Impaired NF-κB Activation and Increased Production of Tumor Necrosis Factor α in Transgenic Mice Expressing Keratin K10 in the Basal Layer of the Epidermis*

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Both the diversity and the precisely regulated tissue- and differentiation-specific expression patterns of keratins suggest that these proteins have specific functions in epithelia besides their well known maintenance of cell integrity. In the search for these specific functions, our previous results have demonstrated that the expression of K10, a keratin expressed in postmitotic suprabasal cells of the epidermis, prevents cell proliferation through the inhibition of Akt kinase activity. Given the roles of Akt in NF-κB signaling and the importance of these processes in the epidermis, a study was made into the possible alterations of the NF-κB pathway in transgenic mice expressing K10 in the proliferative basal layer. It was found that the inhibition of Akt, mediated by K10 expression, leads to impaired NF-κB activity. This appears to occur through the decreased expression of IKKβ and IKKγ. Remarkably, increased production of tumor necrosis factor α and concomitant JNK activation was observed in the epidermis of these transgenic mice. These results confirm that keratin K10 functions in vivo include the control of many aspects of epithelial physiology, which affect the cells not only in a cell autonomous manner but also influence tissue homeostasis.

The keratins are the main components of the intermediate filament cytoskeleton in epithelial cells. The functions of these proteins were clarified when human epithelial fragility syndromes were attributed to mutations of epidermal keratin genes (for reviews see Refs. 1–4). However, this shared function does not clearly explain the great diversity of these proteins, which suggests they may have additional family member-specific functions.

In the search for possible specific keratin functions, a study was made of keratin K10. This protein is expressed in postmitotic differentiating keratinocytes in epithemis in vivo (5), and its expression is severely reduced in hyperproliferative situations, including skin tumors (6, 7). It has been shown that the expression of this keratin inhibits cell proliferation in cultured cells and in transgenic mice (8–10). The modulation of cell growth by keratin K10 is linked to the retinoblastoma (pRb) protein and the molecular machinery controlling cell cycle progression during G1, in particular cyclin D1 expression (8, 9). This activity appears to be promoted by the sequestration of Akt to the keratin cytoskeleton, mediated by keratin K10 through its amino terminus. This leads to decreased Akt kinase activity (9). More recently these results have been amplified to include in vivo situations (10). Transgenic mice were generated in which human keratin K10 gene expression was targeted to the basal layer of the epidermis by using the bovine keratin K5 promoter (11). These animals displayed severe alterations in the epidermis, including decreased proliferation, which results in hypoplastic epidermis, associated with impaired activation of Akt kinase activity in the skin. Finally, by using chemical skin carcinogenesis protocols, it was demonstrated that K10 expression reduces the formation of tumors in vivo (10). These results are in agreement with the recently described fundamental roles of Akt kinase in mouse skin carcinogenesis (12).

Akt is a serine/threonine kinase that plays an important role not only in tumorigenesis but also in many aspects of cell physiology (reviewed in Refs. 13–16). This kinase phosphorylates many cellular substrates involved in the control of cell proliferation, apoptosis, and metabolism. Its role in activating Akt in NF-κB signaling has recently been demonstrated, which may be an essential antiapoptotic event. However, the mechanism responsible for the activation of NF-κB by Akt remains uncertain and may depend on the cell type analyzed (17–19).

The canonical activation of NF-κB requires the phosphorylation of the inhibitory IκB protein by a high molecular weight complex that includes IκKα, IκKβ, and IκKγ1 proteins. This allows the NF-κB to move to the nucleus and activate target genes. In this context, it has been shown that Akt may increase the transactivation of the p65 subunit of NF-κB, without interfering with its nuclear localization (20–22). Similarly, the increased transactivation of p50 subunit by Akt phosphorylation has been reported (23). Furthermore, the phosphorylation of IκK proteins by Akt, leading to increased phosphorylation and thus degradation of IκB proteins, has been widely reported (24–27). Finally, in breast cancer lines, it has been suggested that Akt mediates the calcipain degradation of IκB protein instead of the consensus ubiquitin pathway (28). Collectively, all these studies demonstrate the importance of Akt in activating...

