Requirement of NF-κB/Rel for the development of hair follicles and other epidermal appendices

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SUMMARY

NF-κB/Rel transcription factors and IκB kinases (IKK) are essential for inflammation and immune responses, but also for bone-morphogenesis, skin proliferation and differentiation. Determining their other functions has previously been impossible, owing to embryonic lethality of NF-κB/Rel or IKK-deficient animals. Using a gene targeting approach we have ubiquitously expressed an NF-κB super-repressor to investigate NF-κB functions in the adult. Mice with suppressed NF-κB revealed defective early morphogenesis of hair follicles, exocrine glands and teeth, identical to Eda (tabby) and Edar (downless) mutant mice.

INTRODUCTION

NF-κB transcription factors control the expression of a large number of genes that are essential for innate and adaptive immunity, for regulation of apoptosis and cellular proliferation (Baeuerle and Henkel, 1994). NF-κB is composed of hetero- or homodimers of the related p50, p52, p65 (RelA), Rel or RelB proteins, which are sequestered by cytoplasmic IκB proteins (Verma et al., 1995). Cellular stimulation by pro-inflammatory cytokines and other agents activates an IκB kinase (IKK) complex to phosphorylate the IκBs, triggering their polyubiquitination, proteasomal degradation and release of NF-κB (Karin and Ben-Neriah, 2000). Targeted inactivation of individual NF-κB/Rel genes has revealed an essential function in the immune system. Mouse mutants that lack NF-κB1 (p105/p50), NF-κB2 (p100/p52), Rel or RelB exhibit disturbed proliferation and activation of B and T cells, and impaired immunoglobulin secretion, or suffer from multiorgan inflammation (Attar et al., 1997; Gerondakis et al., 1998). Aside from immunity, an essential role has also been documented for osteoclast maturation (Franzoso et al., 1997). A further important function is an anti-apoptotic activity of NF-κB in various cell types. Lack of p65 (RelA) results in hepatocyte apoptosis and embryonic death (Beg et al., 1995).

The prevailing form of the IKK complex is an IKKα-IKKβ kinase heterodimer associated with IKKγ/NEMO (Karin, 1999). Although the highly related IKKα and IKKβ proteins are both able to phosphorylate IκBα in vitro, the physiological function of both isoforms differs grossly. Gene ablation disclosed that, similar to p65, IKKβ is essential for NF-κB activation by pro-inflammatory stimuli and antagonizes tumor necrosis factor α (TNFα)-induced apoptosis (Li et al., 1999b; Li et al., 1999c; Tanaka et al., 1999). By contrast, IKKα is required for development of the epidermis and gene loss results in epidermal hyperproliferation (Hu et al., 1999; Li et al., 1999a; Takeda et al., 1999). This function, however, is completely independent of NF-κB and does not require the kinase activity of IKKα, as has been shown by complementation of IKKα-deficient keratinocytes (Hu et al., 2001).

Owing to the functional redundancy of NF-κB/Rel subunits in the single or double knockouts and to embryonic lethality observed for RelA-, IKKβ- or IKKγ-deficient mice, the physiological role of NF-κB/Rel in the adult organism has remained widely obscure. We have examined the pathophysiological consequences of a systemic NF-κB/Rel suppression using a gene targeting approach by ubiquitous expression of a constitutively active IκBα mutant. Mice with suppressed NF-κB activity survive to adulthood and display macrophage dysfunction and alamylosplasia, the lack of secondary lymphoid organs. NF-κB suppression results in severe defects in the early steps of the development of epidermal appendices, including hair follicles, tear and sweat glands. In wild-type mice these structures reveal strong NF-κB transcriptional activity, as we demonstrate with β-galactosidase.
reporter mice. This includes the multipotent stem cell-containing bulge region in hair follicles, which responds to morphogenic signals for hair follicle generation. NF-κB suppression results in strongly increased apoptosis in subregions of developing hair follicles. The epidermal phenotype is analogous to hypohidrotic (anhydrotic) ectodermal dysplasia (HED) in humans, and identical to phenotypes of Eda, Edar or crinkled (Cr) mice. The Eda and Edar genes are related to the TNF multigene family of ligands and receptors, respectively. Our data are consistent with the model that NF-κB is required for Edar to transmit Eda signals and to protect against apoptosis.

