

## Functional Redundancy of the Nuclear Factor $\kappa$ B Inhibitors $I\kappa B\alpha$ and $I\kappa B\beta$

By Janet D. Cheng,<sup>\*</sup> Rolf-Peter Ryseck,<sup>\*</sup> Ricardo M. Attar,<sup>\*</sup> Donna Dambach,<sup>†</sup> and Rodrigo Bravo<sup>\*</sup>

From the <sup>\*</sup>Department of Oncology and the <sup>†</sup>Department of Experimental Pathology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543-4000

### Summary

The transcription factor NF- $\kappa$ B is sequestered in the cytoplasm by the inhibitor proteins of the  $I\kappa B$  family. Each member of the  $I\kappa B$  exhibits structural and biochemical similarities as well as differences. In an effort to address the functional redundancy of two closely related  $I\kappa B$  molecules,  $I\kappa B\alpha$  and  $I\kappa B\beta$ , we generated knock-in mice by replacing the  $I\kappa B\alpha$  gene with the  $I\kappa B\beta$  gene. The knock-in mice do not express  $I\kappa B\alpha$ , but express a T7-tagged  $I\kappa B\beta$  under the promoter and regulatory sequence of *ikba*. Unlike the  $I\kappa B\alpha$ -deficient mice, which display severe postnatal developmental defects and die by postnatal day 8, homozygous knock-in mice survive to adulthood, are fertile, and exhibit no apparent abnormalities. Furthermore, thymocytes and embryonic fibroblasts from the knock-in animals exhibit an inducible NF- $\kappa$ B response similar to that of wild-type animals. These results indicate that  $I\kappa B\alpha$  and  $I\kappa B\beta$  share significant similarities in their biochemical activity, and that they acquired their different functions from divergent expression patterns during evolution.

Key words: nuclear factor  $\kappa$ B •  $I\kappa B$  • transgenic mice • knockout mice • hematopoiesis

Nuclear factor  $\kappa$ B (NF- $\kappa$ B)<sup>1</sup> plays an important role in regulating genes involved in inflammatory and immune responses. In vertebrates, NF- $\kappa$ B consists of homo- or heterodimers of the subunits p50, p52, RelA, RelB, and cRel. These subunits share a highly conserved NH<sub>2</sub>-terminal sequence termed the Rel-homology domain (RHD), which is required for DNA binding, dimerization, nuclear localization, and interaction with the inhibitor  $I\kappa B$  molecules (for review see references 1–5). In resting cells, NF- $\kappa$ B is sequestered in the cytoplasm by the inhibitor  $I\kappa B$ s. Cytoplasmic retention is achieved via interaction between a conserved sequence motif known as the ankyrin repeats in the  $I\kappa B$ s and the RHDs of NF- $\kappa$ B subunits. Upon cell stimulation by a variety of agents, specific serine residues on the  $I\kappa B$ s are phosphorylated, signaling for ubiquitination which in turn targets  $I\kappa B$ s for proteasome-mediated degradation. NF- $\kappa$ B released from the inhibitor translocates into the nucleus and activates transcription of target

genes, which include those involved in inflammatory, immune, and acute phase responses.

In mammalian cells, the  $I\kappa B$  family consists of  $I\kappa B\alpha$ ,  $I\kappa B\beta$ ,  $I\kappa B\epsilon$ , Bcl-3, p105, and p100 (for review see references 2–4). Knockout mice studies have revealed the critical roles that some of these  $I\kappa B$  molecules play in development and immune responses. For example, the knockout mice of  $I\kappa B\alpha$  have increased basal NF- $\kappa$ B activity in hematopoietic organs. Extensive granulopoiesis, dermatitis, and death by postnatal day 8–10 are observed in these mutant animals (5, 6). In contrast, p100-deficient mice displayed gastric hyperplasia and an impaired proliferative response in lymphocytes (7). Bcl-3-deficient mice develop normally but are incapable of antigen-specific antibody response (8, 9). These differences in phenotypes suggest that each  $I\kappa B$  family member plays a unique and nonredundant role in regulating NF- $\kappa$ B activity in the hematopoietic system.

Among the  $I\kappa B$  members,  $I\kappa B\alpha$  and  $I\kappa B\beta$  are the most prominent and have been extensively characterized.  $I\kappa B\alpha$  and  $I\kappa B\beta$  were first identified as two fractions from HeLa extracts that had similar kinetics of activities (10). In addition to the ankyrin motif,  $I\kappa B\alpha$  and  $I\kappa B\beta$  also have similar phosphoacceptor sites in their NH<sub>2</sub> termini that mediate signal-induced degradation and a similar specificity of in-

<sup>1</sup>Abbreviations used in this paper: EMSA, electrophoretic mobility shift assay; ES, embryonic stem; MEF, mouse embryo fibroblast, NF- $\kappa$ B, nuclear factor  $\kappa$ B.