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1 The abbreviations used are: IκK, IκB kinase; TNFα, tumor necrosis factor α; JNK, c-Jun terminal kinase; IL-6, interleukin-6; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; wt, wild type; JNK, c-Jun NH2-terminal kinase; TRAF2, TNF receptor-associated factor 2; EMSA, electrophoretic mobility shift analysis; GST, glutathione S-transferase; BoD, bromodeoxyuridine; RIP, receptor-interacting protein; TRADD, tumor necrosis factor-associated death domain.
ing NF-κB signaling, but also emphasize the controversy in identifying the molecular mechanisms responsible for such activation.

The importance of NF-κB signaling in epidermis has been highlighted in recent years (reviewed in Ref. 29). This stems from findings obtained in transgenic mice ectopically expressing a non-degradable IκBα protein (IκBαM), or which overexpress p50 and/or p65 subunits in the epidermis (30). In these animals, increased NFκB activity leads to epidermal hyperplasia and growth inhibition, probably in association with p21WAF1/CIP1 expression (30, 31). The repression of NF-κB activity, on the other hand, produces epidermal hyperplasia (30) and may cause spontaneous squamous cell carcinomas in transgenic mice (32). Nevertheless, we have observed increased endogenous NF-κB activity during chemically induced mouse skin tumorigenesis or upon UV treatment of mouse skin (33, 34). This apparent discrepancy might be explained in that the overexpression of regulatory molecules used in earlier transgenic studies may differ from those involved in endogenous activity. In this regard, one has to take into account that dominant negative IκBα expression does not generate a true NF-κB null phenotype and that nuclear accumulation of Bcl-3 and its interaction with other NF-κB dimers is independent of inhibition by other IκB proteins and IKK stimulation. In agreement with this, neither tumors nor phenotypic alterations were observed in the absence of IκBα overexpression in epithelium (32). This suggests that a threshold in the level of expression of this protein is required to produce the phenotype. More recently, using a knock in approach, which does not produce massive overexpression, it has been shown that the repression of NF-κB promotes severe epidermal defects affecting hair follicle development and increased apoptosis but not increased proliferation or abnormalities in differentiation (35). Finally, the epidermal-specific ablation of IKKβ, which is accompanied by a deficiency in NF-κB activation, results in a TNF-mediated inflammatory skin disease but does not led to hyperproliferation or impaired keratinocyte differentiation (36).

Another line of information on the roles of NF-κB signaling in keratinocytes comes from the strong phenotypes observed in the epidermis of mice lacking the IκKα or IκKβ subunits of the IKK complex (37-41). In the case of IκKα, the abnormalities are not only due to the possible alterations of NF-κB signaling but also to the decreased production of a yet-unidentified soluble factor capable of inducing keratinocyte differentiation (42).

Given the above importance of Akt modulation of NF-κB activity in a range of systems, and the importance of NF-κB in epidermal physiology, an investigation was made into the possible alterations in this pathway that might arise as a consequence of keratin K10 ectopic expression in the basal layer of the epidermis. A dramatic decrease was seen in NF-κB activity in both transgenic skin and cultured mouse keratinocytes. This decrease is attributed to the inhibition of IKK activity associated with decreased expression of IκKα and IκKγ. Remarkably, the transgenic animals showed aberrant overproduction of TNFα, and concomitant increased JNK activity, which may account for some of the phenotypic characteristics observed in K5hK10 transgenic mice.

**Materials and Methods**

**Transgenic Construction and Generation of Transgenic Mice**—The plasmid b5k5K10 was used to generate transgenic mice in a C57BL/10 × BALB/c F1, and (C57BL/10 × DBA/2) F1 genetic background as previously described (10). The presence of the transgene was analyzed by Southern blots. Homozygous and heterozygous mice were identified using a PhosphoImager scanner (Bio-Rad) as reported (10). Primary keratinocyte cultures were established isolating keratinocytes from newborn mice cultured in Eagle's minimal medium containing 8% Chelex-treated serum and 0.03 mM Ca2+ as previously described (43).