MATERIALS AND METHODS

Generation of transgenic mice

The IκBαΔN cDNA encoding amino acids 71-317 of IκBα (Krauppman et al., 1996) was inserted in frame into the β-catenin targeting vector (Huelsken et al., 2000; Huelsken et al., 2001). In the loxPκBΔAN construct, two loxP sites were fused in frame to the start codon of the gene for β-catenin. The loxP sites flank a stop codon, preventing IκBδAN translation, unless removed by Cre recombination. The loxP sites were derived from the plasmid pAMA (Zang et al., 1996), kindly provided by Dr Fred Sablitzky. Heterozygous β-cat<sup>aD</sup>κBΔAN (<sup>b</sup>β-cat<sup>aD</sup>κBΔAN<sub>N</sub> 129/Ola embryonic stem (ES) cells were produced by electroporation and analyzed by Southern blotting. Two independent heterozygous ES cell clones of each targeting construct were used to generate chimeric mice by blastocyst injection. Heterozygous mutant animals were bred into C57B1/6 background. Homozygotes are not viable as a consequence of bi-allelic β-catenin inactivation (Haegel et al., 1995; Huelsken et al., 2000). After birth, cδIκBΔAN animals were readily detected phenotypically. cδIκBΔAN mice were identified by PCR. (IκBα<sub>N</sub>truncal;Z mice were produced as described (Schmidt-Ullrich et al., 1996).

Electrophoretic mobility shift assay (EMSA) and western blotting

ES cells and mouse embryonal fibroblasts (MEFs) were washed with phosphate-buffered saline (PBS) and lysed in extraction buffer (20 mM Hepes (pH 7.0), 0.15 mM EDTA, 0.15 mM EGTA, 10 mM KCl, 0.15 mM spermidine, 1% NP40, 0.4 M NaCl, 10% glycerol and protease inhibitors). After 30 minutes on ice, extracts were centrifuged for 30 minutes at 86,000 g. Supernatants were used for western and EMSA analysis. Organs of mice were quick-frozen in liquid N<sub>2</sub> and lysed by douncing in extraction buffer. ES cells were treated with 75 ng/ml phorbol ester (PMA) (Sigma) for 1 hour. MEFs were treated with 5 ng/ml IL-1β (Promega) or 50 ng/ml TNFα (Biomol) for the times indicated. EMSA was performed as previously described (Krauppman et al., 1996). The following antibodies were used for western blot analysis: p65 (#sc109, #sc8008, Santa Cruz) and IκBα (C-15, #sc203; C-21, #sc371, Santa Cruz). The polyclonal rabbit anti-β-catenin antibody is directed against the C-terminal part of the protein.

Histology and in situ hybridization (ISH)

For liver analysis, embryos were removed and snap-frozen in OCT compound with subsequent storage at −80°C. Cryosections (10 μm) were made. TUNEL assays were performed using the ApopTag® Plus system from Interson Company. For analysis of tissue from adult mice, tissue was removed from perfused mice (4% paraformaldehyde (PFA)) and fixed in 4% PFA for up to a week, depending on the size of the tissue. Tissue containing bone, such as the inner ear and feet was decalcified in 4% EDTA/1×PBS for a week at 4°C. After a series of dehydration steps in ethanol (30%-100%) the tissue was embedded in Technovit 7100 plastic (Heraeus Kulzer). Sections (5 μm) were cut and stained using the Hematoxylin (Delafield’s)-EosinY method. Whole-mount in situ hybridization was performed as described (Huelsken et al., 2000). The single-stranded RNA probe was labeled (Huelsken et al., 2000). The single-stranded RNA probe was labeled (Huelsken et al., 2000). The single-stranded RNA probe was labeled (Huelsken et al., 2000). The single-stranded RNA probe was labeled (Huelsken et al., 2000). The single-stranded RNA probe was labeled (Huelsken et al., 2000). The single-stranded RNA probe was labeled (Huelsken et al., 2000). The single-stranded RNA probe was labeled (Huelsken et al., 2000). The single-stranded RNA probe was labeled (Huelsken et al., 2000).

Fig. 1. Generation of mice with systemic NF-κB suppression. (A) Integration of κBΔAN (Krauppman et al., 1996) into the β-catenin locus by homologous recombination. κBΔAN lacks the phosphorylation and ubiquitination sites, which are required for signal-induced degradation (Krauppman et al., 1996). The murine β-catenin locus (exons, white boxes) and targeting vectors are shown. κBΔAN and loxPκBΔAN cDNAs were inserted in frame to the start codon in the second exon of the β-catenin gene and replaced exons 3 to 6, resulting in a β-catenin null allele (Huelsken et al., 2000). In κBΔAN, a stop codon inserted in frame is flanked by loxP sites (black arrowheads; Zhang et al., 1996). (B) Western blot of κBΔAN (D5-C7) and κBΔAN (D7-H9) ES cell clones using an antibody directed against the IκBα C-terminus (C-21), ns, non-specific. IκBδAN protein is detected only in the ES cells carrying the κBΔAN transgene. (C) EMSA of κBΔAN ES clones (G2 and G3), and κBΔAN (B7 and A3) before Cre-mediated recombination as controls. ES clones were treated with PMA and specific complexes inhibited with anti-p65 antibody, as indicated. NF-κB activity can no longer be induced by PMA in κBΔAN ES cells clones. (D) Embryonic fibroblasts (MEFs) of wild-type (WT) and κBΔAN littermates (IκBΔAN) were stimulated with IL1β and TNFα for the times indicated and extracts were assayed by EMSA. In κBΔAN fibroblasts, DNA-binding activity of NF-κB is severely blocked after stimulation with TNFα and completely impaired after IL1β stimulation. (E) The same extracts were analyzed in Western blots for IκBα and β-catenin proteins, as indicated. As expected, endogenous IκBα is degraded after stimulation leading to the observed variations of the protein in wild-type and κBΔAN fibroblasts. De novo synthesis of IκBα protein, which depends on active nuclear NF-κB complexes, is delayed in κBΔAN fibroblasts, as NF-κB translocation is strongly suppressed. (F) A wild-type littermate compared with a κBΔAN mouse (right). (G) Increased apoptosis in the embryonic liver of κBΔAN mice. Cryosections of embryonic livers of wild-type and κBΔAN embryos at E12.5, E14.5 and at birth, P0, as indicated, were analyzed by in situ TUNEL assay.

