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Nfkbiz regulates the proliferation and differentiation of keratinocytes

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Abstract

Nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) inhibitor zeta (Nfkbiz) is a nuclear inhibitor of NF-κB (IκB) protein that is also termed as molecule possessing ankyrin repeats induced by lipopolysaccharide, interleukin-1-inducible nuclear ankyrin repeat protein, or IκBζ. We found previously that disrupting the Nfkbiz gene resulted in atopic dermatitis-like lesions in mice, suggesting an important role for Nfkbiz in the skin. In this study, we examined the cellular function of Nfkbiz in keratinocytes. Immunohistochemical analyses for Ki-67 revealed that Nfkbiz^{-/-} keratinocytes were hypoproliferative. In skin from Nfkbiz^{-/-} mice, the expression of the keratinocyte differentiation markers K10 and filaggrin were reduced, although that of K14 was unchanged. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay revealed that the frequency of apoptosis was comparable between control and Nfkbiz^{-/-} keratinocytes. Interestingly, the subcellular localization of the NF-κB subunits and the transcriptional activity of NF-κB were not changed in Nfkbiz^{-/-} keratinocytes. These findings indicate a novel possible role of Nfkbiz in controlling the proliferation and differentiation of epidermal keratinocytes through NF-κB-independent mechanisms.

Key Words: IκBζ, INAP, Mail, NF-κB, skin

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Introduction

Nuclear factor of kappa light polypeptide gene enhancer in B cells (NF- κ B) inhibitor zeta (Nfkbiz) is an inhibitor of nuclear factor- κ B (I κ B) that localizes to the nucleus. It is also known as molecule possessing ankyrin repeats induced by lipopolysaccharide, interleukin-1-inducible nuclear ankyrin repeat protein, and I κ B γ ^{5,10,29}. Nfkbiz acts as a transcriptional regulator of various genes in response to inflammatory stimuli such as lipopolysaccharide and IL-1²⁸. We reported previously that mice with systemic Nfkbiz gene disruption (Nfkbiz^{-/-}) showed atopic dermatitis-like lesions at 4–8 weeks of age²⁵. We also found that Nfkbiz was constitutively expressed and strongly induced by IFN- γ in epidermal keratinocytes^{8,15}. However, the role of Nfkbiz in epidermal keratinocytes and the reason behind atopic dermatitis in Nfkbiz^{-/-} mice are still largely unknown.

Nuclear factor- κ B (NF- κ B) is a complex formed by homo- and hetero-dimerization of the NF- κ B family molecules RelA, RelB, c-Rel, p50, and p52. NF- κ B, and its regulatory molecules such as I κ B α and IKK α , are important for not only cellular inflammatory responses but also skin physiology¹⁶. Accumulating evidence indicates that inactivation of NF- κ B increases keratinocyte proliferation, while activation of NF- κ B induces keratinocyte differentiation^{6,12,19,23,24,26,27,30}. Because Nfkbiz interacts with the p50 NF- κ B subunit and increases its activity in macrophages^{22,28}, it is possible that Nfkbiz is involved in skin physiology and pathology by affecting NF- κ B activity in epidermal keratinocytes.

In the present study, we examined the roles of Nfkbiz in epidermal keratinocytes. Specifically, we sought to determine whether the disruption of Nfkbiz changed the proliferative activity and differentiation status of keratinocytes, and whether the function of Nfkbiz in keratinocytes depended on the activity of NF- κ B. We addressed these issues by performing immunohistochemical analyses for cell proliferation markers, keratinocyte

differentiation markers, and subunits of NF- κ B in the skin of Nfkbiz^{-/-} mice. The experiments revealed defects in the proliferation and differentiation of Nfkbiz^{-/-} keratinocytes, but showed that the localization and activity of NF- κ B were unchanged in these cells. Therefore, we suggest that Nfkbiz has a novel NF- κ B-independent function in skin homeostasis.

Materials and Methods

Animals: Nfkbiz^{tm1Mamo} (hereafter Nfkbiz^{-/-}) mice (backcrossed with C57BL/6 at least five generations) and wild-type littermates were maintained under specific-pathogen-free conditions²⁵. Experimental procedures and the care of animals were in accordance with the Hokkaido University Regulations on Animal Experimentation and approved by the Institutional Animal Care and Use Committee.

