

## I $\kappa$ B $\alpha$ Deficiency Results in a Sustained NF- $\kappa$ B Response and Severe Widespread Dermatitis in Mice

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**The ubiquitous transcription factor NF- $\kappa$ B is an essential component in signal transduction pathways, in inflammation, and in the immune response. NF- $\kappa$ B is maintained in an inactive state in the cytoplasm by protein-protein interaction with I $\kappa$ B $\alpha$ . Upon stimulation, rapid degradation of I $\kappa$ B $\alpha$  allows nuclear translocation of NF- $\kappa$ B. To study the importance of I $\kappa$ B $\alpha$  in signal transduction, I $\kappa$ B $\alpha$ -deficient mice were derived by gene targeting. Cultured fibroblasts derived from I $\kappa$ B $\alpha$ -deficient embryos exhibit levels of NF- $\kappa$ B1, NF- $\kappa$ B2, RelA, c-Rel, and I $\kappa$ B $\beta$  similar to those of wild-type fibroblasts. A failure to increase nuclear levels of NF- $\kappa$ B indicates that cytoplasmic retention of NF- $\kappa$ B may be compensated for by other I $\kappa$ B proteins. Treatment of wild-type cells with tumor necrosis factor alpha (TNF- $\alpha$ ) resulted in rapid, transient nuclear localization of NF- $\kappa$ B. I $\kappa$ B $\alpha$ -deficient fibroblasts are also TNF- $\alpha$  responsive, but nuclear localization of NF- $\kappa$ B is prolonged, thus demonstrating that a major irreplaceable function of I $\kappa$ B $\alpha$  is termination of the NF- $\kappa$ B response. Consistent with these observations, and with I $\kappa$ B $\alpha$  and NF- $\kappa$ B's role in regulating inflammatory and immune responses, is the normal development of I $\kappa$ B $\alpha$ -deficient mice. However, growth ceases 3 days after birth and death usually occurs at 7 to 10 days of age. An increased percentage of monocytes/macrophages was detected in spleen cells taken from 5-, 7-, and 9-day-old pups. Death is accompanied by severe widespread dermatitis and increased levels of TNF- $\alpha$  mRNA in the skin.**

Members of the Rel transcription factor family regulate the expression of numerous genes, including many of those involved in immune and inflammatory responses (for reviews, see references 4, 22, and 54). The known mammalian Rel proteins—NF- $\kappa$ B1 (p50/p105), NF- $\kappa$ B2 (p52/p100), RelA (p65), c-Rel, and RelB—contain a conserved 300-amino-acid Rel-homology domain, which is involved in protein-protein interaction, DNA binding, and nuclear localization. Nearly all possible homodimers and heterodimers have been observed (classical NF- $\kappa$ B is a heterodimer of p50 and p65). These dimers are capable of binding to the  $\kappa$ B DNA binding site—a 10- or 11 bp sequence that conforms to a loose consensus. Rel proteins are expressed in most (perhaps all) cells, although their individual levels may vary with the cell type. With few exceptions, the various dimers are localized in the cytoplasm of unstimulated cells. A wide variety of agents (e.g., phorbol ester, lipopolysaccharide [LPS], and cytokines) induce the nuclear translocation of Rel proteins, initiating expression of numerous genes.

Rel dimers are held in the cytoplasm by members of the I $\kappa$ B family of inhibitor proteins, all of which contain five to seven copies of the “ankyrin repeat”—a 33-amino-acid motif that is particularly abundant in the protein ankyrin. The inhibitor

I $\kappa$ B $\alpha$  is capable of binding to most (perhaps all) dimers, thereby blocking their nuclear localization signals (6, 20, 34). Upon stimulation of the cell, I $\kappa$ B $\alpha$  undergoes phosphorylation and rapid degradation (5, 9, 16, 26, 41, 57), resulting in release of the dimer. In vitro I $\kappa$ B $\alpha$  is able to inhibit DNA binding by many (but not all) dimers, suggesting a similar role for I $\kappa$ B $\alpha$  in vivo (62, 63). The recent finding of nuclear I $\kappa$ B $\alpha$  in stimulated cells supports this hypothesis (2).

Like I $\kappa$ B $\alpha$ , the recently described I $\kappa$ B $\beta$  also binds to many dimers and undergoes phosphorylation and degradation upon stimulation (59). Compared with induction of degradation of I $\kappa$ B $\alpha$ , which responds to a wide variety of activating agents, induction of I $\kappa$ B $\beta$  degradation appears more specific. Whereas LPS and interleukin-1 (IL-1) treatment resulted in I $\kappa$ B $\beta$  degradation, phorbol myristate acetate and tumor necrosis factor alpha (TNF- $\alpha$ ) treatment did not. Also unlike I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  is a stable protein whose synthesis is not upregulated by NF- $\kappa$ B (see below).

Both p50 and p52 are synthesized as high-molecular-weight precursors (p105 and p100, respectively) (21, 31, 48) which are proteolytically processed to yield the mature N-terminal DNA-binding protein and an ankyrin repeat-containing C terminus that is rapidly degraded. These precursors are able to dimerize with other family members (38, 39, 42, 46). In such heterodimers, the long C-terminal portion of the precursor evidently blocks the nuclear localization signal of each subunit, resulting in cytoplasmic localization (8, 27, 37). Since processing of the precursors is relatively slow even in the presence of activating agents (19, 38), these dimers are not a major source of rapidly induced DNA binding activity. Finally, an alternately spliced form of p105, I $\kappa$ B $\gamma$ , which consists solely of the long C-terminal ankyrin repeat-containing domain, has been seen in

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some cells. I $\kappa$ B $\gamma$  is able to bind various dimers and retain them in the cytoplasm (29).

Rel/NF- $\kappa$ B dimers are regulated not only at the level of cytoplasmic retention but also at the level of synthesis. The genes encoding p105, p100, and c-Rel all contain  $\kappa$ B binding sites in their promoter regions, and stimulation of cells leads to increased synthesis of these proteins (14, 24, 25, 36, 58). However, synthesis of p65—the most potent activator of transcriptional activity—is not upregulated by Rel/NF- $\kappa$ B dimers. Just as p105, p100, and c-Rel are upregulated by NF- $\kappa$ B, so too is I $\kappa$ B $\alpha$ . Very soon after stimulation of cells, the level of I $\kappa$ B $\alpha$  mRNA increases dramatically, because of  $\kappa$ B binding sites in the promoter of the I $\kappa$ B $\alpha$  gene (9, 12, 13, 17, 30, 35, 49, 57). The I $\kappa$ B $\beta$  gene, on the other hand, is not upregulated by Rel/NF- $\kappa$ B (59).