of dehydorination steps in ethanol (30%-100%) the tissue was embedded in Technovit 7100 plastic (Heraeus Kulzer). Sections (5 μm) were cut and stained using the Hematoxylin (Delafield’s)-EosinY method. Pictures were taken with a Zeiss Axiophot camera.

Whole-mount in situ hybridization was performed as described (Huelsken et al., 2000). The single-stranded RNA probe was labeled with digoxigenin (DIG-UTP according to the manufacturer’s instructions (Roche). The probe for dowsless used comprised nucleotides 302-1038 (AF160502). Subsequently embryos were embedded in Technovit 7100 and 12 μm sections were cut. Sections were counterstained with 0.01% pyroninG. Whole-mount X-Gal staining was performed as described (Schmidt-Ullrich et al., 1996).

Pathogen challenges and nitrate production in macrophages

Leishmania major V121 promastigotes were injected into the left hind footpad and thickness was recorded weekly using a caliper. The difference with the uninfected foot was plotted as the means±s.d. Nitrite production was measured in culture supernatants of macrophages derived from bone marrow using DMEM/10% FCS/20% L929-conditioned medium. Cells were harvested after 6 days and cultured for a further 72 hours at 1.25×10<sup>6</sup> cells per cavity in 48-well plates in DMEM/10% FCS substituted with 100 U/ml IFNγ and increasing doses of TNFα. Nitrite was determined colorimetrically by the Griess reaction. Leishmania major antigens were produced by repeated freezing and thawing of the cells (five times). Lysate corresponding to 10<sup>6</sup> cells/ml was used to restimulate spleen cells.
RESULTS

Generation of mice with suppressed NF-κB function

NF-κB activity is controlled by signal-induced degradation of its cytoplasmic inhibitor IκBα. Deletion of an N-terminal destruction box of IκBα creates a super-repressor, IκBαΔN, which inhibits NF-κB release (Krappmann et al., 1996). IκBαΔN cDNA was integrated into the ubiquitously expressed gene for β-catenin (Huelsken et al., 2000; Huelsken et al., 2001) by homologous recombination in embryonic stem (ES) cells, generating the cIκBαΔN allele (Fig. 1A). In a second construct, loxP IκBαΔN, an inserted stop codon, flanked by two loxP sites, prevents translation of IκBαΔN, unless it is removed by Cre-mediated recombination (Fig. 1A). ES cell clones carrying the cIκBαΔN allele expressed IκBαΔN, which was not detectable in cloxP IκBαΔN ES cells (Fig. 1B). NF-κB DNA binding activity could be induced by PMA in cloxP IκBαΔN cells, while no induction was observed in cIκBαΔN cells (Fig. 1C). Mouse strains carrying the mutant alleles cIκBαΔN or cloxP IκBαΔN...
were established by ES cell technology. In \( c^{IKBa\Delta N} \) mice, the inhibitor was expressed in all tissues analyzed, with the exception of skeletal muscle (data not shown). Embryonic fibroblasts (MEF) were isolated from \( c^{IKBa\Delta N} \) animals and the induction of NF-\( \kappa \)B by cytokines was examined. TNF-\( \alpha \)-mediated stimulation of NF-\( \kappa \)B was severely impaired while IL-1\( \beta \) stimulation was completely blocked (Fig. 1D). The degradation of endogenous I\( \kappa \)B\( \alpha \) in response to IL-1\( \beta \) and TNF\( \alpha \) was normal in \( c^{IKBa\Delta N} \)-positive MEFs, while I\( \kappa \)B\( \alpha \Delta N \), lacking degradation signals, remained unchanged (Fig. 1E). As \( \beta \)-catenin is regulated at the level of protein stability, \( \beta \)-catenin protein amounts remained at wild-type levels in all tissues of \( c^{IKBa\Delta N} \)-positive mice (Fig. 1E; data not shown), in spite of mono-allelic expression. No changes were seen in the expression levels of I\( \kappa \)B\( \beta \), I\( \kappa \)B\( \epsilon \) or p105/p50 in organ extracts of \( c^{IKBa\Delta N} \) mice (data not shown).