teraction with the RelA and cRel complexes (11, 12). Given these similarities, it seemed surprising that I $\kappa$ B $\beta$  could not compensate for I $\kappa$ B $\alpha$  in the I $\kappa$ B $\alpha$ -deficient mice. Two potential explanations are: first, in spite of the similarities in structure, these two proteins have different *in vivo* biochemical activities and, therefore, they cannot replace each other's function. Alternatively, they are *in vivo* biochemically equivalent, but differential expression patterns have made them functionally incapable of compensating for one another. In an attempt to discriminate between these two possibilities, we have replaced I $\kappa$ B $\alpha$  with I $\kappa$ B $\beta$  using homologous recombination in embryonic stem cells in order to determine whether I $\kappa$ B $\alpha$  can be functionally replaced by I $\kappa$ B $\beta$ . The targeting event brought the integrated I $\kappa$ B $\beta$  gene under the control of the *ikba* promoter and at the same time introduced a null mutation in *ikba*. Here we report that the "knock-in" mice develop to adulthood without apparent abnormalities, are fertile, have no increase in their basal NF- $\kappa$ B activity, and can elicit NF- $\kappa$ B responses. These observations contrast strongly with those obtained with the I $\kappa$ B $\alpha$ -deficient mice, which present extensive granulopoiesis, dermatitis, and death by postnatal day 8–10 (5, 6). Thus, our data demonstrate that *in vivo* I $\kappa$ B $\alpha$  can be functionally replaced by I $\kappa$ B $\beta$ , and that these two molecules have acquired different functions by differential tissue pattern expressions and responses to NF- $\kappa$ B inducers.

## Materials and Methods

**Generation of the I $\kappa$ B $\alpha$  Knock-in Mice.** To generate mice with the replacement of I $\kappa$ B $\alpha$  by I $\kappa$ B $\beta$ , the targeting vector pPNT-abki was constructed using the pPNT vector (13). Murine *ikba* and *ikbb* genomic clones were used for vector construction. A 5' 4.7-kb NotI–SalI and a 3' 9.5-kb HindIII–NotI restriction fragment of *ikba* genomic sequence were cloned into the respective sides of the *neo* cassette in the pPNT targeting vector. A pair of oligonucleotide linkers containing the bacterial phage T7 Tag, and a 9.5-kb NcoI–SalI genomic restriction fragment containing the entire coding sequence of *ikbb*, were inserted downstream of the translation initiation of *ikba*. To ensure efficient translation initiation from the T7 Tag sequence, the endogenous translational initiation sequence of *ikbb* was deleted. Additionally, to ensure that the presence of the *pgk-neo* cassette would not interfere with transcription of the recombined *ikbb*, loxp sites were inserted flanking the 5' and 3' ends of the *pgk-neo* cassette to allow cyclic AMP responsive element-mediated excision of this marker. Since our studies (see below) indicated that transcription of the knock-in allele was not affected, the *pgk-neo* cassette was not removed subsequently. The resulting vector pPNT-abki was linearized by NotI and electroporated into murine CJ7 embryonic stem (ES) cells. Neomycin- and gancyclovir-resistant cells were selected and screened by Southern blot analysis using a 3' external probe of the *ikba* gene. DNA from a homologously recombined locus yields an 11-kb EcoRV fragment, whereas the wild-type DNA yields a 15-kb fragment. In addition, hybridization using several internal probes, including neomycin, confirmed that the entire genomic region of the I $\kappa$ B $\beta$  gene was integrated into the I $\kappa$ B $\alpha$  locus. Correctly targeted ES cells were injected into blastocysts or aggregated with morula of ICR mice. Male chimeras were mated with ICR females to obtain germline transmission of the mutated allele.

**Western Blot Analysis, Electrophoretic Mobility Shift Assay, and Immunoprecipitation.** Single cell suspensions of thymocytes or splenocytes were prepared from 4–6-wk-old mice according to standard procedures (14) in RPMI 1640 containing 10% heat-inactivated FCS. Cells were incubated with 20 ng/ml of PMA and 1  $\mu$ g/ml of PHA, or 5 ng/ml mouse TNF at 37°C for the indicated periods of time before harvest. Preparation of cytoplasmic and nuclear extracts was performed as previously described (15). For immunoprecipitation, a total of  $5 \times 10^5$  ES cells or  $6 \times 10^6$  thymocytes were incubated with 0.5 mCi/ml of [ $^{35}$ S]methionine in methionine-free DMEM containing 10% dialyzed FCS for 8 h at 37°C. Cells were lysed in radioimmunoprecipitation assay buffer and immunoprecipitation was performed as previously described (16). Western blot analysis using equal amounts of cytoplasmic extracts was carried out using standard protocols. For electrophoretic mobility shift assay (EMSA), nuclear extracts were tested for binding to the palindromic  $\kappa$ B-specific probe (17) or an octamer-specific probe (18).