Histological analyses: For immunohistochemical analyses, fresh samples of truncal skin isolated from 3-day-old mice were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and snap-frozen in liquid nitrogen. Cryosections (5 μ m) were fixed in cold acetone for 10 min and stained with specific antibodies by using the streptavidin-biotin immunoperoxidase technique, as described previously¹⁵. The positive signals were developed using 3,3'-diaminobenzidine (DAB). Anti-cytokeratin-14 (K14) (Covance, Denver, PA, USA), anti-cytokeratin-10 (K10) (Covance), anti-filaggrin (Covance), anti-Ki-67 (DAKO, San Diego, CA, USA), anti-I κ B α (Sigma-Aldrich, St. Louis, MO, USA), anti-RelA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-c-Rel (Santa Cruz Biotechnology), anti-p50 (Santa Cruz Biotechnology), and anti-p52 (Santa Cruz Biotechnology) were used. Dilution conditions are shown in Table 1. A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction kit (TAKARA, Tokyo, Japan) was used to detect apoptotic cells.

Table 1. Dilution conditions of antibodies

Antibody	Dilution
K14	1 : 8000
K10	1 : 8000
filaggrin	1 : 2000
Ki-67	1 : 25
I κ B α	1 : 500
RelA	1 : 100
c-Rel	1 : 100
p50	1 : 800
p52	1 : 100

Quantification of DAB intensity in tissue: The intensity of DAB staining was measured on digital images stained for specific differentiation markers (K14, K10, and filaggrin) using ImageJ software (NIH, Bethesda, MD, USA). A DAB intensity of 50 (range 0–255) was established as the threshold for distinguishing pixels of specific immunoreactive tissue markers from those of the background. DAB intensity represented the average brightness of all specific immunoreactive markers in the skin section. This value was calculated from all pixels with DAB intensities $\geq$ 50. The mean value was calculated from five areas (200 μm^2 each) of the image.

Cell culture: Primary keratinocytes were isolated from neonatal mice sacrificed within 3 days of birth as described previously⁸⁾. In brief, the truncal skin was excised and disinfected using 70% ethanol. After overnight 1000 U/ml dispase (Godoshusei, Tokyo, Japan) treatment for separating the epidermis and dermis, the epidermis was treated with 0.05% trypsin (Life Technologies, Carlsbad, CA, USA) for 10 min at 37°C to disaggregate the keratinocytes. Trypsin was neutralized with phosphate-buffered saline containing 5% Chelex-treated fetal calf serum. After washing twice with serum-free phosphate-buffered saline, the keratinocyte suspension was centrifuged for 5 min at $1,000 \times g$ to pellet the cells. The keratinocytes were resuspended and grown in a serum-free keratinocyte growth

medium (KGM2 Bullet kit; Cambrex, Walkersville, MD, USA) containing 20 μM CaCl₂, $1 \times$ human keratinocyte growth supplement, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin. After 2 weeks, cells were fixed with cold acetone for 10 min, permeabilized with 0.3% Triton X-100, and stained with anti-K14 antibody by using the streptavidin-biotin immunoperoxidase technique. Positive signals were developed using DAB.

Statistical analysis: Significant differences between means were assessed by Student's *t*-test with $P < 0.01$ considered significant.

Results

Proliferation of keratinocytes

Because it is known that ablation of I κ B α in keratinocytes results in accelerated proliferation of skin cells¹⁹⁾, we investigated the proliferative activity of epidermal cells from Nfkbiz^{-/-} mice. The frequency of Ki-67-positive cells was significantly lower in Nfkbiz^{-/-} than in control mice (Fig. 1a, b). The thickness of the epidermis was comparable between Nfkbiz^{-/-} and control mice. Quantitative analysis revealed that the number of Ki-67-positive cells was 90% less in Nfkbiz^{-/-} than in control mice (Fig. 1c). Primary cultured keratinocytes isolated from Nfkbiz^{-/-} mice also showed a proliferative defect (Fig. 1d).