**Sub-critical hepatocyte apoptosis by I\( \kappa \)B\( \alpha \Delta N \)-mediated NF-\( \kappa \)B suppression**

Heterozygous \( c^{IKBa\Delta N} \) mice were viable, but were not obtained in the expected Mendelian ratio, indicating that a proportion was lost during embryogenesis. The livers of \( c^{IKBa\Delta N} \) embryos showed increased apoptosis between embryonic day 12.5 (E12.5) and E14.5 (Fig. 1G). In 20% of pregnancies at E14.5,
completely anemic or partially resorbed $c^{IKK\beta\Delta N}$-positive embryos with almost complete liver destruction were seen, accounting for the observed non-Mendelian ratio. However, at birth (P0) apoptotic cells were rare and detected at the same frequency as in wild-type mice (Fig. 1G). Histological analysis of adult livers did not show any gross abnormalities. Massive liver apoptosis, defects in liver hematopoiesis and death in utero have previously been reported for mice deficient in p65, IKK$\beta$ and IKK$\gamma$ (Gerondakis et al., 1999; Karin and Ben-Neriah, 2000; Makris et al., 2000; Rudolph et al., 2000; Schmidt-Supprian et al., 2000). In $c^{IKK\beta\Delta N}$ mice, residual NF-$\kappa$B activity may suffice to restrict hepatocyte apoptosis.

Impaired hair follicle development and absence of eccrine glands implicate NF-$\kappa$B as an essential component of the EDA/EDAR pathway

Animals which reached adulthood were small and thin, had shaggy fur, no hair on the tail and behind the ears, fewer vibrissae and slanted eyes (Fig. 1F). In wild-type mice, four hair types, monotrich, awl, auchenes and zigzag, are found,
whereas in \(c^{\text{loxPI}}\) mice only a monotrich-awl intermediate was detected (Fig. 2A). By contrast, uninduced \(c^{\text{loxPI}}\) mice could not be distinguished phenotypically from wild-type mice. However, when mating \(c^{\text{loxPI}}\) animals with deleter-Cre mice, which produce the Cre enzyme ubiquitously (Schwenk et al., 1995), the offspring had the same phenotype as \(c^{\text{loxPI}}\) mice (see Table 1 for phenotype summary).

The hair defects in \(c^{\text{loxPI}}\) mice resulted from impaired hair follicle formation. Newborn mice produced very few hair follicles, developing at a slower rate (Fig. 2B, top). The reduced number of hair follicles persisted throughout adulthood (Fig. 2B, bottom). No anlagen for hair follicles were seen in the tail of \(c^{\text{loxPI}}\) mice (Fig. 2B, middle). In the footpads, plicae digitalis (deeply indented transversed folds) and sweat glands were absent (Fig. 2C and data not shown). Furthermore, outgrowth of molars and incisors was delayed and did not reach normal length in adult animals (Table 1 and data not shown).

Although \(c^{\text{loxPI}}\) mice opened the eyes only after 2.5-3 weeks after birth, the eye-bulb, conjunctiva and the cornea developed normally. However, \(c^{\text{loxPI}}\) mice revealed narrowed palpebral fissures and thickened margins of the eyelids, caused by a hyperproliferative epidermis of the eyelid margin (Fig. 2D). After about 1 month, mice developed conjunctivitis. Hyperproliferation of the corneal stroma, keratinization of the corneal epithelium, neof ormation of blood vessels and granulocyte infiltration could be readily detected after 6 months (not shown). Histological sections disclosed a complete absence of Meibomian glands in all \(c^{\text{loxPI}}\) mice analyzed (Fig. 2D). The Meibomian glands produce the lipid layer of the tear film, preventing its evaporation. Insufficient development of the Harderian glands (data not shown; Table 1) may further facilitate evaporation. As a consequence, the eyes dehydrated with time and, owing to a reduced immune response (see below), the mice additionally acquired severe keratoconjunctivitis sicca, eventually resulting in blindness in all cases.