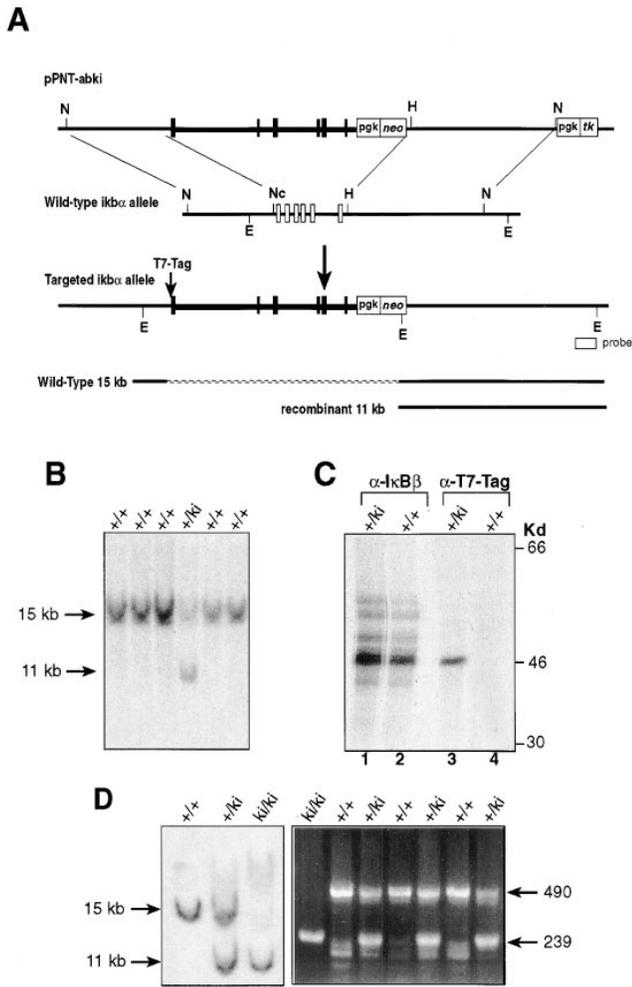
**Histology and Flow Cytometry Analysis.** Mouse tissues were immersion fixed in 10% buffered formalin and embedded in paraffin blocks. Sections were stained with hematoxylin and eosin. Flow cytometry analysis with single cell suspension from thymus, spleen, and bone marrow was performed using commercially available antibodies with a flow cytometer (Becton Dickinson, San Jose, CA).  $3 \times 10^5$  cells were first incubated with 1  $\mu$ g of the antibodies and then incubated with PE- or FITC-conjugated antibodies.

## Results

**I $\kappa$ B $\alpha$  Knock-in Mice.** The murine I $\kappa$ B $\alpha$  gene was replaced by the I $\kappa$ B $\beta$  gene in ES cells by homologous recombination with the gene-targeting vector pPNT-abki (Fig. 1 A). The targeting vector deleted the entire coding sequence of the I $\kappa$ B $\alpha$  gene and replaced it with the genomic sequence encoding the murine I $\kappa$ B $\beta$  gene. To distinguish the I $\kappa$ B $\beta$  introduced by homologous recombination from the endogenous I $\kappa$ B $\beta$ , a 34-bp nucleotide sequence encoding the bacterial phage T7-Tag was placed in front of the ATG start codon of the I $\kappa$ B $\beta$  gene in the targeting vector. This targeting vector was transfected into CJ7 ES cells, and ES cell clones carrying the replacement of the endogenous I $\kappa$ B $\alpha$  gene were identified by Southern blot analysis (Fig. 1 B). The resulting knock-in allele was abbreviated as +/ki or ki/ki for heterozygotes or homozygotes, respectively.

To ensure that the homologous recombination resulted in replacement of the I $\kappa$ B $\alpha$  gene with a functional I $\kappa$ B $\beta$  gene, an immunoprecipitation experiment was performed in the ES cells. Extracts from [ $^{35}$ S]methionine labeled wild-type (+/+) and recombined (+/ki) ES cells were immunoprecipitated with antiserum against I $\kappa$ B $\beta$  (Fig. 1 C, lanes 1 and 2), and then with antiserum against the T7-Tag epitope (Fig. 1 C, lanes 3 and 4). The T7-Tag antiserum precipitated a protein that is similar in molecular weight to the I $\kappa$ B $\beta$  protein in the recombinant ES cells but not in the wild-type cells, indicating that the introduced I $\kappa$ B $\beta$  was expressed and can be distinguished from the endogenous I $\kappa$ B $\beta$  using antibodies against the T7-Tag epitope.

**The Knock-in Animals Develop Normally and Present no Changes in NF- $\kappa$ B Activity.** Aggregation chimeras giving germline transmission were obtained from two targeted ES