**Histological Analysis**—Dorsal skin samples and tumors were fixed either in formalin or ethanol and embedded in paraffin prior to sectioning. 4-μm sections were cut and stained with hematoxylin-eosin. Immunodetection of dermal inflammatory cells including T lymphocytes (anti-CD3ε), granulocytes (anti-Ly-6G/Gr-1), and macrophages (anti-CD11b/Mac1) was performed in frozen sections using fluorescein isothiocyanate (FITC)-labeled specific antibodies prepared from Calbiochem and diluted 1/100, followed by horseradish-peroxidase-labeled anti-rabbit antibody (Jackson ImmunoResearch Laboratory, diluted 1:2000). Positive staining was determined using diaminobenzidine as a substrate (diaminobenzidine kit, Vector, Burlingame, CA) following the manufacturer's recommendations. Sections were then counterstained with hematoxylin and mounted. For ultrastructural analysis, skin samples were fixed in 2.5% glutaraldehyde in PBS and postfixed in 1% osmium tetroxide prior to dehydration and embedding in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate.

**Plasmas and Transfection**—The 8xEc-B-TK-CAT plasmid reporter gene used for transfection assays contained eight copies of the 1x Ec-B binding site. NF-κB binding sites of the TNFα promoter were cloned in pCDNA3 under the cytomegalovirus promoter/enhancer to generate a true NF-κB reporter. The repression of NF-κB activity, on the other hand, produces epidermal hyperplasia (30) and may cause spontaneous squamous cell carcinomas in transgenic mice (32). Nevertheless, we have observed increased endogenous NF-κB activity during chemically induced mouse skin tumorigenesis or upon UV treatment of mouse skin (33, 34). This apparent discrepancy might be explained in that the overexpression of regulatory molecules used in earlier transgenic studies may differ from those involved in endogenous activity. In this regard, one has to take into account that dominant negative IκBα expression does not generate a true NF-κB null phenotype and that nuclear accumulation of Bcl-3 and its interaction with other NF-κB dimers is independent of inhibition by other IκB proteins and IKK stimulation. In agreement with this, neither tumors nor phenotypic alterations were observed in the absence of IκBα overexpression in epithelium (32). This suggests that a threshold in the level of expression of this protein is required to produce the phenotype. More recently, using a knock in approach, which does not produce massive overexpression, it has been shown that the repression of NF-κB promotes severe epidermal defects affecting hair follicle development and increased apoptosis but not increased proliferation or abnormalities in differentiation (35). Finally, the epidermal-specific ablation of IKKβ, which is accompanied by a deficiency in NF-κB activation, results in a TNF-mediated inflammatory skin disease but does not led to hyperproliferation or impaired keratinocyte differentiation (36).

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20 mM Tris-HCl, pH 7.5, 1 µg/ml aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM dithiothreitol, and 1 mM Na3VO4). The kinase activity for Akt and IKK complexes was determined by immunoprecipitation of the endogenous kinase proteins using anti-Akt1/2 antibody or a mixture of different IKKα antibodies (Santa Cruz Biotechnology). Histone H2B was used as a substrate for Akt, and full-length IkBa (Santa Cruz Biotechnology) for IKK in *in vitro* kinase assays (essentially as described in Refs. 12 and 45). Jun kinase assay was performed essentially as described (46) upon immunoprecipitation of JNK using GST-c-Jun (kindly provided by Dr. J. S. Gutkind) as substrate.

*Northern Blots—*Total RNA from freshly harvested mouse epidermis and frozen tumors was isolated by guanidine isothiocyanate-phenolchloroform extraction. Northern blots containing total RNA (15 µg/lane) were probed for expression of TNFα, IL-6, and IL-1 employing DNA probes prepared by random primed reactions using the complete sequences. The membranes were also hybridized with a keratin K14 cDNA probe to verify that equal amounts of mRNA were loaded and transferred.

*TNF Determination—*The quantification of TNFα in mouse serum and in the culture supernatant from primary keratinocytes was performed using an ELISA kit (R&D) following the manufacturer’s recommendations.