\(c^{\text{loxPI}}\) mice did not show the hyperplasia of the suprabasal squamous layer of the epidermis observed in \(ikk\alpha\) and \(\gamma\) knockout or K14-\mIxB\alpha\ models (Kaufman and Fuchs, 2000). However, a requirement of NF-\kxB for hair follicle and exocrine gland formation is supported by blue staining of these structures in mice expressing a NF-\kxB responsive \(\beta\)-galactosidase (\(Ig\kappa\)sconalacZ) transgene (Schmidt-Ullrich et al., 1996) (Fig. 3A-F; data not shown). These mice did not show any X-Gal staining in the epidermal layers (Fig. 3).
Embryos revealed β-galactosidase activity as early as E12 for vibrissae and E15 for pelage hair (R. S.-U., unpublished). In vibrissal and pelage follicles of adult (Igk)3conalacZ mice, strong X-Gal staining was detected in the matrix (Fig. 3B,C). Vibrissal follicles also showed β-galactosidase activity in the hair shaft (Fig. 3C) and in the bulge (Fig. 3D). Similarly, a discrete region of pelage hair follicles, likely to be the bulge, stained blue (Fig. 3A). This indicates a role for NF-κB in de novo hair follicle formation, as the bulge region contains multipotent stem cells, which respond to morphogenic signals to generate a new hair follicle after each hair cycle. X-Gal staining was also observed in hair follicles of the tail (Fig. 3E), in sweat glands (Fig. 3F), Harderian glands and Meibomian glands (data not shown).

Importantly, the complex epidermal phenotype of cκBaΔN mice is identical to that of Eda and Edar mutant mice. These genes encode a new member of the TNF ligand and its receptor, respectively, which both specify the fate of epidermal appendices. Mutations of the homologous genes account for HED in humans.

In situ hybridization (ISH) did not show any Edar expression in pelage follicles of cκBaΔN mice at E15.5 (Fig. 4A, dl-ΔN1), when compared with wild-type mice (Fig. 4A, dl-w.t.1). As expected, no placodes were seen in cκBaΔN mice at this stage (Fig. 4A, dl-ΔN2), while placodes of wild-type mice were developing normally, showing expression of Edar (Fig. 4A, dl-w.t.2). However, developing vibrissal follicles are present in cκBaΔN mice and do show Edar expression – identical to wild-type animals (Fig. 4B) – indicating that Edar is not regulated by NF-κB in vibrissae. Preliminary ISH data of E17.5 embryos also showed Edar expression in body hair follicles in cκBaΔN mice (data not shown). TUNEL assays showed an increased rate of apoptosis in many pelage (Fig. 4C, ΔN1) and vibrissal follicles (Fig. 4C, ΔN2). Taken together, these results demonstrate that NF-κB is essential for Eda and Edar signaling, and the presence of apoptosis implies that NF-κB

### Table 1. Phenotype of cκBaΔN mice in comparison with Eda and Edar mice

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<th>Trait</th>
<th>cκBaΔN mice</th>
<th>Eda and Edar mice</th>
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<tr>
<td>Life-span</td>
<td>Max. 1 year, less than 6 months in 50%</td>
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<tr>
<td>Size</td>
<td>50%-70% of wild type</td>
<td>Normal</td>
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<tr>
<td>Reproduction</td>
<td>Normal</td>
<td>Reduced litter size</td>
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<td>CNS</td>
<td>Equilibrium problems</td>
<td>Lethargy, shakes, displacement activities</td>
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<td>Skeleton and teeth</td>
<td>Domed skull (osteopetrosis)</td>
<td>Hunched posture</td>
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<td>Immune system</td>
<td>No peripheral lymph nodes</td>
<td>Abnormal incisor positioning*</td>
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<td></td>
<td>Leukocytosis</td>
<td>Kinked tail tips*</td>
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<tr>
<td>Liver and gut</td>
<td>Increased embryonal hepatocyte apoptosis</td>
<td>Impaired formation of Peyer’s patches</td>
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<td>Immune system</td>
<td>No peripheral lymph nodes</td>
<td>Loosened epithelial structure of small intestine</td>
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<td></td>
<td>Leukocytosis</td>
<td>Lethal bleedings</td>
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<td>Hair</td>
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<td>Patchy alopecia in older animals*</td>
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<td>No hair on tail and behind ears*</td>
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<td>One intermediate hair type*</td>
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<td>Thickened margin of eyelids*</td>
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<td>Absence or reduced size of sweat glands*</td>
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<td>Atrophy of Harderian glands*</td>
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<td>Reduced number of intestinal goblet cells</td>
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*Defects shared with Eda and Edar mice.

All phenotypic features of cκBaΔN mice were also observed for offspring of cκBaΔN×del-Cre matings. The hair alterations of cκBaΔN mice, except for lack of tail hair, were also seen in offspring of cκBaΔN×K14-Cre matings. cκBaΔN mice were indistinguishable from wild-type animals. The severity of organ defects in cκBaΔN mice increased in more pure-bred cκBaΔN mice of C57Bl/6 or of 129/Ola background.
also acts as a survival factor in the EDA/EDAR pathway. Our data demonstrate a requirement of NF-κB for epithelial-derived organogenesis. Thus, impaired NF-κB activation is a crucial event in anhydrotic ectodermal dysplasia.

