig. 7D, lanes 1–3). However, in the presence of  $\text{TNF}\alpha$ , a significant decrease in the levels of  $\text{I}\kappa\text{B}\beta$  was evident in  $\text{I}\kappa\text{B}\alpha^{-/-}$  (Fig. 7D, lanes 4–6) or  $\text{I}\kappa\text{B}\alpha^{+/+}$  embryonic fibroblasts (not shown). Moreover, identical levels of  $\text{I}\kappa\text{B}\beta$  were detected in wild-type and  $\text{I}\kappa\text{B}\alpha^{-/-}$  embryonic fibroblasts, indicating that the absence of  $\text{I}\kappa\text{B}\alpha$  did not lead to altered regulation of  $\text{I}\kappa\text{B}\beta$  (data not shown). The unaltered level of  $\text{I}\kappa\text{B}\beta$ , the low amount of  $\text{I}\kappa\text{B}\alpha$  in wild-type cells, and the degradation of  $\text{I}\kappa\text{B}\beta$  after stimulation suggest that  $\text{I}\kappa\text{B}\beta$  is mainly re-

sponsible for signal-dependent NF- $\kappa\text{B}$  activation in embryonic fibroblasts and probably in many other nonhematopoietic cell types.

*I $\kappa\text{B}\alpha$  is essential for the postinduction repression of NF- $\kappa\text{B}$  activity*

Sustained NF- $\kappa\text{B}$  activation typically requires the constant presence of a stimulus, with the removal of an inducing agent leading to the disappearance of nuclear NF- $\kappa\text{B}$  (Hohmann et al. 1991). To determine whether  $\text{I}\kappa\text{B}\alpha$  was required for the post-stimulation repression of NF- $\kappa\text{B}$  activity, embryonic fibroblasts were treated with  $\text{TNF}\alpha$  for 30 min, after which cells were placed in medium without  $\text{TNF}\alpha$  for various periods. One hour after removal of  $\text{TNF}\alpha$ , wild-type cells showed a dramatic de-



**Figure 7.** Signal-dependent NF- $\kappa\text{B}$  activation and postinduction repression in embryonic fibroblasts. (A–C)  $\text{I}\kappa\text{B}\alpha^{+/+}$  or  $\text{I}\kappa\text{B}\alpha^{-/-}$  embryonic fibroblasts were treated with  $\text{TNF}\alpha$  (A), PMA (B), or LPS (C) for the indicated periods. Nuclear extracts were obtained after treatment of cells and tested for binding to an MHC class I  $\kappa\text{B}$  DNA probe. The mobilities of the major inducible binding activities are shown by arrows. (D) A Western blot analysis of p105 and  $\text{I}\kappa\text{B}\beta$  levels in embryonic fibroblasts. Embryonic fibroblasts were treated with  $\text{TNF}\alpha$  for the indicated periods after which cytoplasmic extracts were obtained and analyzed using p105 or  $\text{I}\kappa\text{B}\beta$ -specific antisera. The mobilities of the two proteins are indicated arrows. (E)  $\text{I}\kappa\text{B}\alpha^{+/+}$  or  $\text{I}\kappa\text{B}\alpha^{-/-}$  embryonic fibroblasts were treated with  $\text{TNF}\alpha$  for 30 min after which medium without  $\text{TNF}\alpha$  was added to the cells. Nuclear extracts were obtained after various periods after removal of  $\text{TNF}\alpha$  as indicated. NF- $\kappa\text{B}$  is shown by an arrow.

crease in nuclear NF- $\kappa$ B levels (Fig. 7E, lanes 1–5). However, I $\kappa$ B $\alpha$ <sup>-/-</sup> cells maintained high levels of nuclear NF- $\kappa$ B for  $\leq$ 4 hours (Fig. 7E, lanes 6–10). These results demonstrate that although I $\kappa$ B $\alpha$  is not necessary for activation of NF- $\kappa$ B in embryonic fibroblasts, it is necessary for postinduction repression, suggesting a general role for I $\kappa$ B $\alpha$  even in cell types where it is not a predominant species.