Regulation of Rel proteins is clearly complex. Synthesis of some (but not all) family members is autoregulated, as is synthesis of some (but not all) of the inhibitors. The inhibitors respond rapidly (I $\kappa$ B $\alpha$  and in some cases I $\kappa$ B $\beta$ ) or more slowly (p105 and p100) to inducing agents, and they respond to a wide variety of agents (I $\kappa$ B $\alpha$ ) or to a more restricted subset (I $\kappa$ B $\beta$ ). To begin to dissect the key roles played by each of the inhibitors, we have generated mice deficient in the I $\kappa$ B $\alpha$  gene. Here we show that cytoplasmic retention and rapid nuclear translocation of NF- $\kappa$ B can occur in the absence of I $\kappa$ B $\alpha$  but that the absence of I $\kappa$ B $\alpha$  results in a sustained NF- $\kappa$ B response. I $\kappa$ B $\alpha$  deficiency in mice resulted in a severe and widespread form of dermatitis, histological alterations in the liver and spleen, an increased level of monocytes/macrophages, and death within 7 to 10 days after birth.

#### MATERIALS AND METHODS

**Construction of the I $\kappa$ B $\alpha$  gene-targeting vector and generation of I $\kappa$ B $\alpha$ -deficient mice.** A 5' 3.8-kb *Nco*I DNA fragment and a 3' 3.6-kb *Hind*III-*Xho*I DNA fragment of the I $\kappa$ B $\alpha$  gene were cloned into either side of the pGK-neo<sup>r</sup> expression cassette. The herpes simplex virus thymidine kinase expression cassette was inserted at the 3' end of the I $\kappa$ B $\alpha$  gene at an *Xho*I site, resulting in an I $\kappa$ B $\alpha$  gene-targeting vector designated pI $\kappa$ B-KO. The deleted portion of the I $\kappa$ B $\alpha$  gene contained exons encoding amino acids 13 to 317, which comprises the entire 3' portion of I $\kappa$ B $\alpha$ , including the ankyrin repeat domain. pI $\kappa$ B-KO was linearized, introduced into murine 129Sv embryonic stem (ES) cells (W9.5) by electroporation, and selected with neomycin and ganciclovir as described previously (33). ES cells containing a deleted I $\kappa$ B $\alpha$  allele were identified by Southern analysis of ES cell DNA using *Xho*I digestion and a flanking probe (Fig. 1, Probe A). The DNA fragment indicative of the homologous recombination event is 12 kb long, while the endogenous *Xho*I DNA fragment is 14 kb long. Chimeric mice were generated by microinjection of the ES cells with a deleted I $\kappa$ B $\alpha$  gene into C57BL/6 donor blastocysts and subsequent implantation into (C57BL/6 X CBA) F1 hybrid foster mothers (55). Once mouse lines were established, a simpler Southern screening method using *Hind*III digestion and an internal probe (Fig. 1, Probe B) was devised. With *Hind*III digestion, the diagnostic DNA fragment for the null allele is 4 kb long (labeled R in Fig. 1), while the endogenous *Hind*III DNA fragment is 5.5 kb long (labeled WT in Fig. 1).

**Establishment of PMEF cultures.** Primary mouse embryo fibroblast (PMEF) cultures were established from 13- to 15-day-old embryos as described previously (1), and their genotype with respect to the I $\kappa$ B $\alpha$  gene was determined by Southern analysis as described above.

**Preparation of cellular extracts for Western blotting (immunoblotting).** Whole-cell extracts were prepared by lysis in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 0.25 mM phenylmethylsulfonyl fluoride) followed by several passages of the lysate through a 25-gauge needle to shear the DNA. For nuclear and cytoplasmic cell fractions, PMEF cultures in 6-cm-diameter dishes were rinsed in ice-cold hypotonic buffer (HB; 25 mM Tris [pH 7.6], 1 mM MgCl<sub>2</sub>, 5 mM KCl) and lysed in 1 ml of HB containing 0.25% Nonidet P-40. The dishes were scraped to remove adherent cytoskeletal material and trapped nuclei. After vortexing, the lysate was centrifuged at 500  $\times$  g for 5 min; the supernatant constitutes the cytoplasmic fraction. After being washed once in 0.5 ml of HB, the nuclear pellet was resuspended in a solution containing 0.2 ml of 20 mM Tris (pH 8.0), 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, and 25% glycerol; vortexed; and then agitated vigorously at 4°C for 30 min. The extract was centrifuged at 900  $\times$  g for 5 min; the supernatant constitutes the nuclear extract. Protein concentrations were determined by using the Bio-Rad Protein Assay, with bovine serum albumin for standardization. Extracts

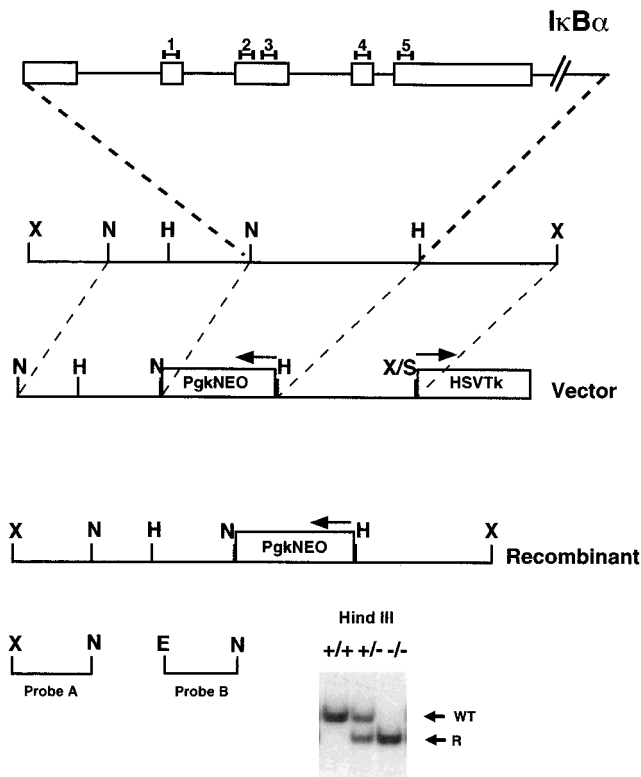


FIG. 1. Construction of the I $\kappa$ B $\alpha$  gene targeting vector. The second line represents a portion of the murine I $\kappa$ B $\alpha$  gene, and the top line expands the region between the indicated *Nco*I (N) and *Hind*III (H) sites. Boxes in the top line represent I $\kappa$ B $\alpha$  coding sequence, and regions encoding individual ankyrin repeats are numbered. The relevant portion of the targeting vector is shown in the third line, and the recombinant is shown in the fourth. Probe A was used in initial screening experiments. Later, screening was performed with *Hind*III digestion and probe B. Under these conditions, the wild-type allele gives a 5.5-kb fragment (WT), while the null allele gives a 4-kb fragment (R). X, *Xho*I; S, *Sal*I; E, *Eco*RV; NEO, neomycin expression cassette; HSVtk, herpes simplex virus thymidine kinase expression cassette; +/+, wild type; +/-, heterozygous for I $\kappa$ B $\alpha$ -null allele; -/-, homozygous for I $\kappa$ B $\alpha$ -null allele.