NF-κB suppression results in macrophage dysfunction, chronic otitis media and deafness

The cIκBαΔN mice showed equilibrium problems (Table 1) and initially inner ear defects were assumed. But the inner ear structure and hair cells showed no abnormalities. However, hearing tests revealed deafness starting 4-6 weeks post partum. An analysis of the middle ear revealed chronic otitis media (Fig. 5A). The main pathogen was identified as Staphylococcus aureus in all mice tested, while wild-type littermates were free of pathogens. As macrophages play an important role in clearing S. aureus infections (Thomas et al., 2000), their functional capacity was tested in the Leishmania major infection model. Similar to Rel−/− mice (Gerondakos et al., 1999), cIκBαΔN mice infected with L. major developed significantly more severe lesions than wild-type littermates (Fig. 5B). Furthermore, strongly decreased NOS2 activity was detected in IFNγ- and TNFα-stimulated 1xBαΔN-positive bone marrow macrophages (Fig. 5C). However, splenocytes of cIκBαΔN mice produced the same or even higher amounts of L. major-specific IFNγ than control cells (Fig. 5D). Thus, increased infection susceptibility is caused by reduced NOS2 activity in cIκBαΔN macrophages, and not by type I T-helper-cell deficiencies. Otitis media may be caused by a combined effect of NF-κB inhibition on macrophages and on EDAR signaling, affecting mucous gland function. In fact, NF-κB activity was detected in the mucous membrane of the middle ear of Igκ seconalacZ mice (data not shown). In Edar/Eda mice most mucous-producing glands in the nasopharyngeal area, in the Eustachian tubes and in the tympanic cavity are missing (Gruneberg, 1971).

NF-κB is required for secondary lymphoid organ development

As a further major deficiency, systemic NF-κB suppression resulted in alymphoplasia. cIκBαΔN mice were devoid of peripheral lymph nodes and possessed only small numbers of minute axilar/brachial and superficial cervical lymph nodes (Fig. 6A). The lack of draining popliteal lymph nodes is consistent with a more than 100-fold increased parasite burden observed in spleens of L. major infected cIκBαΔN mice (see above). Mesenteric lymph nodes developed normally. Peyer’s patches (PPs) were absent (10% of cases) or severely reduced in size and numbers (90% of cases) (Fig. 6B). Spleens of cIκBαΔN mice did not show any macroscopic abnormalities, although preliminary data indicate abnormal segregation of T- and B-cell areas (not shown). Defects in secondary lymphoid organ development have been found in mice deficient in various TNF family members or their receptors, TNFRα/TNFR1, lymphotixin α or lymphotixin β, lymphotixin receptor and OPGL/RANK, and in AlyAly mice, which carry a mutation in the Nik (NF-κB-inducing-kinase) gene (Dougall et al., 1999; Fagarasan et al., 2000; Fu and Chaplin, 1999; Kong et al., 1999; Shinkura et al., 1999). Our data prove that NF-κB activation is needed for early secondary lymphoid organ development and implicate NF-κB as an essential downstream effector of these signaling pathways.

DISCUSSION

This study demonstrates that NF-κB transcription factors are required for the development of epidermal appendices, including hair follicles, exocrine glands and teeth. These novel physiological functions of NF-κB were identified by establishment of a mouse model which allows the analysis of the consequences of a systemic NF-κB suppression in adult mice. For this purpose we have integrated the signal-unresponsive NF-κB repressor 1xBαΔN into one allele of the β-catenin locus to allow effective and ubiquitous expression. The observed effects were specific for 1xBαΔN and independent of mono-allelic integration into β-catenin, as floxed cIκBαΔN mice revealed no defects, unless mated with deleter-Cre mice, whose offspring had the same phenotype as cIκBαΔN animals. Furthermore, it has previously been shown that mice with a heterozygous β-catenin gene inactivation have a wild-type phenotype (Haegel et al., 1995; Huelsken et al., 2000).

As predicted, cytokine-induced NF-κB in ES cells and in fibroblasts of 1xBαΔN-expressing mice was strongly suppressed, and as expected from p65-, IKKβ- or γ-deficient mice, cIκBαΔN animals revealed pronounced hepatocyte apoptosis. However, in contrast to these knockouts, cIκBαΔN mice survived, perhaps as residual NF-κB activity restricted apoptosis to a non-lethal level. Residual NF-κB activity in cIκBαΔN mice may also account for the absence of some of the phenotypes observed in NF-κB single or double knockouts. In particular, 1xBα has limited affinity for RelB and therefore 1xBαΔN should inhibit RelB complexes less sufficiently. Thus, multiorgan inflammation, observed in RelB-deficient mice (Weih et al., 1995), was not observed. In addition to early lethality, functional redundancy in the single or double NF-κB knockouts may have obscured important novel functions detected in this study.