## Discussion

I $\kappa$ B $\alpha$ <sup>-/-</sup> mice display elevated levels of nuclear NF- $\kappa$ B in various cell types. Thus, the absence of I $\kappa$ B $\alpha$  is sufficient to cause nuclear translocation of NF- $\kappa$ B, and no other signal-dependent events than those leading to the degradation of I $\kappa$ B $\alpha$  are necessary for nuclear translocation of NF- $\kappa$ B. Nuclear NF- $\kappa$ B results in elevation of mRNA levels of only a subset of NF- $\kappa$ B-regulated genes suggesting that up-regulation of NF- $\kappa$ B levels may be sufficient to activate some but not all responsive genes. Thus, the signal-dependent induction of mRNAs of genes that contain  $\kappa$ B sites may also require the induction of other transcription factors. However, the results presented here suggest that the genes for G-CSF, MIP-2, and VCAM-1 can be activated transcriptionally in a non-signal dependent manner by simply increasing nuclear NF- $\kappa$ B levels. A consequence of the up-regulation of mRNAs of these and other genes may be the extensive granulopoiesis and skin abnormalities seen in I $\kappa$ B $\alpha$ <sup>-/-</sup> mice. Although thymocytes were used as a source for RNA in this study, other cell types such as macrophages or stromal cells could be more important for the production of these cytokines. The wide range of tissues that show NF- $\kappa$ B elevation suggest that most, if not all, cell types will have some degree of NF- $\kappa$ B activation.

Interestingly, dramatically different levels of NF- $\kappa$ B activation occur in particular cell types from the I $\kappa$ B $\alpha$ <sup>-/-</sup> mice. For example, thymocytes show an extensive dramatic elevation in nuclear NF- $\kappa$ B, whereas embryonic fibroblasts show only a slight increase. In addition, we find that higher levels of I $\kappa$ B $\alpha$  are normally present in thymocytes than in fibroblasts. Thus, the differences in nuclear NF- $\kappa$ B levels in individual I $\kappa$ B $\alpha$ <sup>-/-</sup> cell types probably reflects the constitutive presence in the nucleus of the portion of NF- $\kappa$ B that is ordinarily associated with I $\kappa$ B $\alpha$ . I $\kappa$ B $\alpha$  would then play a major role in the cytoplasmic retention of NF- $\kappa$ B in hematopoietic cells (thymocytes and splenocytes), but a minor role in nonhematopoietic cells (fibroblasts or brain). Increased levels of I $\kappa$ B $\alpha$  in hematopoietic cells may be caused by up-regulation of transcription of the I $\kappa$ B $\alpha$  gene because of the higher constitutive levels of nuclear NF- $\kappa$ B in those cells (Grilli et al. 1993; Scott et al. 1993; Sun et al. 1993; Baeuerle and Henkel 1994). In contrast, the levels of I $\kappa$ B $\beta$  are roughly equivalent in various cells because it is not regulated by NF- $\kappa$ B (Thompson et al. 1995).

### *Phenotypic abnormalities in I $\kappa$ B $\alpha$ <sup>-/-</sup> mice*

I $\kappa$ B $\alpha$ <sup>-/-</sup> mice display extensive granulopoiesis and skin

defects. Skin sections of I $\kappa$ B $\alpha$ <sup>-/-</sup> mice revealed the presence of a poorly defined basal cell layer, a possible consequence of rapid differentiation of these cells. However, it is presently unclear whether keratinocytes in I $\kappa$ B $\alpha$ <sup>-/-</sup> mice are themselves defective or exhibit abnormalities as a consequence of signals provided by other cells, perhaps those of lymphoid or myeloid lineages. Interestingly, several skin diseases in humans, including psoriasis, have been associated with abnormalities in hematopoietic cells. In this regard, I $\kappa$ B $\alpha$ <sup>-/-</sup> mice may provide a useful tool for understanding the interplay between hematopoietic cells and keratinocytes.