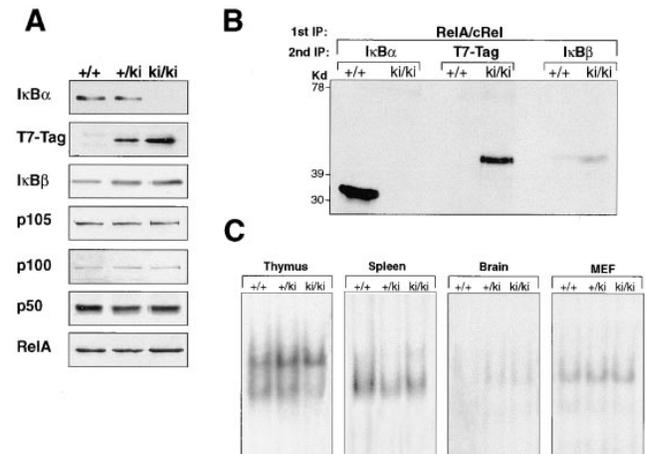


**Figure 1.** Targeted replacement of  $I\kappa B\alpha$  with the  $I\kappa B\beta$  gene. (A) The targeting vector pPNT-abki is shown at the top. Black boxes and thickened lines represent  $I\kappa B\beta$  exons and introns. Open boxes and thin lines represent  $I\kappa B\alpha$  exons and introns. Homologous recombination takes place between the 5' and 3' flanking regions of the *ikba* gene, resulting in replacement of the entire  $I\kappa B\alpha$  locus by  $I\kappa B\beta$ . Additionally, the ATG start codon of the  $I\kappa B\beta$  was also replaced with the T7-Tag sequence. N, NotI; Nc, NcoI; H, HindIII; E, EcoRV. (B) Southern blot analysis of DNA isolated from ES cell lines. EcoRV-digested DNA from cells that have undergone homologous recombination yielded an 11-kb signal when hybridized to an external probe. (C) Immunoprecipitation analysis of recombinant knock-in ES cells. Equal numbers of wild-type and +/ki ES cells were labeled with [ $^{35}$ S]methionine and immunoprecipitated with antiserum against  $I\kappa B\beta$  (lanes 1 and 2) or T7-Tag (lanes 3 and 4). (D) Analysis of homozygous mouse tail DNA. (Left) Southern blot analysis of wild-type, heterozygous, and homozygous mouse tail DNA; (right) PCR analysis of tail DNA using  $I\kappa B\alpha$  primers and neomycin primers. Amplification of  $I\kappa B\alpha$  yields a 490-bp PCR product, whereas amplification of the neomycin gene yields a 239-bp PCR product.

cell lines. Both lines of heterozygous animals (+/ki) were crossed to obtain homozygous mice (ki/ki). Homozygous ki/ki mice were born in normal Mendelian ratio, indicating that replacement of  $I\kappa B\alpha$  with  $I\kappa B\beta$  did not affect embryonic development (Fig. 1 D).

In contrast to the previously described  $I\kappa B\alpha$ -deficient mice, which have severe developmental defects and die by postnatal day 8 (5, 6), both lines of the ki/ki mice developed to adulthood. Examination of 5-wk-old ki/ki mice revealed that the phenotype was both grossly and histologically similar to that of wild-type mice. Additionally, peripheral blood cells and serum biochemical analysis were also similar to those from wild-type animals. In particular, there was no evidence of granulopoiesis or epidermal dysplasia with hyperkeratosis as reported in the  $I\kappa B\alpha$ -deficient mice (data not shown). The lack of granulopoiesis was further confirmed by flow cytometric analysis of bone marrow from wild-type and knock-in animals using the granulocyte/macrophage-specific surface markers Mac-1 and Gr-1. Additionally, a normal distribution of B and T lymphocyte markers (CD4, CD8, TCRab, Thy1.2, CD25, B220, and IgM) and erythroid marker (Ter119) was observed, indicating that the lymphoid and erythroid compartments of these animals are also normal (data not shown).

To determine if replacement of  $I\kappa B\alpha$  by  $I\kappa B\beta$  affected the overall patterns of  $I\kappa B$  and NF- $\kappa B$  gene expression, Western blot analysis was performed. Splenocytes from wild-type (+/+), heterozygous (+/ki), and homozygous (ki/ki) animals were analyzed. No differences in the steady-state levels of p105, p100, p50, and RelA were found between wild-type and knock-in splenocyte extracts (Fig. 2 A). As expected,  $I\kappa B\alpha$  protein was absent in the homozygous ki/ki animals, whereas the T7-Tag antibody detected a protein that migrated to the identical position as the  $I\kappa B\beta$  protein in +/ki



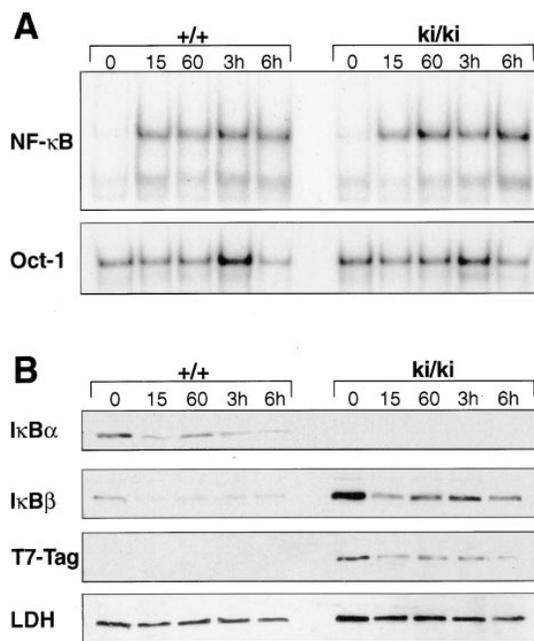
**Figure 2.** Expression of NF- $\kappa B$ / $I\kappa B$  proteins and NF- $\kappa B$  binding activity in the knock-in mice. (A) Western blot analysis of splenocytes from wild-type, heterozygous, and homozygous mice with antisera against members of the NF- $\kappa B$  and  $I\kappa B$  family. (B) Immunoprecipitation of cRel- and RelA-associated  $I\kappa B$  proteins. Homozygous (ki/ki) and wild-type (+/+) thymocytes were labeled with [ $^{35}$ S]methionine and immunoprecipitated with RelA and cRel antiserum in nondenaturing conditions. The immunoprecipitates were then denatured and sequentially reprecipitated with  $I\kappa B\alpha$ , T7-Tag, and  $I\kappa B\beta$  antisera. (C) EMSA for basal NF- $\kappa B$  activity in thymus, spleen, brain, and MEFs of the knock-in mice. Whole cell extracts were incubated with a palindromic  $\kappa B$ -specific probe.

and ki/ki splenocyte extracts. Consistent with the presence of extra I $\kappa$ B $\beta$  molecules in the heterozygous and homozygous animals, an increase was observed in the level of I $\kappa$ B $\beta$  protein in these animals compared with wild-type animals.