### RESULTS

#### Decreased NF-κB Activity in bK5hK10 Transgenic Mice—

Transgenic mice ectopically expressing K10 in the basal layer of the epidermis display severe epidermal abnormalities associated with decreased proliferation in close conjunction with Akt kinase activity inhibition (10). Given the importance of Akt in the activation of NF-κB (see the introduction), the present study was designed to investigate NF-κB binding activity in the epidermis of transgenic mice through electrophoretic mobility shift analysis using a κB-labeled oligonucleotide. In non-transgenic samples, two retarded complexes were observed (Fig. 1A, lanes 1 and 2) that were identified as p50-p50 homodimers and p50-p65 heterodimers by supershift experiments using specific antibodies (not shown; see also Ref. 34). Severely decreased DNA binding activity was observed in heterozygous transgenic mice (Fig. 1A, lanes 3 and 4). The κB binding activity in homozygous animals was barely detectable (Fig. 1A, lanes 5 and 6). Interestingly, the inhibition of Akt kinase activity runs in parallel with the increased K10 expression observed in heterozygous and homozygous transgenic mice (Fig. 1A′) (not shown, see also Ref. 10). Given that we used whole skin extracts for these experiments, and to rule out the possibility that other cell types were actually responsible for the observed effect, similar analysis were performed using primary keratinocytes derived from wt and bK5hK10 transgenic mice. In addition, we also tested whether stimulation with IL-1α could induce increased DNA binding in these cells. IL-1α treatment led to increased NF-κB activity in non-transgenic keratinocytes but not in keratinocytes derived from transgenic animals (Fig. 1B). Western blot analysis against keratin K14 (Fig. 1B′) also demonstrated that this effect was not due to different amounts of keratinocyte protein in the assays. These data show that the expression of K10 leads to a dramatic reduction in NF-κB activity in keratinocytes and impedes the activation of this complex on stimulation.

Experiments were then performed to see whether this decreased NF-κB activity was due to K10 expression and K10-mediated Akt inhibition. PB mouse keratinocytes were transfected with a NFκB reporter element (8xκB-CAT) plus empty vector, K10, or K10 plus an expression vector for wt Akt (Fig. 1C). It has previously been shown that co-expression of wt Akt is sufficient to override K10-induced cell cycle arrest in keratinocytes (10). The expression of K10 produced a severe inhibition of NF-κB transcriptional activity induced either by IL-1β or TNFα (Fig. 1, C and C′, respectively), whereas co-expression of Akt almost completely abolished such inhibition (Fig. 1, C and C′). The quantification of TNFα in mouse serum and in the culture supernatant from primary keratinocytes was performed using an ELISA kit (R&D) following the manufacturer’s recommendations.

![](http://www.jbc.org/) **Fig. 1. Expression of human keratin K10 in the epidermis of transgenic mice results in impaired NF-κB signaling.** A, whole cell extracts from non-transgenic, heterozygous, and homozygous bK5hK10 transgenic mice were analyzed by EMSA using a labeled κB oligonucleotide. The composition of the complexes is indicated on the right. A′, *in vitro* Akt kinase activity of the same protein extracts. B, cultured primary keratinocytes obtained from non-transgenic and homozygous transgenic mice were analyzed by EMSA as in A. Where indicated, the keratinocytes were also stimulated by incubation (10 min) in the presence of IL-1α (10 ng/ml). Note that in transgenic epidermis and primary keratinocytes there is a dramatic inhibition of NF-κB signaling. No stimulation was observed. B′, the same protein extracts shown in B were analyzed by Western blot against K14 to rule out the possibility that the observed differences were due to different protein concentrations. C and C′, the K10-mediated inhibition of Akt is responsible for impaired NF-κB signaling. PB keratinocytes were transfected with the quoted plasmids. 48 h after transfection cells were stimulated as indicated with TNFα or IL-1β (both 10 ng/ml for 10 min). Chloramphenicol acetyltransferase assays were performed using ELISA (Roche Molecular Biochemicals), and the activities were normalized to β-galactosidase activity. Data were taken from duplicate experiments and are shown as mean ± S.D.
and C). Finally, to determine whether the inhibition of Akt activity might be sufficient to account for the decreased NF-κB activity, the reporter plasmid was co-transfected with a dominant negative form of Akt, resulting in almost complete inhibition of NF-κB activity in response to TNFα or IL-1β (Fig. 1, C and C). Together, these results demonstrate that K10-mediated Akt inhibition leads to impaired NF-κB basal activity and NF-κB activation in cultured keratinocytes and in epidermis in vivo.