were fractionated on SDS-polyacrylamide (10 or 12%) gels and transferred to Immobilon-P or Amersham ECL membranes by semidry blotting or Trans-Blotting (Bio-Rad). Filters were blocked with 5% (wt/vol) dried milk in TBS (20 mM Tris [pH 7.6], 0.14 M NaCl, 0.1% Tween-20), probed with antisera (diluted 1:1,000 or 1:2,000 in milk-TBS), and analyzed by using the Amersham ECL system according to the manufacturer's protocol.

**Electrophoretic mobility shift assay.** Confluent T75 flasks were treated (or not treated) with TNF- $\alpha$  and lysed in HB plus 0.25% Nonidet P-40, as described above. Pelleted nuclei were extracted with a solution containing 50 mM HEPES (pH 7.5), 0.25 M NaCl, 5 mM EDTA, and 0.1% Nonidet P-40. Portions (5 to 10  $\mu$ l) of the nuclear extracts (60  $\mu$ l total) were tested for DNA-binding activity as previously described (18), by using a <sup>32</sup>P-labeled oligonucleotide containing the  $\kappa$ B site from the I $\kappa$ B gene. For supershift analysis, 1  $\mu$ l of antiserum was added to the extract before addition of the <sup>32</sup>P-labeled DNA.

**Antisera.** Antisera were raised in rabbits against synthetic peptides, as previously described (32, 41, 42). Antiserum 1157 was raised against an internal peptide (residues 339 to 357) in human p50, and antiserum 1263 was raised against residues 2 to 15 of murine p50. Antiserum 1495 was raised against residues 2 to 17 of murine p52. Antisera 1050, 1051, and 1266 were raised against murine c-Rel peptides (residues 438 to 455, 498 to 513, and 573 to 586, respectively). Antisera 1207 and 1226 were raised against human p65 peptides (residues 2 to 17 and 537 to 586, respectively). Antiserum 751 was raised against residues 2 to 16 of murine I $\kappa$ B $\alpha$ . Antiserum that recognizes I $\kappa$ B $\beta$  was a kind gift of Sankar Ghosh. Each serum is specific for a particular protein and does not cross-react with other family members.

**Histological and fluorescence-activated cell sorting (FACS) analysis of wild-type and homozygous I $\kappa$ B $\alpha$ -null pups.** Organs were isolated from 5-day-old pups, fixed, sectioned, and stained with hematoxylin and eosin. Skin sections were also stained with Gomori's methenamine-silver stain (which detects fungi),

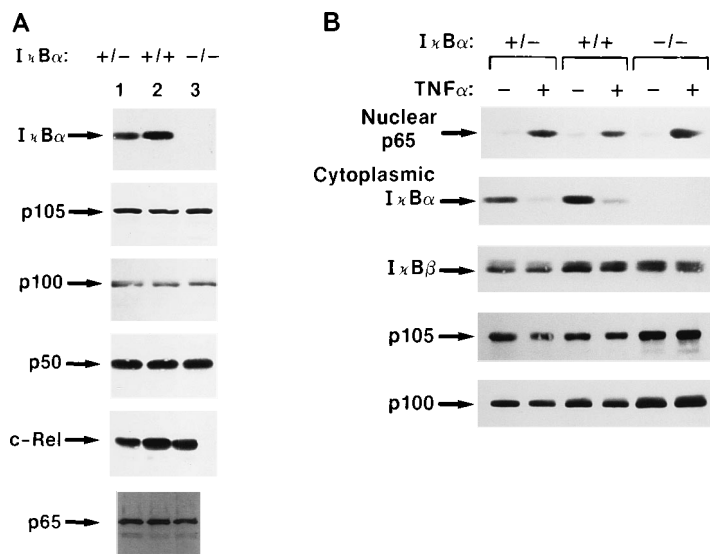


FIG. 2. There are normal levels of Rel family proteins in I $\kappa$ B $\alpha$ -deficient embryo fibroblasts. (A) Western analysis of untreated cells. Whole-cell extracts were fractionated by SDS-polyacrylamide gel electrophoresis and probed with anti-p50/p105 (antiserum 1157), anti-p52/p100 (antiserum 1495), anti-c-Rel (antiserum 1266), anti-p65 (antiserum 1226), or anti-I $\kappa$ B $\alpha$  (antiserum 751). PME $\alpha$  genotypes are indicated above the lanes (+/+, wild type; +/-, heterozygous; and -/-, homozygous for I $\kappa$ B $\alpha$ -null mutation). (B) Treatment of PME $\alpha$  cultures with TNF- $\alpha$ . Cultures were untreated (-) or treated with a combination of TNF- $\alpha$  (human recombinant TNF- $\alpha$  [4-ng/ml] from Boehringer Mannheim) and cycloheximide (10  $\mu$ g/ml) for 45 min (+). Nuclear and cytoplasmic extracts were fractionated by SDS-polyacrylamide gel electrophoresis, and immunoblots were probed with anti-p65 (antiserum 1226), anti-I $\kappa$ B $\alpha$  (antiserum 751), anti-I $\kappa$ B $\beta$  (a gift from Sankar Ghosh), anti-p105 (antiserum 1157), and anti-p100 (antiserum 1495).

Brown and Brenn stain (which detects gram positive and negative bacteria), and periodic acid-Schiff stain per standard procedures.

For immunohistochemistry, skin was removed from euthanized 5- to 6-day-old pups, snap-frozen in isobutanol chilled in liquid nitrogen, embedded by using Tissue-Tek O.C.T. compound (Miles, Inc., Elkhart, Ind.), and cut into 5- $\mu$ m-thick sections on a cryostat. Immunohistochemistry was performed by a VectaStain ABC kit (AK-5002 alkaline phosphatase; Vector Laboratories, Burlingame, Calif.) with keratin monoclonal antibodies (a gift from Birgit Lane) according to the manufacturer's protocol.