To demonstrate that the introduced I $\kappa$ B $\beta$  could complex with NF- $\kappa$ B subunits, coimmunoprecipitations were performed (Fig. 2 B). Wild-type and knock-in thymocytes were labeled with [<sup>35</sup>S]methionine and immunoprecipitated with antiserum against RelA and cRel under native conditions. The immunoprecipitates were denatured and then sequentially immunoprecipitated with I $\kappa$ B $\alpha$ , T7-Tag, and I $\kappa$ B $\beta$  antiserum. Fig. 2 B shows that in wild-type cells a large amount of I $\kappa$ B $\alpha$  was associated with RelA and/or cRel, whereas only a small amount of I $\kappa$ B $\beta$  was associated with these subunits. On the other hand, in homozygous knock-in cells, a large amount of T7-Tag-I $\kappa$ B $\beta$  was associated with RelA and/or cRel. The amount of T7-Tag-I $\kappa$ B $\beta$  in homozygous cells was comparable to that of I $\kappa$ B $\alpha$  in wild-type cells. This indicates that the I $\kappa$ B $\beta$  molecule introduced by homologous recombination was expressed at a similar level to I $\kappa$ B $\alpha$  in the wild-type animals, and that the introduced I $\kappa$ B $\beta$  is capable of interacting with the NF- $\kappa$ B subunits.

In I $\kappa$ B $\alpha$ -deficient animals, a prominent increase in the basal level of NF- $\kappa$ B activity was observed in spleen and thymus, emphasizing the importance of I $\kappa$ B $\alpha$  in controlling the basal NF- $\kappa$ B activity in hematopoietic organs (5, 6, 18). Thus, we determined the basal levels of NF- $\kappa$ B activity in thymus, spleen, brain, and embryonic fibroblasts from control and mutant mice using EMSA. Basal NF- $\kappa$ B activity in ki/ki animals remained relatively similar to that in the wild-type animals in all cell types examined (Fig. 2 C), indicating that the introduced I $\kappa$ B $\beta$  has replaced the role of I $\kappa$ B $\alpha$  in controlling basal levels of NF- $\kappa$ B activity. Furthermore, NF- $\kappa$ B activity in nonhematopoietic cells was not significantly altered, indicating that the presence of extra I $\kappa$ B $\beta$  molecules did not affect the basal machinery controlling NF- $\kappa$ B in these cells.

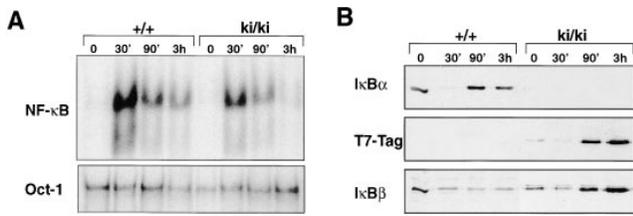
**Signal-dependent NF- $\kappa$ B Response in the Thymus.** A significant feature that distinguishes I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  is the inducibility of I $\kappa$ B $\alpha$  expression by NF- $\kappa$ B. After stimulation with NF- $\kappa$ B inducers, NF- $\kappa$ B accumulates in the nucleus and stimulates *ikba* transcription. Transcriptional stimulation of the I $\kappa$ B $\alpha$  gene is mediated by several  $\kappa$ B enhancer elements present in its 5' flanking region (19, 20) and leads to rapid accumulation of I $\kappa$ B $\alpha$  molecules, which in turn inhibit NF- $\kappa$ B response. It has been proposed that this regulatory loop is responsible for the transient induction of NF- $\kappa$ B activity. In contrast, the 5' upstream region of the I $\kappa$ B $\beta$  gene does not contain functional  $\kappa$ B enhancers, and its transcription is not regulated by NF- $\kappa$ B (11). This fundamental difference in regulation might be part of the functional differences that exist between I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ . Since the I $\kappa$ B $\beta$  gene introduced by homologous recombination was placed under the control of the *ikba* promoter, it should become inducible by NF- $\kappa$ B. To test this inducibility and assess the functional consequence of this induction, we stimulated thymocytes from wild-type and ki/ki animals with PMA and PHA and analyzed NF- $\kappa$ B activity by



**Figure 3.** Signal dependent NF- $\kappa$ B activation in ki/ki thymocytes. (A) NF- $\kappa$ B binding activity from wild-type (+/+) and ki/ki thymocytes stimulated with PMA/PHA. Cells were stimulated for the indicated periods of time and EMSA was performed using the palindromic  $\kappa$ B sequence with 5  $\mu$ g of nuclear extracts. Oct-1 binding activity was used as control. (B) Western blot analysis of +/+ and ki/ki thymocytes stimulated with PMA/PHA. Similar to I $\kappa$ B $\alpha$ , T7-Tag-I $\kappa$ B $\beta$  is induced and reaccumulates after NF- $\kappa$ B stimulation. 20  $\mu$ g of cytoplasmic extracts were used per lane and a sister blot was probed with antilactate dehydrogenase (LDH) antibodies as control for loading.