Differentiation status of keratinocytes

Immunohistochemical analyses for the expression of specific differentiation markers relative to that in control mice revealed comparable expression of K14 (a specific marker for basal cells) (Fig. 2a, b) and decreased expression of K10 (a marker for stratum spinosum) (Fig. 2d, e) and filaggrin (a specific marker for stratum granulosum) (Fig. 2g, h) in Nfkbiz^{-/-}. Quantitative analysis of DAB staining intensity revealed that the expression of K10 and filaggrin in skin from Nfkbiz^{-/-} mice was significantly decreased by 23% and 30%, respectively, relative to that in

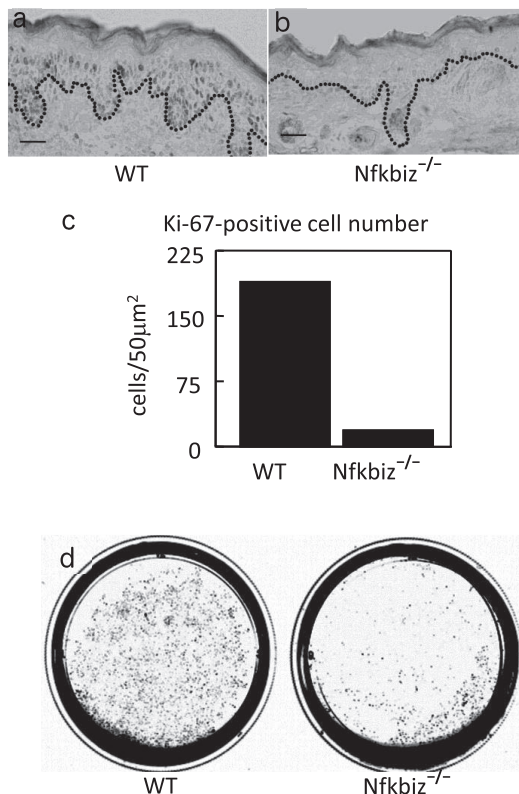


Fig. 1. Proliferation of keratinocytes in Nfkbiz^{-/-} mice. Micrographs of immunohistochemical analysis for the proliferation marker Ki-67 in (a) wild-type (WT) control and (b) Nfkbiz^{-/-} mice. Three mice were analyzed in each group. The dots show the basement membrane. (c) The numbers of Ki-67-positive cells within 50 μm² were counted. (d) Primary keratinocytes were cultured for 2 weeks and stained with an antibody specific for K14. Bar: 50 μm

control mice.

Apoptosis of keratinocytes

Epidermal keratinocytes undergo apoptosis when they terminally differentiate, a process known as cornification³⁾. Owing to the changed differentiation status of keratinocytes in Nfkbiz^{-/-} mice, we investigated the frequency of apoptotic cells in the stratum corneum. Interestingly, the number of apoptotic cells was comparable between Nfkbiz^{-/-} and control mice (Fig. 3).

Localization of NF-κB and IκBα

Nfkbiz interacts with the p50, one of the subunits of NF-κB²²⁾. Therefore, we investigated the localization of NF-κB subunits.

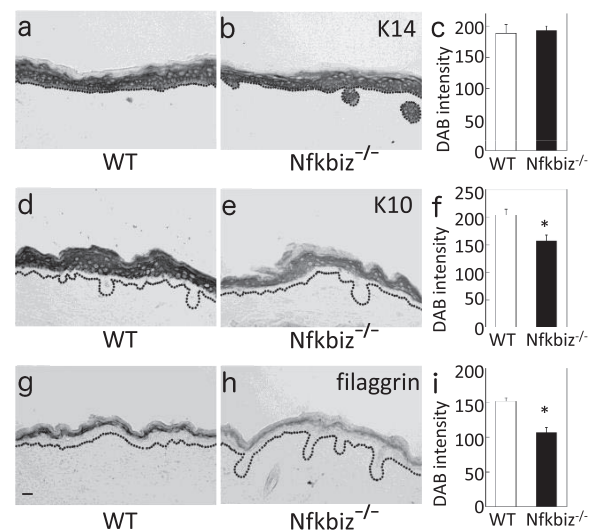


Fig. 2. Immunohistochemical analyses of specific differentiation markers in the skin. Micrographs of immunohistochemical analysis for (a, b) K14, (d, e) K10, and (g, h) filaggrin of wild-type (WT) or Nfkbiz^{-/-} mice. Three mice were analyzed in each group. The dots indicate the basement membrane. DAB intensity was analyzed using ImageJ software (c, f, i). The mean value was calculated from five areas (200 μm² each) of the image. **P* < 0.01 compared with WT. Bar: 50 μm

Immunohistochemical analyses revealed no differences in the localization of NF-κB subunits between Nfkbiz^{-/-} and control mice (Fig. 4a–h). In addition, the expression of IκBα, a typical transcriptional target gene of NF-κB, was comparable between Nfkbiz^{-/-} and control mice (Fig. 4i, j).