NF-κB activity is requisite for formation of secondary lymphoid organs and macrophage-dependent immunity

We found that NF-κB activity is indispensable for formation of secondary lymphoid organs and for macrophage-dependent immunity. Aymphoplasia, the complete absence of peripheral lymph nodes and of Peyer’s patches (PPs), has previously been observed in AlyAly mice, which have a mutation in Nik (Fagarasan et al., 2000; Shinkura et al., 1999), and in mice deficient in NIK (Yin et al., 2001), lymphotixin β (LTβ) (Alimzhanov et al., 1997) or LTβ receptor (Futterer et al., 1998; Rennert et al., 1998). But it has not been detected in any of the NF-κB single or double knockouts reported so far. p105/p100 double knockouts reveal severe structural defects of lymph nodes and thymus, while p100-deficient mice have disorganized B- and T-cell areas in spleen and lymph nodes (Franzoso et al., 1998; Franzoso et al., 1997). However, all these models do form lymph nodes, presumably owing to functional redundancy at an early stage of development.

Our data are complementary to two recent reports revealing that the LTβ pathway activates NF-κB via NIK (Matsushima et al., 2001; Yin et al., 2001). Both find that NF-κB transcriptional activity induced by LTβ is impaired in NIK-deficient and NIK-mutant (AlyAly) fibroblasts. Yet, LTβ-
induced NF-κB DNA-binding activity is not impaired in NIK-deficient cells and the mechanism of action of NIK is not understood (Yin et al., 2001). In IKKα−/− fibroblasts, LTβ also fails to induce IkBα phosphorylation (Matsushima et al., 2001) and an essential role of IKKα for lymphorganogenesis was suggested by the lack of PPs in the embryonic intestine of IKKα−/− mice (Matsushima et al., 2001). However, these studies did not present a physiological evidence for a requirement of NF-κB in lymphorganogenesis.

**NF-κB is required for the development of epidermal appendices**

An overt defect in NF-κB deficient mice is the thin hair, caused by the absence of normal hair types. We further noted a characteristic lack of hair behind the ears and on the tail. A detailed analysis revealed a lack of plical digitalis and sweat glands in the foot pads and other pathological signs, such as delayed molar and incisor outgrowth, lack of Meibomian and Harderian glands, as well as defective hair follicle development in cIκBαAN mice.

In humans, congenital deficiencies of Meibomian glands, sweat glands and hair have been reported for an autosomal or X-linked syndrome, hypohidrotic (or anhydrotic) ectodermal dysplasia, caused by a mutation in the EDAR or EDA genes, respectively (Headon and Overbeek, 1999; Monreal et al., 1999). Humans suffering from this genetic disorder display a phenotype analogous to that of the Edar (autosomal) and Eda (X-linked) mice (Monreal et al., 1999). Eda encodes a novel TNF-family protein and recently it has been shown that it is the ligand of the TNF receptor homolog EDAR (downless) (Headon and Overbeek, 1999; Kumar et al., 2001; Monreal et al., 1999; Yan et al., 2000). Thus, cIκBαAN mice present a further model for HED.

We have used a complementary transgenic mouse model, which allows detection of NF-κB activity via an integrated NF-κB responsive β-gal reporter (IgκxcomalacZ) (Schmidt-Ullrich et al., 1996). Abundant NF-κB activity was detected in these transgenic mice, specifically in hair follicles, Meibomian and Harderian glands, sweat glands, and mucous glands (Fig. 3 and data not shown), all of which are ablated or severely affected in mice with suppressed NF-κB and in Eda or Edar mice. NF-κB activity in developing hair follicles was seen as early as stage I of hair follicle development (E12.5-E15.5). These findings rule out the possibility that NF-κB acts indirectly by affecting gene expression in the mesenchyme or through other distant structures, and underscore the importance of NF-κB activity at an early stage in the development of epidermal appendices. In fact, hair follicles of vibrissae and pelage hair displayed strong NF-κB activity in the matrix and in the bulge region, the latter of which contains stem cells that regenerate hair follicles after each cycle. This may hint to a further role of NF-κB in progression through the hair cycle phases: anagen (active growth phase), catagen (regression) and telogen (resting phase).