FACS analysis was performed on spleens removed from euthanized 5-, 7-, and 9-day-old pups. The splenocytes were dissociated in phosphate-buffered saline by being pipetted up and down through a serological pipette. Cells were analyzed by flow cytometry on a Becton-Dickinson FACSCAN device using the following panel of antibodies (Pharmingen): MAC-1(CD11b) (monocyte/macrophage marker), B220 (pan-B cell marker), Thy-1.2 (T cell marker), immunoglobulin M (B cell marker), immunoglobulin K (B cell marker),  $\alpha$  $\beta$ TCR; (T-cell marker), and NK-1.1 (natural killer cell marker).

**Determination of TNF- $\alpha$  mRNA levels in I $\kappa$ B $\alpha$  +/+, +/-, and -/- pup skin by using the RT-PCR.** Pup organs were homogenized in RNazol B (Cinna/Biotech) with a Polytron tissue homogenizer, and RNA was isolated per the manufacturer's (Cinna/Biotech) protocol. Reverse transcriptase PCRs (RT-PCRs) used 1  $\mu$ g of RNA, as described previously (51). This procedure utilizes an internal control consisting of a synthetic RNA containing primer sites and a poly(A) tail, such that the RT-PCR product from the internal control is different in size from that produced from the endogenous mRNA. The assay was also performed with  $\beta_2$ -microglobulin primers as a control for RNA quality. The resulting RT-PCR products were resolved on 5% (wt/vol) polyacrylamide (19:1 acrylamide/bis ratio) gels with TBE buffer (45), and the gels were dried and exposed to X-ray film.

## RESULTS

**Generation of mice deficient in I $\kappa$ B $\alpha$ .** The I $\kappa$ B $\alpha$  gene was disrupted in murine ES cells by homologous recombination with the targeting vector pI $\kappa$ B-KO, resulting in the replacement of most of the coding sequence for I $\kappa$ B $\alpha$  by a neomycin resistance expression cassette (Fig. 1). Recombinants were selected by antibiotic resistance and confirmed by Southern analysis of DNA. Selected ES cells were microinjected into blastocysts, which were implanted into foster mothers and gave rise to chimeric animals. These were bred with wild-type mice to generate heterozygotes, which were subsequently interbred to

produce single-litter progeny consisting of wild-type, heterozygous, and homozygous null I $\kappa$ B $\alpha$  genotypes.

**Levels of Rel and I $\kappa$ B proteins in murine embryo fibroblasts deficient in I $\kappa$ B $\alpha$ .** To assess the relative importance of I $\kappa$ B $\alpha$  in regulating the NF- $\kappa$ B signal transduction pathway, PME $\alpha$ s were isolated from I $\kappa$ B $\alpha$  wild-type (+/+), heterozygous (+/-), and homozygous null (-/-) 13- to 15-day embryos. If I $\kappa$ B $\alpha$  plays a crucial role in cytoplasmic retention of NF- $\kappa$ B, the null cells would be expected to have constitutive nuclear NF- $\kappa$ B, as well as increased levels of c-Rel, NF- $\kappa$ B1, and NF- $\kappa$ B2, as NF- $\kappa$ B upregulates synthesis of these three family members (14, 24, 25, 36, 58). However, constitutive NF- $\kappa$ B activity was not detected (see below), and Western analysis of whole-cell extracts (Fig. 2A) revealed no difference in steady-state levels of c-Rel, p105, p50, or p100 in all three cell types (p52 was not detected in any of the cells). Levels of p65 were also constant. As expected, I $\kappa$ B $\alpha$  was found in wild-type and heterozygous cells but not in the null cells. If the cytoplasmic role of I $\kappa$ B $\alpha$  is assumed by another inhibitor(s) in the null cells, an increase in its level might be detectable. However, as noted above, no significant increases in the amounts of p105 or p100 were noted (Fig. 2A), and the level of I $\kappa$ B $\beta$  was also unaffected (Fig. 2B).

To test directly whether the null cells contained increased levels of nuclear NF- $\kappa$ B, cell lysates were separated into nuclear and cytoplasmic fractions before immunoblotting. Little or no increase in the level of nuclear p65 in I $\kappa$ B $\alpha$ -deficient cells relative to that in the wild-type or heterozygous cells was detected (Fig. 2B, - lanes). Thus, loss of I $\kappa$ B $\alpha$  did not lead to any significant increase in nuclear p65 levels in unstimulated PME $\alpha$ s, and, just as in normal cells, nearly all p65 was cytoplasmic.

**Activation of NF- $\kappa$ B in I $\kappa$ B $\alpha$ -deficient fibroblasts.** To determine if I $\kappa$ B $\alpha$ -deficient cells could respond to activators of NF- $\kappa$ B, TNF- $\alpha$  was employed as an agonist. It has been reported that I $\kappa$ B $\beta$  is unaffected by TNF- $\alpha$  treatment in 70Z/3



pre-B cells and in Jurkat T cells (59). Cells were exposed to TNF- $\alpha$  and cycloheximide for 45 min, separated into nuclear and cytoplasmic fractions, and analyzed by immunoblotting. Cycloheximide was included in order to prevent synthesis of new inhibitor molecules, thus accentuating any effect of TNF- $\alpha$  on degradation of preexisting inhibitors. The null cells were clearly responsive to TNF- $\alpha$  stimulation, as the amount of nuclear p65 increased to a level comparable to that seen in the wild-type and heterozygous cells (Fig. 2B, + lanes). It is unclear, however, which cytoplasmic inhibitor released the p65. The levels of p105, p100, and I $\kappa$ B $\beta$  did not appear to change during the 45 min of TNF- $\alpha$  treatment, whereas the I $\kappa$ B $\alpha$  levels in the wild-type and heterozygous cells declined dramatically, as expected. I $\kappa$ B $\beta$  was also unaffected after 2 h of treatment (data not shown). Thus, the nuclear p65 must originate from only a small fraction of the I $\kappa$ B $\beta$ , p105, and/or p100 complexes or from complexes with an unknown inhibitor.

These results show that I $\kappa$ B $\alpha$ -deficient PMEFs have normal levels of NF- $\kappa$ B proteins, do not contain constitutive nuclear p65, and do respond to TNF- $\alpha$  treatment. At least in these cells, therefore, I $\kappa$ B $\alpha$ 's role in cytoplasmic retention and release of NF- $\kappa$ B can be assumed by other factors. We next examined whether I $\kappa$ B $\alpha$  might play a role in terminating an NF- $\kappa$ B response. NIH 3T3 cells were used as a test system to monitor the level of nuclear p65 following removal of TNF- $\alpha$  from the medium. We found that the level of nuclear p65 reverted to that seen in unstimulated cells within 30 min after removal of TNF- $\alpha$  (Fig. 3A, lane 3). However, when the cells were treated with cycloheximide during the chase period, the amount of nuclear p65 did not decrease to an unstimulated level (lane 8) but remained elevated. Thus, protein synthesis is required for termination of an NF- $\kappa$ B response, as shown previously (3, 47).