Discussion

In this study, we have shown that disruption of the Nfkbiz gene leads to a lower proliferation rate and abnormal differentiation of epidermal keratinocytes. Evidence of a lower proliferation rate included significantly reduced Ki-67-positive cell population in the skin of Nfkbiz^{-/-} mice and significantly lower total number of Nfkbiz^{-/-} keratinocytes after culturing for 2 weeks. Abnormal differentiation was supported by the finding that expression of the differentiation markers K10 and filaggrin was significantly lower in the skin of Nfkbiz^{-/-} mice than in the

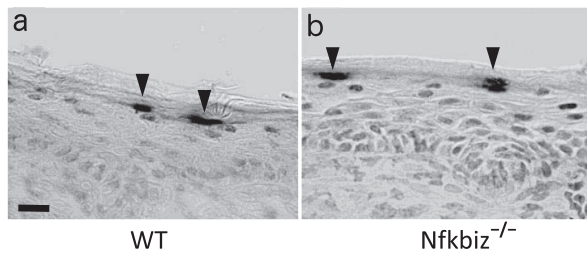


Fig. 3. Apoptosis of keratinocytes in stratum corneum. Apoptotic cells (arrow heads) were detected by the TUNEL reaction. (a) Wild-type (WT) control. (b) *Nfkbiz*^{-/-}. Bar: 20 μ m

skin of control mice.

Nfkbiz^{-/-} mice show atopic dermatitis-like lesions²⁵. Atopic dermatitis is one of the most common chronic inflammatory skin diseases. The pathophysiology of atopic dermatitis is complex and involves numerous genetic and environmental factors². Some recent studies have suggested that abnormalities in the permeability barrier lead to atopic dermatitis^{9,14,20}. The strongest and most widely replicated risk factor for atopic dermatitis is loss-of-function mutations of filaggrin^{17,20}. Therefore, the decrease of filaggrin in *Nfkbiz*^{-/-} keratinocytes may be a cause of atopic dermatitis in *Nfkbiz*^{-/-} mice.

The epidermis consists of five layers: a basal layer, spinous layer, granular layer, transition zone (stratum lucidum), and cornified layer. In this study, we found abnormalities in the basal layer and granular layer. We previously found that *Nfkbiz* was constitutively expressed in epidermal keratinocytes by using whole epidermal samples and primary cultured keratinocytes¹⁵. Although it remains unclear which cell layer is the major source of *Nfkbiz*, the abnormality found in the basal layer may be critical because the basal layer is the cell source for all four overlying layers. Detailed analysis of the epidermal cell layer will provide information regarding the pathological mechanisms of atopic dermatitis in *Nfkbiz*^{-/-} mice.

Nfkbiz^{-/-} mice develop atopic dermatitis at 4–8 weeks of age²⁵. In this study, we analyzed 3-day-old mice that had not yet developed atopic dermatitis. It is still unknown whether