EMSA (Fig. 3 A). Similar to the wild-type thymocytes, ki/ki thymocytes exhibited a rapid increase in NF- $\kappa$ B activity 15 min after PMA/PHA stimulation. The increase persisted for 6 h in wild-type and ki/ki cells, indicating that the knock-in thymocytes were capable of eliciting signal-dependent NF- $\kappa$ B response. Western blot analysis revealed that, similar to I $\kappa$ B $\alpha$  in the wild-type, the T7-Tag-I $\kappa$ B $\beta$  in ki/ki thymocytes reaccumulated 60 min after stimulation. The time course of reappearance for T7-Tag-I $\kappa$ B $\beta$  is similar to that for I $\kappa$ B $\alpha$  in the wild-type, indicating that the I $\kappa$ B $\beta$  gene placed downstream of the I $\kappa$ B $\alpha$  promoter is inducible by NF- $\kappa$ B (Fig. 3 B). However, in spite of the increased level of I $\kappa$ B $\beta$  protein in basal condition and after induction, signal-dependent NF- $\kappa$ B response in the knock-in thymocytes remained relatively similar to the wild-type.

**Signal-dependent NF- $\kappa$ B Response in Mouse Embryo Fibroblasts.** A tissue-specific difference in function has been proposed between I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ . Based on the relative abundance of these two proteins, and on the fact that disruption of I $\kappa$ B $\alpha$  specifically affected NF- $\kappa$ B activity in hematopoietic cells, it has been postulated that I $\kappa$ B $\alpha$  plays a more important role in hematopoietic cells, whereas I $\kappa$ B $\beta$  is more important in nonhematopoietic cells (18). To assess the functional consequence of the replacement in nonhematopoietic cells, we tested NF- $\kappa$ B response in fibroblasts



**Figure 4.** Signal dependent NF- $\kappa$ B activation in ki/ki embryo fibroblasts. (A) NF- $\kappa$ B binding activity of wild-type (+/+) and ki/ki fibroblasts stimulated with TNF- $\alpha$  for the indicated periods of time. EMSA was performed using 5  $\mu$ g of nuclear extracts. Oct-1 binding was used in parallel as control. (B) Western blot analysis of +/+ and ki/ki fibroblasts stimulated with TNF- $\alpha$ . The T7-Tag-I $\kappa$ B $\beta$  reaccumulates in ki/ki fibroblasts after TNF- $\alpha$  stimulation. I $\kappa$ B $\alpha$ , T7-Tag-I $\kappa$ B $\beta$ , and I $\kappa$ B $\beta$  levels were determined on blots with 20  $\mu$ g of cytoplasmic extracts.

from 15-d-old embryos (Fig. 4). Wild-type and ki/ki primary mouse embryo fibroblasts (MEFs) were treated with TNF- $\alpha$  for various periods of time and harvested for EMSA and Western blot analysis. After 30 min of TNF- $\alpha$  treatment, NF- $\kappa$ B was activated in both ki/ki and wild-type MEFs (Fig. 4 A). The level of NF- $\kappa$ B induction was similar, indicating that the knock-in fibroblasts are capable of eliciting a normal NF- $\kappa$ B response. However, NF- $\kappa$ B activity in ki/ki cells diminished to near basal level 3 h after stimulation, whereas NF- $\kappa$ B activity in the wild-type remained prominent at the same time point. A Western blot analysis showed that the T7-Tag-I $\kappa$ B $\beta$  in ki/ki cells was induced after TNF- $\alpha$  stimulation, resulting in accumulation of a significantly higher amount of I $\kappa$ B $\beta$  in the knock-in cells than in the wild-type cells (Fig. 4 B). The time at which the T7-Tag-I $\kappa$ B $\beta$  accumulates dramatically in the cell correlates to the time point at which NF- $\kappa$ B activity becomes noticeably lower in the ki/ki fibroblasts.

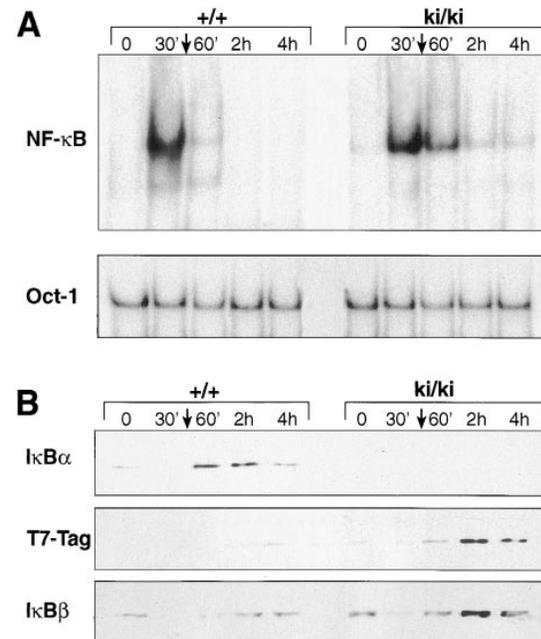
**I $\kappa$ B $\beta$  Is Capable of Postinduction Repression.** Studies using primary fibroblasts from I $\kappa$ B $\alpha$ -deficient embryos have demonstrated the importance of I $\kappa$ B $\alpha$  in postinduction repression. NF- $\kappa$ B activity in fibroblasts lacking I $\kappa$ B $\alpha$  persisted for >2 h after TNF- $\alpha$  removal, whereas NF- $\kappa$ B activity in wild-type fibroblasts quickly returned to basal level (within 30 min after stimulation; references 5, 6). To determine if the I $\kappa$ B $\beta$  introduced by homologous recombination was capable of inhibiting the NF- $\kappa$ B activity after induction, we performed postinduction experiments using the ki/ki fibroblasts (Fig. 5). Wild-type and ki/ki primary embryo fibroblasts were treated for 30 min with TNF- $\alpha$ , after which TNF- $\alpha$  was removed, and the cells were harvested 60, 120, and 240 min later. In the wild-type MEFs, NF- $\kappa$ B activity returned to basal levels nearly 60 min after TNF- $\alpha$  removal (Fig. 5 A). In ki/ki MEFs, the return of NF- $\kappa$ B to basal levels occurs on or after 120 min. A Western blot analysis revealed that, similar to I $\kappa$ B $\alpha$ , the T7-Tag-I $\kappa$ B $\beta$  was upregulated after induction. However, the time at which maximal T7-Tag-I $\kappa$ B $\beta$  accumulated was delayed compared to I $\kappa$ B $\alpha$  (60 min versus 2 h; Fig. 5 B). Peak T7-Tag-I $\kappa$ B $\beta$  accumulation corresponded to the time at which NF- $\kappa$ B activity diminished to basal level.