To determine whether I $\kappa$ B $\alpha$  synthesis is required to extinguish the NF- $\kappa$ B response, the same experiment was performed with wild-type and null PMEFs. Cells were treated with TNF- $\alpha$  for 30 min and then incubated in medium without TNF- $\alpha$  for 30 or 60 min. As in the NIH 3T3 cells, amounts of nuclear p65 in the wild-type cells returned to unstimulated levels within 30 min after removal of TNF- $\alpha$  (Fig. 3B, lane 3). In contrast, no reduction in the nuclear p65 level was seen in the null cells at 30 or 60 min (lanes 7 and 8). In a subsequent experiment, the level of nuclear p65 remained elevated at 2 h following removal of TNF- $\alpha$  (data not shown). These experiments were repeated with a DNA-binding assay in order to show that the nuclear p65 detectable by immunoblotting was functional. TNF- $\alpha$  induced nuclear DNA-binding activity in both the wild-type and null cells (Fig. 3C, lanes 2 and 5). Reactivity with antisera directed toward p50 (lane 8) and toward p65 (lane 9) demonstrated that this binding activity consisted of Rel-NF- $\kappa$ B proteins. As in the immunoblotting experiments, the activity declined dramatically in the wild-type cells within 30 min after removal of TNF- $\alpha$  (lane 3). In contrast, there was only a modest reduction in DNA-binding activity in the null cells (lane 6). These results indicate that I $\kappa$ B $\alpha$  plays a crucial role in regulating nuclear levels of NF- $\kappa$ B following withdrawal of an inductive stimulus.

**Pathology of I $\kappa$ B $\alpha$ -deficient mice.** I $\kappa$ B $\alpha$ -homozygous null pups are overtly normal at birth, and their growth and development are indistinguishable from those of their wild-type and heterozygous littermates for the first 3 days. Thereafter the I $\kappa$ B $\alpha$ -deficient pups stop gaining weight, and by 6 days after birth they weigh approximately one-third as much as their littermates. The pups develop a dry flaky skin, and failure to thrive proceeds inexorably to death, usually within 7 to 10 days after birth. Necropsy and histological analysis failed to reveal

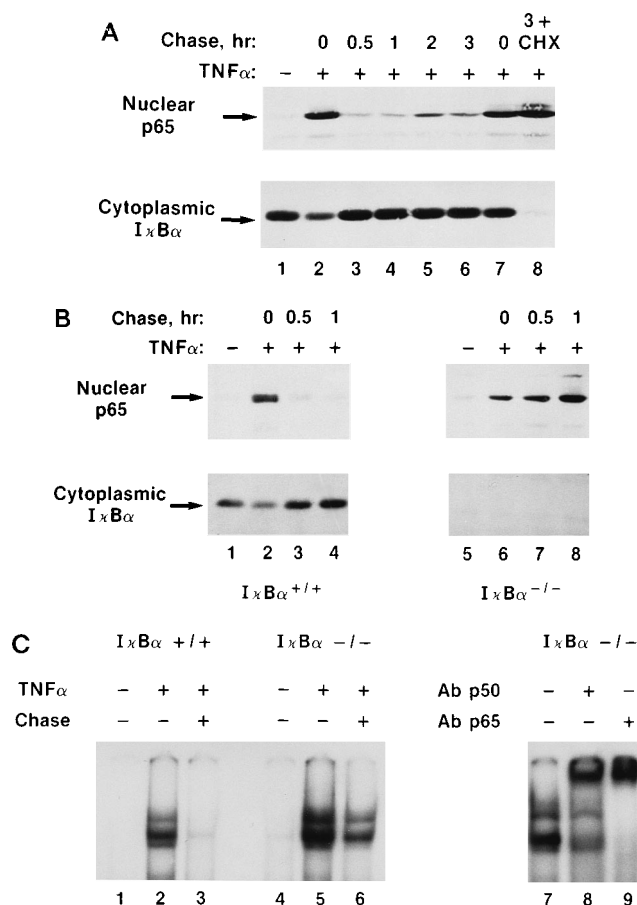


FIG. 3. The effect on nuclear p65 of removing TNF- $\alpha$ . (A) Nuclear p65 in NIH 3T3 cells. Cells in 6-cm-diameter dishes were untreated (-) or treated (+) with TNF- $\alpha$  (4 ng/ml) for 30 min (lanes 2 to 6 and 8) or 3.5 h (lane 7). The TNF- $\alpha$ -containing medium was then removed and replaced with medium without TNF- $\alpha$ , and cells were incubated for the indicated times (chase). The culture analyzed for lane 8 contained cycloheximide (CHX; 10  $\mu$ g/ml) during the chase period. Nuclear and cytoplasmic extracts were analyzed by immunoblotting with anti-p65 (antisera 1226) and anti-I $\kappa$ B $\alpha$  (antisera 751). (B) Nuclear p65 in wild-type and I $\kappa$ B $\alpha$  -/- PMEFs. Cells in 6-cm-diameter dishes were untreated (-) or treated (+) with TNF- $\alpha$  for 30 min and then chased in fresh medium for 30 or 60 min, as indicated. Nuclear and cytoplasmic extracts were analyzed by immunoblotting, as for panel A. (C) Nuclear DNA-binding activity in wild-type and I $\kappa$ B $\alpha$  -/- PMEFs. Cells were untreated or treated with TNF- $\alpha$  for 30 min, as indicated, and then harvested or chased in fresh medium for 30 min. Nuclear extracts were analyzed for DNA-binding activity (lanes 1 to 7). For lanes 8 and 9, anti-p50 (antisera 1263) and anti-p65 (antisera 1207), respectively, were added to the extract prior to incubation with the  $^{32}$ P-labeled  $\kappa$ B oligonucleotide. Ab, antibody.

developmental abnormalities and confirmed that the mice were able to suckle and apparently digest milk in a normal manner. Brown fat deposits appeared normal for the size of the animal. Blood smears did not reveal significant differences from wild-type counterparts, suggesting normal hematopoiesis (data not shown).