Ectopic Expression of K10 Leads to Decreased IKKβ and IKKγ Expression in Skin—A study was then undertaken to determine whether the decreased NF-κB activity found in the transgenic mouse epidermis was due to altered expression of different NF-κB family members (Fig. 2A). No major alterations in p65 (RelA) or p50 protein levels were detected by Western blotting (Fig. 2A), and a decrease in only p50 was observed in whole skin extracts from homozygous transgenic mice (Fig. 2A). Because the NF-κB activity is dependent on the activity of the IKK complex, the expression of IKKα, IKKβ, and IKKγ was analyzed. Surprisingly, in homozygous transgenic mice there was a significant decrease in IKKβ and IKKγ levels (Fig. 2A'). Because these two components of the IKK complex are essential for IκB phosphorylation and for NF-κB activation (47, 48), IκB kinase activity was evaluated in the transgenic mouse extracts (45). Almost complete inhibition of the IκB kinase activity was seen in homozygous mouse extracts (Fig. 2A'). These results strongly indicate that the decrease in Akt activity elicited by K10 also results in a dramatic inhibition of IκB kinase activity.

The decreased protein levels of the IKK components and the concomitant inhibition of IKK activity are very striking. It was therefore, through experiments similar to those shown in Fig. 1 (C and C'), determined whether expression vectors for IKKβ or IKKγ were able to rescue the K10-induced inhibition of NF-κB activity. IKKβ co-expression partially restored the NF-κB inhibition elicited by K10, whereas IKKγ co-expression totally rescued it (Fig. 2, B and B'). These results strongly indicate that the reduction of IKKβ and IKKγ in transgenic epidermis is probably responsible for the K10-induced impaired activation of NF-κB activity. In addition, the partial rescue observed with IKKβ also indicates that a residual amount of IKKγ must be
present upon K10 expression, because this protein is essential for IκB kinase activity (48).

Inhibition of Akt Leads to Decreased IKKγ Expression—The above commented results prompted us to analyze if the observed decrease in Akt activity might be responsible of the decrease in IKKβ and IKKγ proteins. To this, PB keratinocytes were infected with retrovirus coding for dominant negative Akt, wt Akt, or empty retroviral backbone. Forty-eight hours after infection protein extracts were obtained and used for Western blot determination of IKKα, IKKβ, IKKγ, and Akt as well as Akt phosphorylated in Ser-473. The results (Fig. 3) demonstrate that the inhibition of Akt leads to a significant decrease in IKKβ and IKKγ proteins, whereas IKKα remained unaffected. This indicates that the observed decrease in these two proteins in skin extracts from bK5hK10 transgenic mice can be attributed to the inhibition of Akt mediated by the expression of K10. In addition, these data are of great importance, because, to our knowledge, this is the first evidence suggesting that Akt activity might regulate the protein level of these two components of the IKK complex.

Phenotypic Alterations in Epidermis of Transgenic Mice Are Different to Those of IKKγ-deficient Mice—IKKγ is an X-linked gene both in humans and mice. Male mice lacking IKKγ are embryonic lethal (37, 38). Early after birth, heterozygous females show a strong phenotype in skin closely related to that seen in patients with the hereditary disorder retinitis pigmentosa (37, 38). In particular, the epidermis is characterized by a generalized edema with increased intercellular spaces, severe alterations in differentiation, and increased proliferation and apoptosis (37, 38). Given the decreased IKKγ protein levels observed in the epidermis of bK5hK10 transgenic mice, one might expect that they would correlate with phenotypic alterations similar to those of IKKγ-deficient mice. However, as reported previously (10), heterozygous animals show no overt alterations, and homozygous mice exhibited severe, although different, abnormalities to those reported for IKKγ-deficient females. By day 21 after birth, homozygous bK5hK10 transgenic mice show an epidermal phenotype characterized by a clear decrease in epidermal thickness caused by reduced proliferation as a consequence of K10 expression in proliferative keratinocytes, along with an increased stratum corneum (data not shown, see Ref. 10). Electron microscopy analysis also shows flattened and degenerative basal cells but no intercellular edema in transgenic mice (Fig. 4A′, see also Ref. 10). In the present work, prominent irregularities of the basal membrane of the epidermal cells were observed (Fig. 4A′). Another characteristic feature of these animals is their progressive generalized phenotype. Transgenic animals were markedly underweight, became very frail, and showed signs of impaired movement and wasting 3–5 weeks after birth (Fig. 4B). In addition, the skin of these transgenic mice was characterized by a severe reduction of the proliferation of the epidermal cells,
as demonstrated by BrdUrd incorporation experiments (Fig. 4C, see also Ref. 10). In contrast, we detected increased proliferation of the dermal cells in these animals compared with non-transgenic littermates. Finally, a severe reduction in the amount of dermal adipose tissue was also observed (Fig. 4, compare D with E). This causes the bending of hair follicles (arrow in Fig. 4D) due to the 2- to 4-fold decrease in the distance between the epidermis and the muscle layer. None of these alterations are present in animals lacking the IKKγ subunit of the IκB kinase complex (37, 38). On the other hand, most of these features are characteristics of animals overexpressing TNFα in epidermal cells (49) or as a consequence of IkBα inactivation (50), which also show increased production of this cytokine (50). Consequently, the expressions of TNFs and related cytokines were studied by Northern blotting. Homozygous mice showed a dramatic increase in the mRNA levels of TNFα, IL-6, and IL-1 (Fig. 5A). To further confirm this, we measured the levels of TNFα in the serum of these animals and found a significant increase in circulating levels (Fig. 5B). Finally, experiments were performed to determine whether the keratinocytes were the source of these increased levels of TNFα. For this, the production and release of TNF-α by primary keratinocytes derived from non-transgenic and transgenic mice were analyzed. Elevated levels of TNF-α were found in the supernatant of cultured primary transgenic keratinocytes (Fig. 5B).