Therefore, in the context of the I $\kappa$ B $\alpha$  promoter, I $\kappa$ B $\beta$  can be induced by NF- $\kappa$ B and is competent in postinduction repression, although the time course of repression is slower than that of I $\kappa$ B $\alpha$  in the wild-type cells.

## Discussion

Using the knock-in approach, we have generated mice that carry a replaced I $\kappa$ B $\alpha$  gene. A T7-Tag-modified I $\kappa$ B $\beta$  gene was expressed under the promoter and the 5' regulatory sequence of the I $\kappa$ B $\alpha$  gene. As a result, the *ikba* allele was inactivated, and the introduced *ikbb* was expressed and regulated like *ikba*. This approach allowed us to investigate the functional similarity of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  under physiological conditions. The knock-in mice survived to adulthood and did not display any of the abnormalities that are present in the I $\kappa$ B $\alpha$ -deficient mice. Basal levels of NF- $\kappa$ B activity in the hematopoietic organs of the knock-in mice were not elevated like those in the I $\kappa$ B $\alpha$ -deficient mice. Furthermore, signal-induced NF- $\kappa$ B response in thymocytes was also normal. These results indicate that I $\kappa$ B $\beta$  can functionally compensate for the absence of I $\kappa$ B $\alpha$ .

The notion that I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  are biochemically equivalent is unexpected. Although many similarities exist between these two molecules, significant biochemical differences have also been reported. For example, I $\kappa$ B $\alpha$  degradation is induced by a wide variety of NF- $\kappa$ B inducers,



**Figure 5.** Postinduction repression of NF- $\kappa$ B in ki/ki embryo fibroblasts. (A) NF- $\kappa$ B binding activity of wild-type (+/+) and ki/ki fibroblasts treated with TNF- $\alpha$  for 30 min, after which cells were washed and medium without TNF- $\alpha$  was added to the cells for the indicated period. Nuclear extracts were prepared at the indicated periods after removal of TNF- $\alpha$ . A loading control using the Oct-1 probe was also performed. (B) Western blot analysis of +/+ and ki/ki fibroblasts treated with TNF- $\alpha$ . 20  $\mu$ g each of the total cell extracts were analyzed for levels of I $\kappa$ B $\alpha$ , T7-Tag-I $\kappa$ B $\beta$ , and the endogenous I $\kappa$ B $\beta$  proteins.

but only a subset of these inducers effects I $\kappa$ B $\beta$  degradation (11, 21). The kinetics of degradation is also different between the two molecules. In general, I $\kappa$ B $\alpha$  has a more rapid degradation than I $\kappa$ B $\beta$ . Differences in the basal phosphorylation state, degradation mechanism (21), and affinity to RelA (22) have also been shown for these two molecules. These observations formed the basis of hypotheses that the biochemical differences of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  contribute to functional differences for these two molecules.

One prominent functional difference that has been proposed for the I $\kappa$ Bs is the unique "chaperone" role of I $\kappa$ B $\beta$  (23). I $\kappa$ B $\beta$  molecules resynthesized after stimulation have been found to be hypophosphorylated. The hypophosphorylated I $\kappa$ B $\beta$  molecules can bind to NF- $\kappa$ B complexes, but do not mask the nuclear localization signals of NF- $\kappa$ B. Thus, they serve as chaperones for NF- $\kappa$ B complexes, protecting them from inhibition by the resynthesized I $\kappa$ B $\alpha$  molecules and, therefore, maintaining the persistent activation of NF- $\kappa$ B. However, recent reports have provided evidence against this model. For example, Miyamoto et al. have reported that inhibition of I $\kappa$ B $\beta$  degradation by proteasome inhibitors does not have any effect on the constitutive activity of NF- $\kappa$ B in WEHI cells (21). Prolonged nuclear localization of NF- $\kappa$ B complexes also is not necessarily associated with long-term depletion of I $\kappa$ B $\beta$  (24). Moreover, mice deficient in I $\kappa$ B $\beta$  have been also generated (Attar, R.M., manuscript in preparation). NF- $\kappa$ B responses elicited by various extracellular stimuli, along with various immune responses in these mice, are indistinguishable from those in wild-type mice, suggesting that persistent NF- $\kappa$ B activation can take place in the absence of I $\kappa$ B $\beta$ . Furthermore, NF- $\kappa$ B activity in thymocytes remained relatively unchanged in the knock-in mice, indicating that the resynthesized I $\kappa$ B $\beta$ s did not chaperone more NF- $\kappa$ B complexes into the nucleus, although a large excess of T7-Tag-I $\kappa$ B $\beta$  is produced after stimulation (Fig. 3). It is surprising that such a large amount of I $\kappa$ B $\beta$  did not affect NF- $\kappa$ B activity. It appears as if the NF- $\kappa$ B complexes in the knock-in cells are protected from the resynthesized T7-Tag-I $\kappa$ B $\beta$  just as the NF- $\kappa$ B complexes in the wild-type cells are protected from the resynthesized I $\kappa$ B $\alpha$ . Possibly, a separate mechanism exists that is independent of the phosphorylation status of I $\kappa$ B $\beta$  for maintaining the persistent activation of NF- $\kappa$ B.