The elevated neutrophil counts in the peripheral blood of I $\kappa$ B $\alpha$ <sup>-/-</sup> animals are probably attributable to increased granulopoiesis in the bone marrow. Elevated production of cytokines such as G-CSF in I $\kappa$ B $\alpha$ <sup>-/-</sup> mice may lead to increased differentiation of hematopoietic stem cells along the granulocyte pathway. No evidence of bacterial infections were found in the I $\kappa$ B $\alpha$ <sup>-/-</sup> mice, suggesting that elevated granulopoiesis is not secondary to infection. Although the precise physiological defects that result in death of these animals remains unclear, the progressive runting suggests that constitutively elevated cytokines may be a precipitating cause.

### *Delayed onset of phenotypic abnormalities in p50<sup>-/-</sup>I $\kappa$ B $\alpha$ <sup>-/-</sup> mice*

The increased survival of mice deficient for both p50 and I $\kappa$ B $\alpha$  over mice deficient in I $\kappa$ B $\alpha$  alone, and the reduced levels of constitutive NF- $\kappa$ B activity in these mice, implicates constitutive nuclear NF- $\kappa$ B activity as the major cause of lethality in I $\kappa$ B $\alpha$ <sup>-/-</sup> mice. In contrast to other organismal systems, suppression of a mouse phenotype caused by mutation at one locus by elimination of a gene at another locus has rarely been shown previously. Crosses between mouse models with null mutations in related genes, such as the src protein tyrosine kinase (Lowell et al. 1994) or insulin-like growth factor family (Liu et al. 1993), typically have led to an augmented phenotype, a consequence of the elimination of compensation or redundancy of gene function by other family members. In a few instances, suppression of a mammalian phenotype has been reported, such as the reduction in lymphoproliferation in lpr mice lacking class I MHC (Maldonado et al. 1995). Interestingly, genetic background has been shown recently to dramatically affect the mutant phenotype of mice deficient in the keratin 8 gene (Baribault et al. 1994) and mice deficient in the epidermal growth factor (EGF) receptor (Sibilia and Wagner 1995; Threadgill et al. 1995), presumably because of the effect of unidentified background genes. Given the incomplete suppression of the I $\kappa$ B $\alpha$ <sup>-/-</sup> phenotype by a null p50 allele, it is likely that the appearance of phenotypic abnormalities in the I $\kappa$ B $\alpha$ <sup>-/-</sup> mice can be mediated by other NF- $\kappa$ B subunits. Further combinatorial analysis of mice deficient in multiple NF- $\kappa$ B/Rel and I $\kappa$ B family members will almost certainly provide further insight into the regulation and physiology of the NF- $\kappa$ B/Rel signal transduction pathway.



### Divergent functional roles for $I\kappa B\alpha$ and $I\kappa B\beta$ in fibroblasts

$I\kappa B\alpha$  has been thought to be the key regulator of signal-dependent NF- $\kappa B$  activation. However, our results demonstrate that  $I\kappa B\alpha$  is not essential for NF- $\kappa B$  induction in embryonic fibroblasts. In these cells,  $I\kappa B\beta$  is degraded specifically in response to TNF $\alpha$  suggesting that it may be primarily responsible for NF- $\kappa B$  activation in embryonic fibroblasts and perhaps other nonhematopoietic cell types. Previous studies have concluded that TNF $\alpha$  or PMA can lead to the degradation of  $I\kappa B\alpha$  but not  $I\kappa B\beta$ , suggesting that these proteins are targeted in a signal-dependent manner (Thompson et al. 1995). However, our results suggest that there may be little specificity for targeting particular forms of  $I\kappa B$  proteins in response to specific signals because both  $I\kappa B\alpha$  and  $I\kappa B\beta$  are degraded after TNF $\alpha$  treatment. The levels of different forms of  $I\kappa B$  proteins in different cell types may be the major determinant of which form plays a more significant role in NF- $\kappa B$  activation. The relatively low levels of  $I\kappa B\alpha$  protein present in embryonic fibroblasts, in contrast to thymocytes, suggest that  $I\kappa B\beta$  may be primarily responsible for the cytoplasmic retention and activation of NF- $\kappa B$  in those cells.