To further confirm that keratinocytes are the source of TNFα in vivo, two experiments were performed. First, we monitored the localization of TNFα and IL-6 in skin samples from transgenic and non-transgenic mice using specific antibodies. We observed a positive TNFα staining in the non-transgenic samples located deep in the dermis, close to the muscle layer (Fig. 6A), and a few scattered areas more close to the epidermal-dermal border (Fig. 6A'). On the contrary, in transgenic samples TNFα was located primarily at the epidermal-dermal border (Fig. 6B) and in some cases positive staining was also observed in basal keratinocytes (Fig. 6A'). In fact, the dermis of homozygous littermate samples (Fig. 6C), a clear expression was detected in the epidermis of homozygous littermate samples (Fig. 6D). Interestingly, in this case, a stronger reaction was observed in the hair follicles (Fig. 6D).

As a second approach, the possible presence of inflammatory cells in the dermis of transgenic and non-transgenic skin samples was monitored using antibodies against T lymphocytes (CD-3), granulocytes (GR-1), and macrophages (Mac-1) in frozen skin samples. No increase in any of these cell populations was observed in the transgenic samples (Fig. 7, compare A, B, and C with A', B', and C', respectively). In fact, the dermis of transgenic mice displayed lower number of these cell types as compared with non-transgenic littermates. Given that the increased production of cytokines in the transgenic skin (Fig. 6) would allow for the recruitment of inflammatory cells, the observed decrease would reflect a possible alteration in the production and/or maturation of these cells in transgenic mice. In agreement, we have observed defective thymus development.

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**Fig. 5. Increased production of TNFα in bK5hK10 transgenic mice epidermis.** A, the expression of TNFα, IL-1, IL-6 genes in the quoted mouse skin RNA extracts were analyzed by Northern blot using the corresponding cDNA probes. Note the increased expression in the homozygous bK5hK10 transgenic mice. The loading was normalized by the corresponding cDNA probes. B, the levels of secreted TNFα in serum and primary keratinocyte culture supernatants were quantified by ELISA.

**Fig. 6. Localization of TNFα and IL-6 in transgenic mice skin.** Formalin-fixed, paraffin-embedded sections from non-transgenic (A, A', and C) and homozygous bK5hK10 transgenic mice littermates (B, B', and D) were stained for TNFα (A, A', B, and B') or IL-6 (C and D) expression using specific rabbit polyclonal antibodies. Bars in A–D = 100 μm; bars in A', B' = 50 μm.
in bK5hK10 homozygous transgenic mice (not shown).  

Together, these results demonstrate that the expression of K10 in the basal layer of the epidermis leads to the production of higher levels of TNF-α, which might explain some of the features of the phenotype of these animals. However, it is worth mentioning that such increased levels were not sufficient to produce liver apoptosis in vivo (not shown).