A major histological alteration was detected in the skin, with more subtle changes in the liver and spleen. A striking widespread dermatitis was consistently observed in skin sampled from various sites and was characterized by marked acanthosis (epidermal hyperplasia), hyperkeratosis, an absence of keratohyalin granules in the stratum granulosum, and the presence of numerous small to multiple and coalescing subcorneal and intracorneal neutrophilic microabscesses (Fig. 4). A diffuse epithelial transmigration of neutrophils was seen, and the

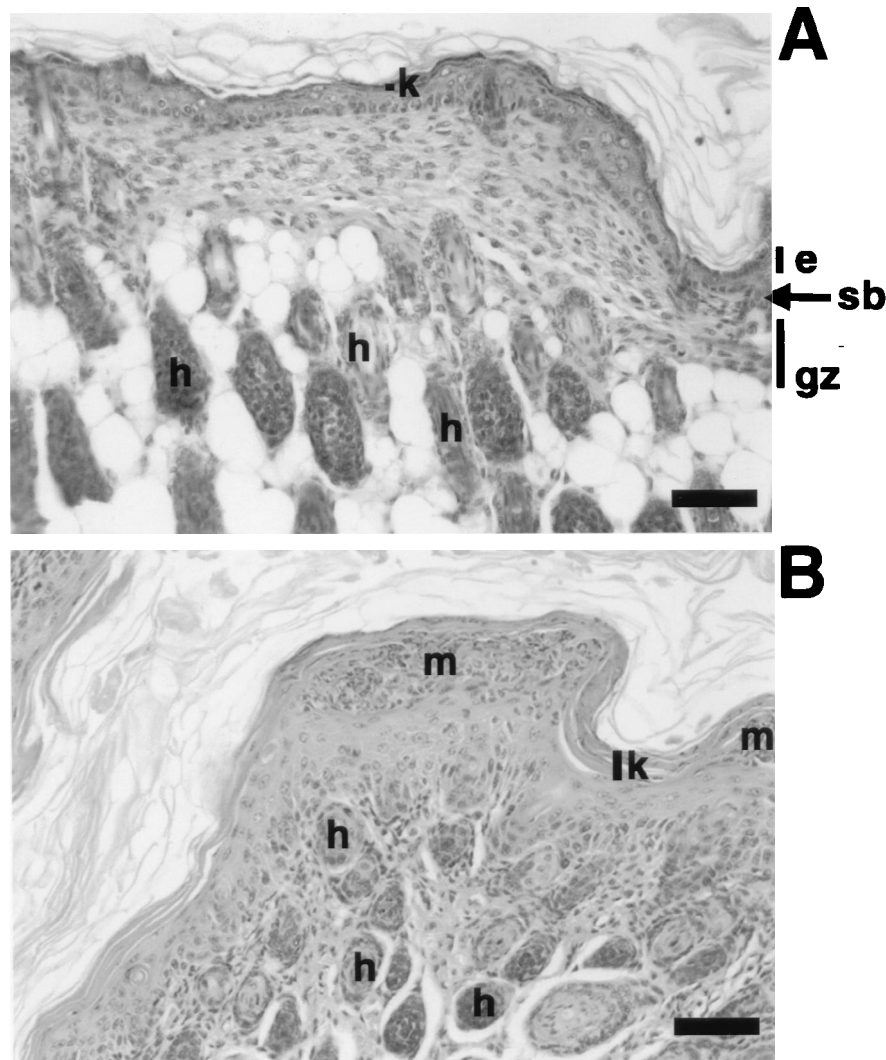


FIG. 4. Histological analysis of the skin from wild-type and homozygous  $I\kappa B\alpha$ -null pups. Tissues from 5-day-old pups were fixed, and histological analysis was performed by using standard methods and hematoxylin and eosin staining. (A) Skin cross section from a wild-type pup. The lettering on the right identifies the various layers of the skin. e, epidermal region; sb, stratum basale (a layer of cuboidal keratinocytes); gz, Grenz zone (region subjacent to the epidermis); h, hair follicle. The bar labeled k at the surface of the skin indicates the depth of the keratinized layer of cells. (B) Skin cross section from an  $I\kappa B\alpha$ -deficient pup. Note that the ordered structure of the epidermis, stratum basale, and Grenz zone is disrupted by the infiltrating leukocytes. This is especially evident in the region between the hair follicles (h), where adipose cells (clear-staining cells) visible in panel A have been replaced by infiltrating neutrophils. Note also that the depth of the keratinized layer (k) is greater than that in panel A. The severe degree of inflammation is evidenced by microabscesses (m) which form on the epidermis over the entire body surface of the  $I\kappa B\alpha$ -deficient pup.

Grenz zone (area immediately subjacent to the epidermis and labeled gz in Fig. 4) was filled with mononuclear cells, principally macrophages with fewer neutrophils. A graduation from microabscessation to dermal ulceration was apparent. Staining for bacteria, fungi, and yeasts did not identify microorganisms associated either with epidermal lesions or with leukocytes (data not shown), indicating that the dermatitis was unlikely to have been a consequence of infection. An immunohistochemical analysis of skin taken from 5-day-old  $I\kappa B\alpha$   $+/+$  and  $-/-$  pups was performed with antibodies to keratins 10, 14, and 16. Antibodies to keratin 14 and keratin 16 both showed increased staining in the suprabasal layer of the skin taken from  $I\kappa B\alpha$ -deficient pups, compared with skin from their wild-type siblings, while no increase in the expression of keratin 10 was noted (data not shown). Expression of keratins 14 and 16 is indicative of proliferating keratinocytes, while keratin 10 is differentiation specific and is localized to the nonproliferative

layer of the skin. These results indicate that proliferative keratinocytes were increasing in proportion relative to differentiated cell types.

Enhanced splenic extramedullary hematopoiesis was present in mice lacking  $I\kappa B\alpha$  (data not shown). Myelopoiesis was also enhanced, while erythropoiesis appeared moderately depressed. Hepatic myelopoietic areas adjacent to portal triads and terminal portal venules were enlarged, and diffusely distributed perisinusoidal erythropoietic islands were virtually absent (data not shown). However, hematopoietic alterations were considered to be secondary to granulopoietic stimuli arising from severe diffuse dermatitis and were limited to animals sacrificed close to the expected time of death.

A FACS analysis of spleen cells from wild-type, heterozygous, and homozygous pups from 5-, 7-, and 9-day-old litters was performed with a panel of antibodies specific for different hematopoietic cells (see Materials and Methods). This analysis

TABLE 1. Levels of splenic monocytes/macrophages are elevated in  $I\kappa B\alpha$ -deficient mice<sup>a</sup>

Genotype	% of cells MAC-1 <sup>+</sup>
+/+	5.8
+/-	3.1
+/-	4.1
+/-	5.1
+/-	5.4
+/-	4.1
+/-	3.8
-/-	16.8
-/-	39.5
-/-	26.8
-/-	47.7

<sup>a</sup> Splenocytes from a single 7-day-old mouse litter were subjected to FACS analysis using an antibody to the monocyte/macrophage marker MAC-1 (CD11b). Values represent the percentages of total cells stained by the antibody. The difference between the  $I\kappa B\alpha$ -deficient population (-/-) and the  $I\kappa B\alpha$ -containing population (+/+ and +/-) is highly significant ( $P < 0.01$  by the 1-tailed Student *t* test). Similar results were obtained with spleen cells from 5- and 9-day-old mice.

revealed a relatively normal frequency and distribution of the majority of hematopoietic cells in all the mice. The exception was that in the  $I\kappa B\alpha$ -deficient mice the proportion of MAC-1<sup>+</sup> monocytes/macrophages (as a percentage of the total number of cells analyzed) was significantly increased at all ages examined (Table 1). Forward and side light scatter values (44, 53) for the macrophages in the  $I\kappa B\alpha$ -null pups were slightly, but consistently, elevated, suggesting increased physiological activation of these cells. This increase in activation of the macrophages was consistent with the acute dermatitis present in the homozygotes at these ages. It is possible that these changes in the macrophages arose as a direct consequence of loss of  $I\kappa B\alpha$  or as an indirect effect caused by another cell population acting on the macrophages.