Hair follicles and other epidermal appendices originate as ectodermal placodes that result from the interaction of ectoderm with the underlying mesenchyme. The stages of hair follicle development take place at different time points (Hardy, 1992). The EDAR pathway is essential for the initiation of the development, when the placode is formed, starting at E14-E16 (Headon and Overbeek, 1999), although there are regional variations in timing (e.g. body, tail, whiskers, limb skin) (Headon and Overbeek, 1999; Wilson et al., 1994). At this stage, which is inhibited in Eda and Edar mice and also in the cIκBαAN mice, the cells of the placode are committed to differentiate into an epidermal appendix, which can still give rise to a hair follicle or an exocrine gland. The second stage, starting at E17, which commits the placode to make a hair follicle (Hardy, 1992) and depends on Lef-1/Wnt signaling (DasGupta and Fuchs, 1999; Kishimoto et al., 2000), is not inhibited. Interestingly, Edar, Eda and cIκBαAN mice do develop hair follicles at this stage, but only the awl type. As mentioned above, the coat of mice consists of four hair types: monotrichs or guard hairs, awls, zigzags and the rare auchen. The three main types, monotrichs, awl and zigzag have been traced to three different waves of hair follicle development in embryonic skin. The formation of monotrich and zigzag follicles (and possibly auchenises) is initiated at E14 and depends on EDA/EDAR. These hair types are not present in either Eda and Edar mice (Vielkind and Hardy, 1996) or in cIκBαAN mice. However, awl hairs do not develop until E16 and perhaps depend only on Wnt signaling. This is the reason why Eda, Edar and cIκBαAN mice exclusively have awl-type hairs in their coat.

EDA expression was unchanged throughout the epidermis in cIκBαAN mice (data not shown) and thus did not require NF-κB activity. At day E15 body hair follicle formation was suppressed in cIκBαAN mice and no EDAR-expressing placodes could be detected. However, EDAR was expressed in vibrissal follicles, which showed abundant NF-κB activity in the β-galactosidase stain. This may suggest that NF-κB is required downstream, not upstream, of Edar. However, for body hair follicles, we may not exclude the possibility that the upregulation of EDAR at the site of placode formation around E14 could in part be induced by NF-κB. But at E13, when EDAR is still expressed evenly throughout the basal layer of the epidermis (Headon and Overbeek, 1999), no NF-κB activity can be detected in the same cell layer of IgκxcomalacZ mice (data not shown). This suggests that EDAR expression is not regulated by NF-κB.

As a further intriguing finding, suppression of NF-κB (at E17) resulted in marked accumulation of apoptotic cells in hair follicles of pelage hair and vibrissae, indicating that NF-κB acts downstream of Edar to inhibit apoptosis. Thus, NF-κB may also play an important role in the homeostasis of hair follicles. In line with this, EDAR contains in its intracellular domain a death domain (Kumar et al., 2001). Similar to TNFR1, overexpressed EDAR activates NF-κB and JNK but involving a different interaction with signaling molecules, since it does not appear to sequester TRADD, FADD or TRAF2 in the same way as TNF-R1. Furthermore, when overexpressed, EDAR induces apoptosis in a caspase-independent manner (Kumar et al., 2001).

Intriguingly, cIκBαAN mice did not show the hyperplasia of the suprabasal squamous and cornified layers of the epidermis observed in IKKα−/− mice. The IKKα-deficient epidermis is thickened, owing to hyperproliferation of basal layer cells and blocked keratinocyte differentiation (Hu et al., 1999; Li et al., 1999a; Takeda et al., 1999). Very similarly, the epidermis of transgenic mice expressing a keratin 14 promoter-driven mouse IkBα mutant produced a hyperplastic epithelial basal layer (Seitz et al., 1998). It was therefore originally assumed
that IKKα was responsible for NF-κB activation in keratinocytes via an unknown inducing signal. However, as has recently been shown, hyperproliferation and blocked differentiation in the IKKα−/− epidermis can be rescued by kinase-inactive IKKα mutants. Furthermore, the skin function of IKKα is completely independent of NF-κB activity and involves the production of a soluble factor that induces keratinocyte differentiation (Hu et al., 2001). We also observed the hair alterations of cIkBαΔN mice, except for lack of tail hair, in identical form in offspring of cJosPlaBαΔN matings with animals expressing Cre under control of a keratin 14 promoter, but without the thickened epidermis seen by Seitz et al. (R. S.-U., unpublished). Furthermore, we did not detect any β-galactosidase activity in the epidermal layers, indicating that NF-κB is not activated in the suprabasal layer. The reason underlying this discrepancy must await further analysis and may be related to different expression levels of the super-repressor.

IKKγ knockout mice are yet another example of IKK involvement in skin development. The gene for IKKγ is located on the X chromosome and, hence, heterozygous female animals deficient in IKKγ present a patchy skin phenotype. These mice manifest changes in skin pigmentation, hyperproliferation of the suprabasal and cornified layers of the epidermis and granulocyte infiltrations in the epidermis, also observed in individuals with incontinentia pigmenti (IP; Makris et al., 2000; Schmidt-Supprian et al., 2000; Smahi et al., 2000). The alterations in pigmentation and granulocyte infiltration were not observed in IKKα−/− mice (Hu et al., 1999; Li et al., 1999a; Takeda et al., 1999). Nevertheless, IKKγ and IKKγ both seem to affect the differentiation of keratinocytes (Kaufman and Fuchs, 2000).


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Role of NF-{kappa}B in epidermal organogenesis


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