Activation of the JNK Pathway in Transgenic Mouse Epidermis—The pro-inflammatory cytokine TNF-α plays an important role in several cellular events such as septic shock, induction of other cytokines, cell proliferation, differentiation, and apoptosis. In response to TNF treatment, the transcription factor NF-κB and c-Jun terminal kinase (JNK) are activated in most cells. Because it was observed that bK5hK10 transgenic mice keratinocytes do not respond to TNF-α either in vivo or in vitro (Figs. 1 and 2), an investigation was made into the possible stimulation of JNK as a consequence of increased production of TNF-α in these keratinocytes. JNK activity was analyzed by an in vitro kinase assay using GST-c-Jun as a substrate. The results showed increased JNK activity in the homozygous mice (Fig. 8A). In agreement, increased phosphorylation of ATF2 and phosphorylated JNK-1 was also observed (Fig. 8A).

The binding of TNF-α to its receptors TNFR-1 or TNFR2 induces receptor aggregation and results in the recruitment of a number of cytoplasmic proteins to these complexes. TRAF2 is one of these signaling complexes. It interacts directly with TNFR2 and indirectly through TRADD with TNFR1. TRADD also recruits the death domain kinase RIP to these complexes.

Interestingly, TRAF2 and RIP are differentially involved in the modulation of NF-κB and JNK signals upon TNF stimulation (51–54). Consequently, we studied the expression of TRADD, TRAF2, and RIP in the epidermal extracts of non-transgenic, heterozygous, and homozygous bK5hK10 transgenic mice. No differences were seen in the expression of these molecules among the different genotypes (Fig. 8B), therefore, ruling out the possibility that the different NF-κB and JNK activities were due to altered expression of these signaling intermediates.

**DISCUSSION**

The functional diversity of the keratins is a matter of debate. This report focuses on the possible functions of keratin K10, a protein expressed in non-proliferative suprabasal skin keratinocytes, which in addition is down-regulated during hyperproliferative situations. Using cultured cells and transgenic mouse models we have previously demonstrated that the expression of keratin K10 inhibits cell cycle progression through its ability to bind and inhibit the activation of Akt and PKCζ (8–10). More remarkably, transgenic mice expressing hK10 in the basal proliferative keratinocytes are almost completely resistant to skin tumorigenesis (10). This is in agreement with our recent observations indicating a fundamental role for Akt in the process of mouse skin carcinogenesis (12). Akt kinase is involved in many aspects of cell physiology, including the regulation of NF-κB signaling. Because this pathway is also very important in the regulation of epidermal functions, we sought to analyze the possible alterations in NF-κB in the epidermis of bK5hK10 transgenic mice.

Decreased NF-κB activity was seen in the skin and primary keratinocytes derived from bK5hK10 transgenic mice (Fig. 1). Moreover, such inhibition was dependent on K10-induced re-
pression of Akt activity (Fig. 1). One of the main phenotypic alterations in these mice is the decreased proliferation in vitro and in vivo (10). This seems to be in disagreement with the results shown by other authors who indicate that NF-κB acts in epidermis to arrest cell proliferation (reviewed in Ref. 29). However, it is important to notice that this antiproliferative function has been inferred from transgenic mouse models overexpressing different molecules that interfere with NF-κB activity. It is therefore possible that this effect is owed to such overexpression. Recent knock-in and epidermal-specific null animal models have provided data to support this hypothesis (35). Moreover, increased endogenous NF-κB activity during mouse skin carcinogenesis has been observed (33).

The present data clearly indicate that decreased NF-κB activity can be reversed by Akt co-expression, suggesting that the inhibition of this kinase is responsible for the observed impaired NF-κB signaling observed in vitro and in vivo. The experimental evidence suggesting such functional relationship is very ample and still growing (see the introduction). However, the molecular mechanism responsible for such a connection has not been fully demonstrated. Here we show that the inhibition of the molecular mechanism responsible for this process has not been sufficiently demonstrated. We show that the inhibition of the IkB kinase complex is involved. In fact, the co-expression of either IKKβ or IKKγ was, respectively, able to rescue NF-κB inhibition partially or totally (Fig. 2). More strikingly, it was observed that in animals expressing higher amounts of K10, and thus with the strongest inhibition of Akt kinase and NF-κB activities, there was a dramatic reduction IKKβ and IKKγ levels. These data are further confirmed by the use of retroviral constructs coding for wt or dominant negative Akt. We have found that the inhibition of Akt activity leads to a decreased expression of IKKβ and IKKγ proteins (Fig. 3). To our knowledge, this is the first evidence suggesting the involvement of Akt in the modulation of IKK expression. The molecular mechanism underlying this process will be studied in the future. Finally, the fact that IKKγ co-expression can partially rescue NF-κB activity clearly points to the possibility that IKKγ levels were reduced but not completely absent, because this protein is absolutely necessary for this process (37, 38).