**Elevated TNF- $\alpha$  mRNA levels in skin of  $I\kappa B\alpha$ -deficient mice.** Overexpression of TNF- $\alpha$  in the skin, with concomitant increases in levels of IL-1 $\alpha$  and IL-1 $\beta$ , has been shown to result in severe dermatitis (11). An analysis of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and gamma interferon mRNA levels was performed. RNA was isolated from the spleens, livers, kidneys, and skin of  $I\kappa B\alpha$  +/+, +/-, and -/- mice and subjected to RT-PCR analysis (51). An increase in the TNF- $\alpha$  levels in the skin of  $I\kappa B\alpha$  homozygous -/- pups relative to those in the skin of

heterozygous or wild-type mice was detected (Fig. 5). The TNF- $\alpha$  mRNA level was also elevated in spleen tissue from the  $I\kappa B\alpha$  -/- pups, but this elevation was also seen in the spleens of the  $I\kappa B\alpha$  +/- and +/+ animals and therefore was not considered significant. Similar analyses did not reveal significant differences between wild-type and  $I\kappa B\alpha$ -deficient pups in IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, or gamma interferon mRNA levels in any of the organs examined. Whether NF- $\kappa B$  activates TNF- $\alpha$  expression directly (15, 52) or indirectly cannot be determined from these results. Dermal TNF- $\alpha$  expression in the  $I\kappa B\alpha$ -deficient pups offers one possible explanation for the presence of infiltrating leukocytes in the skin of these pups, i.e., induction of secondary chemotactic cytokines.

## DISCUSSION

Phosphorylation and rapid degradation of  $I\kappa B\alpha$  upon cellular stimulation allow NF- $\kappa B$  to translocate to the nucleus and activate gene transcription (5, 9, 57). As one of the genes activated by NF- $\kappa B$  encodes  $I\kappa B\alpha$  (9, 12, 13, 17, 30, 35, 49, 57), it has been suggested that the rapid increase in the level of  $I\kappa B\alpha$  protein terminates the NF- $\kappa B$  response. However, direct evidence of an absolute requirement for  $I\kappa B\alpha$  in termination of the response has been lacking. Here we present data suggesting that cytoplasmic retention and rapid nuclear translocation of NF- $\kappa B$  can occur in the absence of  $I\kappa B\alpha$  but that  $I\kappa B\alpha$  is an absolute requirement for normal termination of the NF- $\kappa B$  response. When wild-type cells were treated with TNF- $\alpha$  and then chased in the absence of TNF- $\alpha$ , nuclear p65 (as assayed by immunoblotting) and NF- $\kappa B$  (as assayed by DNA binding) disappeared within 30 min. In the  $I\kappa B\alpha$ -deficient cells, however, nuclear p65 persisted for at least 2 h following removal of the TNF- $\alpha$ . DNA-binding activity levels also remained elevated. This phenomenon can be duplicated in  $I\kappa B\alpha$ -containing NIH 3T3 cells if the TNF- $\alpha$  chase is carried out in the presence of cycloheximide. In these cells, the level of nuclear p65 was undiminished after 3 h, confirming that protein (presumably  $I\kappa B\alpha$ ) synthesis is required for clearance of NF- $\kappa B$  from the nucleus. Thus, termination of an NF- $\kappa B$  response appears to be a critical function of  $I\kappa B\alpha$ , and at least in these fibroblastic cells, other  $I\kappa B$  proteins cannot compensate for the loss of  $I\kappa B\alpha$ .

These results correlate well with the previously published work (cited above) showing that  $I\kappa B\alpha$  mRNA accumulates rapidly upon NF- $\kappa B$  nuclear translocation. Similarly, dexamethasone (an immunosuppressant) has been shown to up-regulate transcription of the  $I\kappa B\alpha$  gene, with the result that little or no nuclear NF- $\kappa B$  accumulates following an inductive signal (3, 47). The mechanism of  $I\kappa B\alpha$  action is not yet clear, however. It is possible that during the rapid synthesis of new  $I\kappa B\alpha$ , some molecules diffuse into the nucleus and actively remove NF- $\kappa B$  from DNA. Nuclear  $I\kappa B\alpha$  has, in fact, been observed following stimulation of HeLa cells with TNF- $\alpha$  or IL-1 (2). Alternatively (or additionally), nuclear DNA-binding activity may have a short half-life independent of  $I\kappa B\alpha$ , and the elevated level of cytoplasmic  $I\kappa B\alpha$  may simply serve to prevent any further translocation of NF- $\kappa B$  to the nucleus. The failure of the  $I\kappa B\alpha$ -deficient cells in the present study to clear nuclear NF- $\kappa B$  after removal of TNF suggests a direct role for  $I\kappa B\alpha$  in the nucleus, as does the requirement for new protein synthesis, but further work is required to test this hypothesis.

In agreement with recent results of Beg et al. (7), we found little or no constitutive DNA-binding activity in cultured fibroblasts from  $I\kappa B\alpha$ -deficient embryos. In some cells, therefore,  $I\kappa B\alpha$ 's role in cytoplasmic retention of Rel family proteins can

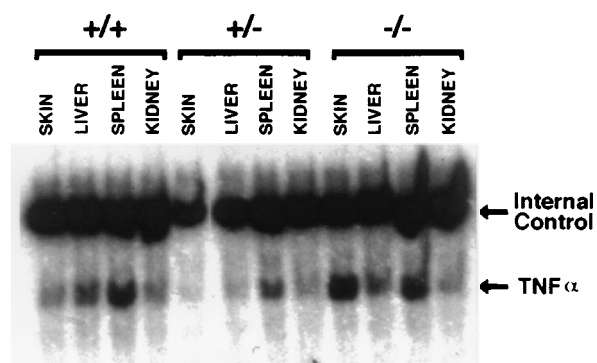


FIG. 5. Determination of TNF- $\alpha$  mRNA levels in  $I\kappa B\alpha$  +/+, +/-, and -/- pup skin by using the RT-PCR. The genotypes of the pups (+/+, wild type; +/-, heterozygous; and -/-, homozygous) and the organs from which the RNA was isolated are indicated above the lanes. The bands representing the internal control and TNF- $\alpha$ -generated PCR products are indicated by arrows.