In light of the fact that the mice lacking I $\kappa$ B $\alpha$  but expressing I $\kappa$ B $\beta$  controlled by the *ikba* promoter regulatory elements appear healthy, we conclude that the two I $\kappa$ B molecules are biochemically equivalent, and that certain biochemical features reported on I $\kappa$ B $\beta$ , such as hypophosphorylation or slow degradation, do not contribute significantly to the functional difference between them. We postulate that the difference in relative level of expression between these two molecules is a primary component of their functional difference. It has been reported that these two I $\kappa$ B molecules are expressed differently in different tissues: I $\kappa$ B $\alpha$  mRNA is highly abundant in the spleen, whereas I $\kappa$ B $\beta$  mRNA is mostly abundant in the testis (11). I $\kappa$ B $\alpha$  protein expression is also known to be higher in the

hematopoietic organs, whereas I $\kappa$ B $\beta$  protein is distributed equally between the hematopoietic and nonhematopoietic tissues (5). Consistent with these observations, we are able to immunoprecipitate a much larger amount of I $\kappa$ B $\alpha$  than I $\kappa$ B $\beta$  molecules in our thymocyte preparation (Fig. 2 B and data not shown). Additionally, our Western blots also show that the I $\kappa$ B $\beta$  introduced by homologous recombination is expressed at a higher level than the endogenous I $\kappa$ B $\beta$  molecules in thymocytes (Fig. 3 B). On the other hand, the relative increase in I $\kappa$ B $\beta$  level does not appear to be as high in the knock-in fibroblasts (Fig. 4 B). Although we can not rule out that the differences in stability may have contributed to some of the difference in steady state expression level, our observations suggest that the *ikba* promoter is more active in the hematopoietic system compared with nonhematopoietic systems.

In addition to tissue-specific differences in expression, the autoregulatory feature is also a fundamental difference between the two molecules. It has been proposed that NF- $\kappa$ B-inducible regulation of I $\kappa$ B $\alpha$  gene transcription is important for terminating NF- $\kappa$ B response in nonhematopoietic cells. By performing a postinduction repression experiment similar to that performed in the study of I $\kappa$ B $\alpha$ -deficient mice, we found that the T7-Tag-I $\kappa$ B $\beta$  molecule can serve as an inhibitor similar to I $\kappa$ B $\alpha$  in postinduction repression. This again supports the notion that I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  are biochemically equivalent, and that regulatory  $\kappa$ B enhancer elements upstream of the genes are crucial components of the functional differences between these I $\kappa$ Bs. A more recently identified member of the I $\kappa$ B family, I $\kappa$ B $\epsilon$ , also has this autoregulatory feature (25, 26). The fact that some, but not all, members of the I $\kappa$ B family share this property further implicates the importance of specific regulatory sequences in conferring different functions of these molecules. This auto-regulatory feature appears to be closely linked to tissue-specific difference in expression, as resynthesis of excess T7-Tag-I $\kappa$ B $\beta$  is associated with a different effect in fibroblasts than in thymocytes.

Despite the increase in understanding NF- $\kappa$ B function, the specificity and physiological relevance of the different Rel/NF- $\kappa$ B and I $\kappa$ B proteins remains unclear. The knock-outs and transgenic animals have provided important information on the physiological roles of the individual members (7, 27–35), but functional redundancy probably has masked the full importance of each member. Besides knowledge provided from single and double knockout studies, the knock-in approach provides a powerful tool for analysis of redundancy and the physiological function of individual members. The knock-in approach could be used to dissect the importance of specific domains or structures within a molecule in conferring specificity. Similar to this study, the knock-in approach has been used to reveal redundancy of two other transcription factor families, the Engrailed family (En-1 and En-2; reference 36), and the MyoD family (Myogenin and Myf-5; reference 37). In both cases, replacement of a similar member of the same family resulted in complete rescue of the phenotype from single knockout. Together, these data demonstrate that

many closely related members of a gene family acquire different functions in evolution through divergence of gene expression, rather than through divergence in biochemical function. Because overlapping gene function is likely to be

prevalent in mammals, such approaches are critical for clarifying the unique and overlapping function of members of a family, and for determining the complete repertoire of functions of individual genes.

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Address correspondence to Rodrigo Bravo, Department of Oncology, Bristol-Myers Squibb Pharmaceutical Research Company, PO Box 4000, Princeton, NJ 08543-4000. Phone: 609-252-5744; Fax: 609-252-6051; E-mail: bravo#m#\_rodrigo.prlvms1@msmail.bms.com

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