The phenotype of the high expressing bK5hK10 transgenic mice was, however, clearly different to that reported for IKKγ-deficient mice. These animals die in utero while heterozygous IKKγ females display severe dermatopathy characterized by keratinocyte hyperproliferation, skin inflammation, hyperkeratosis, intercellular edema, and increased apoptosis (37, 38). Of all these characteristics, however, only hyperkeratosis was observed in bK5hK10 mice along with some alterations suggestive of increased apoptosis. In addition, the reduced proliferation observed in keratinocytes, in parallel with increased BrdUrd incorporation in the dermal cells, the flattened appearance of the keratinocytes, the reduction in the subepidermal adipose tissue, and the characteristic progressive phenotype (Fig. 4), were all similar to reported alterations in transgenic mice expressing TNFα in the epidermis (49). Similar alterations have also been described in the epidermis of mice lacking IkBα (50), which also have increased levels of circulating TNFα. In agreement with this, the results show that bK5hK10 keratinocytes produce increased amounts of this cytokine (Figs. 5 and 6). This increased production may be responsible for some of the phenotypic alterations observed in bK5hK10 transgenic mice.

The increased production of TNFα is particularly striking. In fact, it is well established that TNFα, IL-1, and IL-6 genes are predominantly regulated by NF-κB elements (55). In this regard it is worth mentioning that similar increases in TNFα, as well as the increased expression of several genes normally controlled by NF-κB factor, have been reported in mice lacking IKKγ (37), and in epidermal-specific IKKβ-null mice (36). However, in these cases, the increased production of these cytokines has been attributed to the inflammatory cells that invade the tissue (36, 37). In bK5hK10 this is not the main cause, as confirmed by immunofluorescence analysis (Fig. 7). On the other hand, we observed a decreased number of inflammatory cells in the dermis of bK5hK10 homozygous transgenic mice. This might be in agreement with our findings indicating that these animals display severe immunodeficiency due to altered thymus development.2 The decreased expression of IKKγ observed in bK5hK10 transgenic epidermis clearly indicates the existence of alternative mechanisms for the production of TNFα, given that this subunit is essential for NF-κB activation (48). TNF synthesis and secretion are regulated at several points, including the transcriptional and post-transcriptional levels. Among the elements that may control TNFα gene expression, besides NF-κB sites, are Ets, ATF-2/c-jun, Sp1, Elk1, CBP, and p300 (56–59). This clearly points to a central role of JNK activity in the positive modulation of TNFα gene expression. Interestingly, JNK activity is inhibited by Akt kinase through direct binding and phosphorylation of SEK1/MKK4 (46, 60). Consequently, although we do not know at present how TNFα is produced in the absence of normal NF-κB signaling in transgenic keratinocytes, a possible explanation might be that Akt inhibition promoted by K10 expression can lead to the activation of JNK. This would allow the production of TNFα, which, upon binding to TNFR1, might induce increase binding of ATF-2/c-jun to the TNFα promoter, therefore, inducing increased transcription (57). Finally, such increased secretion of TNFα may account for the expression of IL-1 and IL-6 genes. Alternatively, it is tempting to speculate that differentiation-specific keratins, such as K10, could directly modulate TNFα. In this regard, it has been demonstrated that simple epithelial keratins modulate different aspects of TNFα signaling through direct binding with several components of the TNF-dependent network (61–63). Therefore, K10 and K8 and/or K18 could act in opposite manners. These aspects will be the subject of future experiments.

This report provides evidence that the specific expression of keratins in epithelia may affect the transcriptional program executed by these cells, probably through the modulation of signaling molecules. This may lead to abnormal production of cytokines, which results, not only in a cell autonomous effect but in a complete disturbance of tissue homeostasis. In this regard, it is worth mentioning that the recently described effect of K10 loss in adult mice results in hyperproliferation in basal layer of epidermis (64). This observation points to a paracrine effect of keratin expression. Whether this might be attributed to altered expression of cytokines as described here is an attractive possibility that merits future investigation.

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