Although not required for the signal-dependent activation of NF- $\kappa B$  in embryonic fibroblasts,  $I\kappa B\alpha$  is necessary for its subsequent down-regulation. Thus,  $I\kappa B\alpha$  may play a more general role in the down-regulation of NF- $\kappa B$  and NF- $\kappa B$  responsive genes after induction in many cell types. In contrast to  $I\kappa B\beta$ ,  $I\kappa B\alpha$  mRNA synthesis is induced strongly in various cell types with stimulation of cells with NF- $\kappa B$  inducers (Thompson et al. 1995). In addition,  $I\kappa B\alpha$  is evident in nuclei after stimulation of cells with NF- $\kappa B$  inducers, suggesting that newly synthesized  $I\kappa B\alpha$  enters the nucleus and actively removes NF- $\kappa B$  from  $\kappa B$  sites (Arenzana-Seisdedos et al. 1995). The results presented here indicate divergent physiological roles for  $I\kappa B\alpha$  and  $I\kappa B\beta$ , with  $I\kappa B\beta$  being required for the activation of NF- $\kappa B$  and  $I\kappa B\alpha$  being necessary for its postinduction repression.

These studies have initiated an understanding of the relative roles of  $I\kappa B\alpha$  and  $I\kappa B\beta$  in control of NF- $\kappa B$ . NF- $\kappa B$  is thought to play a role in responses of cells and organisms to such events as DNA damage (Jung et al. 1995), oxidative stress, and infectious diseases. Further studies of genetically challenged mice should help elucidate the pathways involved in these responses.

### Materials and methods

#### Generation of $I\kappa B\alpha^{-/-}$ mice

The  $I\kappa B\alpha$  targeting vector was constructed using the pPNT plasmid (Tybulewicz et al. 1991). A human  $I\kappa B\alpha$  cDNA (Haskill et al. 1991) was used to isolate clones from a 129/Sv mouse liver genomic library (Stratagene). Murine  $I\kappa B\alpha$  genomic clones were restriction mapped using the murine  $I\kappa B\alpha$  cDNA sequence (provided by Dr. I. Verma, The Salk Institute, San Diego, CA). We were interested in determining the expression of  $I\kappa B\alpha$  during development. Thus, the  $I\kappa B\alpha$  gene was fused in-frame to the

bacterial *lacZ* gene (derived from the  $\beta$ AP-1 plasmid provided by Dr. J. Darnell, The Rockefeller University, New York) allowing colorimetric detection of  $I\kappa B\alpha$  expression. This was accomplished by ligation of a 1.2-kb *EcoRI*-*PvuII*  $I\kappa B\alpha$  genomic fragment to a *HindIII*-*EcoRI* *LacZ* fragment. The resulting construct contained ~1.2 kb of sequence upstream of the  $I\kappa B\alpha$  translational start site, 13 nucleotides (four amino acids) from the  $I\kappa B\alpha$  coding sequence, and the entire *lacZ* coding sequence joined in-frame to the  $I\kappa B\alpha$  coding sequence. The sequence of the junction was ATGTTTCAGCCAGTATCGATACCG, with the  $I\kappa B\alpha$  sequence underlined. Expression of this  $I\kappa B\alpha$ -*LacZ* fusion protein is readily detected in  $I\kappa B\alpha^{+/-}$  and  $I\kappa B\alpha^{-/-}$  mice. The  $I\kappa B\alpha$ -*lacZ* fragment was transferred to *Bam*HI-*Xba*I-cut pPNT in the opposite transcriptional orientation from the phosphoglycerokinase (PGK)-*neo* gene to generate the plasmid pPNT- $I\kappa B\alpha$ /*LacZ*. An ~7-kb region of homology, 3' to the  $I\kappa B\alpha$ -coding sequence was isolated as a *Kpn*I fragment from an  $I\kappa B\alpha$  genomic clone and inserted into the *Xho*I site of pPNT- $I\kappa B\alpha$ /*LacZ* to generate the  $I\kappa B\alpha$  targeting vector pPNT- $I\kappa B\alpha$ KO. The PGK-*tk* gene is 3' to the *Kpn*I fragment in pPNT- $I\kappa B\alpha$ KO. Homologous recombination of genomic DNA with pPNT- $I\kappa B\alpha$ KO, therefore, would result in the replacement of the  $I\kappa B\alpha$  gene with the bacterial *lacZ* gene.