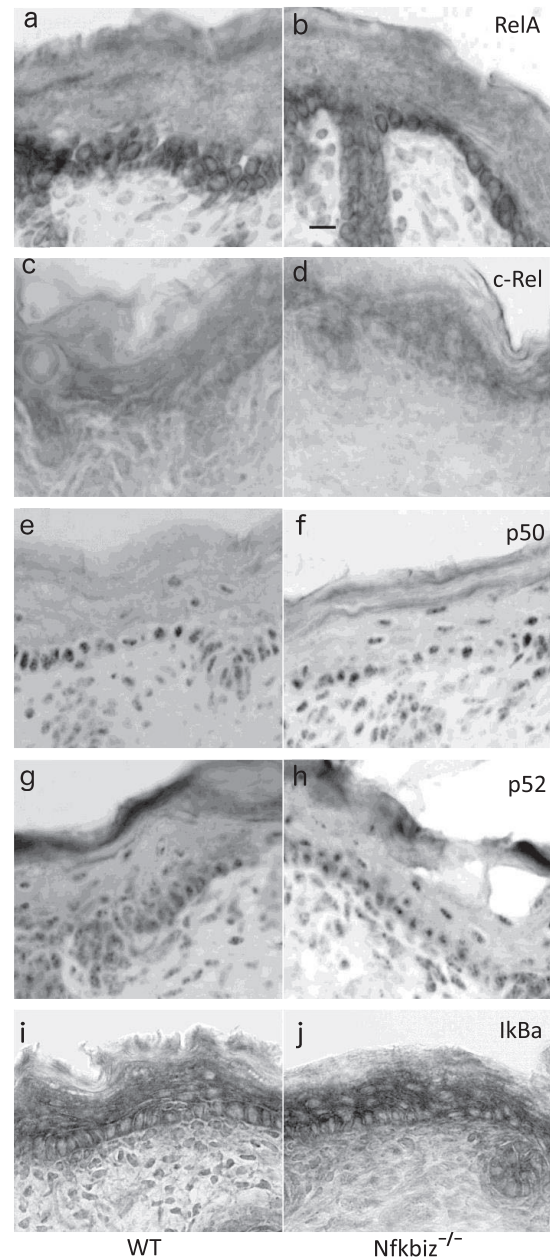


Fig. 4. Immunohistochemical analysis for NF- κ B and I κ B α . Micrographs of immunohistochemical analysis for (a, b) RelA, (c, d) c-Rel, (e, f) p50, (g, h) p52, and (i, j) I κ B α of wild-type (WT) (a, c, e, g, i) and *Nfkbiz*^{-/-} mice (b, d, f, h, j). Bar: 50 μ m. a, c, e, g: Reproduced with permission of the Japanese Society of Veterinary Science from Oonuma T, *et al.*: Role of NF- κ B in Constitutive Expression of MAIL in Epidermal Keratinocytes. *J Vet Med Sci* 2007; 69; 279–284.

dysfunction of *Nfkbiz* at this age is a direct or indirect cause of the atopic dermatitis that develops at 4–8 weeks of age. A time course

analysis of Nfkbiz expression in the epidermis and an analysis of mice with time- and tissue-specific Nfkbiz deletion would answer this question.

Many NF- κ B signaling molecules are involved in keratinocyte physiology and pathology¹⁶. Knockout of I κ B α , an inhibitor of NF- κ B, leads to severe dermatitis in mice^{1,11,19}. In these mice, nuclear localization of RelA is observed and the transcriptional activity of NF- κ B is constitutively upregulated. Rebholz *et al.* showed that the mRNA expression levels of some NF- κ B responsive genes are significantly upregulated in I κ B α ^{-/-} epidermis¹⁹. In contrast, suppression of NF- κ B by a constitutively active I κ B α mutant leads to defective morphogenesis of follicles and other skin appendages²¹. These reports indicate that hyperactivation of NF- κ B in keratinocytes leads to severe dermatitis and that suppression of NF- κ B leads to developmental skin defects. In this context, because Nfkbiz interacts with the p50 NF- κ B subunit and upregulates, rather than suppresses, its activities^{22,28}, the observation of atopic dermatitis in Nfkbiz^{-/-} mice seems to be paradoxical. One possible explanation for this paradox is that Nfkbiz may be involved in skin physiology independently of NF- κ B activity because we did not observe any changes of NF- κ B activity in skin from Nfkbiz^{-/-} mice.

The NF- κ B pathway in keratinocytes appears to be distinct from that in other cell types^{4,18}. There is an NF- κ B signaling molecule, IKK α , that regulates keratinocytes differentiation independently of NF- κ B⁷. Mulero *et al.* reported that phosphorylated and sumoylated I κ B α regulates keratinocyte differentiation by interacting with histone proteins¹³ without NF- κ B. Our findings suggest the presence of a novel NF- κ B-independent mechanism whereby Nfkbiz modulates keratinocyte proliferation and differentiation.

In conclusion, we have shown that Nfkbiz regulates keratinocytes proliferation and differentiation independently of NF- κ B. Thus, a defect in Nfkbiz function in epidermal keratinocytes may cause atopic dermatitis.

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