evidently be assumed by other inhibitors. Since we also showed that the levels of the other inhibitors—I $\kappa$ B $\beta$ , p105, and p100—were indistinguishable in wild-type and I $\kappa$ B $\alpha$ -deficient cells, minor adjustments must be sufficient to compensate for loss of I $\kappa$ B $\alpha$ . The existence of an additional unknown inhibitor whose level does increase substantially in the absence of I $\kappa$ B $\alpha$  cannot be excluded by the present results. In other cells I $\kappa$ B $\alpha$  may play a more important role in cytoplasmic retention of NF- $\kappa$ B. Beg et al. found elevated DNA-binding activity in thymocytes and splenocytes from 3-day-old I $\kappa$ B $\alpha$ -deficient mice (7). This activity could result from inadequate cytoplasmic retention of NF- $\kappa$ B or, alternatively, from failure to quench the response to an earlier stimulus.

The phenotype of I $\kappa$ B $\alpha$ -deficient pups, which has also been reported by Beg et al. (7), is consistent with NF- $\kappa$ B hyperactivity. Development of an apparently sterile, widespread, severe dermatitis in mice otherwise free of recognized murine pathogens suggests inappropriate expression of inflammatory cytokines and/or the presence of chemotactic stimuli within the upper epidermis. Indeed, the skin lesions of I $\kappa$ B $\alpha$ -deficient mice are morphologically related to those of psoriasis, wherein lymphocytic infiltration and keratinocyte proliferation (probably cytokine driven) occur. Abnormal expression of cytokines in the I $\kappa$ B $\alpha$ -null mice could be triggered by NF- $\kappa$ B, as NF- $\kappa$ B serves as an important transcription factor for many cytokine genes. As the skin was the only inflamed organ in the I $\kappa$ B $\alpha$ -null mice, we suggest that the skin may contribute to, or even initiate, the observed phenotype. It is difficult to account for why the skin should be the site of an inflammatory response, but the fact that such a response occurred there may reflect the skin's being the first organ exposed to diverse environmental stimuli, including nonpathogenic microorganisms, physical trauma, and irradiation. It is unlikely that cachexia was the cause of death, as the usual hallmarks of cachexia—decrease in brown fat levels, loss of appetite, and vacuolated hepatocytes—were not observed.

Related dermal phenotypes have been observed in transgenic mice expressing either human TNF- $\alpha$  from a keratin promoter (11), the Epstein-Barr virus latent membrane protein from the polyomavirus promoter (61), or IL-1 $\alpha$  from the keratin 14 promoter (23). Their skin showed signs of inflammation with extensive macrophage and lymphocytic infiltration. For the TNF- $\alpha$  transgenic mice the phenotype was attributed to overexpression of human TNF- $\alpha$  in the skin, together with an increase in murine IL-1 $\alpha$  and IL-1 $\beta$  expression. In the I $\kappa$ B $\alpha$ -deficient animals there was also an increase in TNF- $\alpha$  mRNA levels as detected by RT-PCR. Whether the TNF- $\alpha$  is directly controlled by NF- $\kappa$ B or is a consequence of the dermal inflammation cannot be determined by these experiments. In the latent membrane protein 1 transgenic mice, aberrant expression of keratins 6 and 14, indicative of an increase in the number of hyperplastic keratinocytes, was observed and is consistent with our results. Latent membrane protein 1 has been shown to induce NF- $\kappa$ B by inactivation of I $\kappa$ B $\alpha$  in fibroblasts and B lymphocytes (28), and its expression may also prevent termination of the NF- $\kappa$ B response.

We have attempted to ameliorate the phenotype of the I $\kappa$ B $\alpha$ -deficient pups by introducing the I $\kappa$ B $\alpha$  deficiency into mice lacking IL-6 (40), type 1 IL-1 receptor (34a), TNF- $\alpha$  receptor (p55) (43), or leukemia inhibitory factor (56). None of these backgrounds significantly affected the phenotype of I $\kappa$ B $\alpha$ -deficient mice, except for the I $\kappa$ B $\alpha$ -leukemia inhibitory factor-deficient pups, which appeared to have a more severe phenotype (31a). The inability of the TNF- $\alpha$ -receptor (p55)-null background to compensate for I $\kappa$ B $\alpha$  deficiency may suggest that the TNF- $\alpha$  expression detected in I $\kappa$ B $\alpha$ -deficient

pups is a consequence rather than a cause of the dermal inflammation. However, the physiology of TNF is complex. There are at least three TNFs (TNF- $\alpha$ , - $\beta$ , and - $\gamma$ ) and three receptors (p55, p75, and lymphotoxin  $\alpha$ - $\beta$  receptor), and multiple TNF and/or receptor deficiencies may therefore be required to complement the I $\kappa$ B $\alpha$  deficiency. Considering the large array of genes which are thought to be regulated by NF- $\kappa$ B, the phenotype of the I $\kappa$ B $\alpha$ -deficient pups may very well be multifactorial.

These results add to the accumulating data which suggest that the *in vitro* analysis of signal transduction pathways cannot always be extrapolated to the *in vivo* situation. In support of this contention are results obtained with mice lacking the *nfkb1* and *relb* genes. The NF- $\kappa$ B1-deficient mice developed normally and reached adulthood, indicating that NF- $\kappa$ B1 and, by association, classical NF- $\kappa$ B are not required for development. The major defect noted in these mice was a slightly impaired immune response (50). Expression of some genes thought to require NF- $\kappa$ B was not affected in the NF- $\kappa$ B1-null mice. RelB-deficient mice also developed normally to term, but shortly after birth they developed a severe pathology with inflammation of several organs that was frequently lethal (10, 60). These results indicate that the Rel family proteins have different functions in the immune and inflammatory responses and control different sets of genes. They also suggest that other Rel family members cannot fully compensate for a deficiency in one of the Rel family proteins. Likewise, our results have shown that the loss of I $\kappa$ B $\alpha$  resulted in a severe form of dermatitis. Related I $\kappa$ B genes were unable to compensate for the loss of I $\kappa$ B $\alpha$ , indicating that each may have a unique role in regulation of Rel protein activation. By the use of gene targeting to eliminate components of the Rel family signal transduction pathways, we have begun to elucidate the many complexities and regulatory mechanisms of this essential system. In addition, these studies have also revealed that defects in such a regulatory pathway may make a critical contribution to some forms of chronic inflammation.

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