pPNT- $I\kappa B\alpha$ KO was linearized at a unique *Kpn*I site and electroporated into J1 ES cells (Li et al. 1992). G418 and GANC-resistant colonies were isolated and genotyped by Southern blotting using an external DNA probe (Fig. 1A). Recombinant clones were injected into C57BL6/J blastocysts to obtain chimeric animals. Heterozygous animals were obtained when male chimeric mice displaying >70% coat color chimerism were bred to C57BL6/J females. Heterozygotes were interbred to obtain homozygotes.

#### Cell fractionation and Western blotting

Nuclear and cytoplasmic extracts were obtained as described previously (Beg et al. 1993). Embryonic fibroblasts were lysed in the presence of buffer containing 0.3% NP-40, whereas all other cell types were lysed in a 0.1% NP-40 buffer. Western blot analysis was carried out as described previously. The  $I\kappa B\alpha$  and  $I\kappa B\beta$  antibodies (Rice et al. 1992; Thompson et al. 1995) were provided by Dr. N. Rice and Dr. S. Ghosh, respectively. The p105 antibody has been described previously (Sha et al. 1995).

#### Histological and FACSscan analysis

Mouse tissues were fixed in buffered formalin for 48 hr and then embedded in paraffin. Sections of various tissues were stained with hematoxylin-eosin. FACSscan analysis was carried out as described previously using commercially available antibodies (Sha et al. 1995).

#### Cell culture and EMSA assays

Embryonic fibroblasts were grown in DMEM + 10% calf serum. Embryonic fibroblasts were stimulated in the presence of 10 ng/ml TNF $\alpha$  (GIBCO), 100 ng/ml of PMA (Sigma), or 10  $\mu$ g/ml of LPS (Sigma) for the time periods indicated in the text. Nuclear extracts containing equal amounts of protein were tested for binding to an NF- $\kappa B$ -specific MHC class I hairpin DNA probe (Fujita et al. 1992) or to an octamer-specific probe (Singh et al. 1986). Antibodies against various members of the NF- $\kappa B$  family used here have been described previously (Liou et al. 1994).

### RT-PCR analysis

RT-PCR was carried out as described previously (Beg et al. 1995). The sequence of the  $\beta$ -actin primers have been described. The sequence of other primers used here are: G-CSF (Tsuchiya et al. 1986) (1) 5'-CTGCCAGAGGCGCATGAAGC-3' (2) 5'-TGGCCAGCAACACAGCTCC-3'; VCAM-1 (Araki et al. 1993) (1) 5'-TTGGGAAGCCGGTACAGTCA-3' (2) 5'-GCCT-TGTGGAGGGATGTACAG-3'; MIP-2 (Tekamp-Olson et al. 1990) (1) 5'-CAGACTCCAGCCACACTTCAG-3' (2) 5'-CTT-TGGTTCTTCCGTTGAGGG-3'. RT-PCR assays were carried out in the linear range of the reactions using multiple isolates of tissues from mice.

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