RIP4 Regulates Epidermal Differentiation and Cutaneous Inflammation

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The receptor-interacting protein (RIP) family kinase RIP4 interacts with protein kinase C (PKC) isoforms and is implicated in PKC-dependent signaling pathways. RIP4$^{-/-}$ mice die at birth with epidermal differentiation defects, causing fusions of all external orifices and loss of the esophageal lumen. To further understand RIP4 function in the skin, we generated transgenic mice with epidermal-specific expression of RIP4 using the human keratin-14 promoter (K14-RIP4). The K14-RIP4 transgene rescued the epidermal phenotype of RIP4$^{-/-}$ mice, showing that RIP4 acts autonomously in the epidermis to regulate differentiation. Although RIP4$^{-/-}$ mice share many phenotypic similarities with inhibitor κB kinase (IKK)α$^{-/-}$ mice and stratifin repeated epilation (Sfn$^{Er/Er}$) mice, the K14-RIP4 transgene failed to promote epidermal differentiation in these mutant backgrounds. Unexpectedly, topical treatment of K14-RIP4 mice with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced dramatic, neutrophilic inflammation, an effect that was independent of tumor necrosis factor type 1 receptor (TNFR1/p55) function. Despite their enhanced sensitivity to TPA, K14-RIP4 mice did not have an altered frequency of tumor formation in TPA-promoted skin cancer initiated with 7,12-dimethylbenz[a]anthracene (DMBA). These data suggest that RIP4 functions in the epidermis through PKC-specific signaling pathways to regulate differentiation and inflammation.

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INTRODUCTION

The RIP family members RIP1–RIP7 are a diverse group of proteins that share homology within their serine/threonine kinase domain. Individual members differ by the types of domains linked to their kinase region, and are involved various cellular processes such as cell death, immunity, response to DNA damage, and differentiation (Meylan and Tschopp, 2005).

RIP4 was initially described as a previously unidentified protein found to interact with protein kinase C (PKC)βI and PKCδ in yeast two-hybrid assays (Bahr *et al.*, 2000; Chen *et al.*, 2001). RIP4 contains an N-terminal kinase domain linked to a unique intermediate domain that can be cleaved by caspases, and a C terminus domain harboring ankyrin repeats that may negatively regulate its activity (Meylan *et al.*, 2002). Other groups have reported that overexpression of RIP4 in HEK293T cells activates nuclear factor-κB (NF-κB), c-Jun N-terminal kinase (JNK), and activator protein-1. They also indicate that catalytically inactive versions of RIP4 specifically inhibit signaling induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA)/Ca^{2+} ionophore, suggesting that RIP4 may function primarily in a PKC-dependent signaling pathway (Meylan *et al.*, 2002; Muto *et al.*, 2002; Moran *et al.*, 2003).

RIP4$^{-/-}$ mice die at birth and have fewer skin folds than wild-type littermates, partial fusion of the hind limbs and tail to the body, and fusion of the external orifices, oral cavity, and esophagus. The epidermis of RIP4$^{-/-}$ mice is thickened with expanded spinous and granular layers, and contains an outermost layer of parakeratotic cells instead of enucleated squamous cells. Furthermore, proteins that characteristically mark specific layers of the epidermis are mis-expressed (Holland *et al.*, 2002). Although the phenotype of RIP4$^{-/-}$ animals appears to result from defects restricted to the squamous epithelium, the expression of RIP4 mRNA in adult tissues is widespread (Chen *et al.*, 2001). Therefore, it is unclear whether RIP4 functions outside of the epidermal compartment, as this may have been masked by the severity of the epidermal phenotype.

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Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; Er, repeated epilation; IKK, inhibitor κB kinase; K14-RIP4, epidermal-specific expression of RIP4 using the human keratin-14 promoter; NF-κB, nuclear factor-κB; PKC, protein kinase C; RIP, receptor-interacting protein; Sfn, stratifin; TNFR1/p55, tumor necrosis factor type 1 receptor; TPA, 12-O-tetradecanoylphorbol-13-acetate

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The phenotypes of RIP4$^{-/-}$ animals are remarkably similar to two other mouse mutants, inhibitor κB kinase (IKK)α$^{-/-}$ and stratifin repeated epilation (Sfn$^{Er/Er}$) mice, which also die at birth with fusion of external orifices and a thickened epidermis (Hu *et al.*, 1999; Li *et al.*, 1999, 2005; Takeda *et al.*, 1999; Fisher, 2000; Holland *et al.*, 2002; Herron *et al.*, 2005). Similar to RIP4$^{-/-}$ mutants, the epidermis of IKKα$^{-/-}$ and Sfn$^{Er/Er}$ mice has an expanded spinous layer and an outermost layer containing parakeratotic cells. However, the epidermis from RIP4$^{-/-}$ and Sfn$^{Er/Er}$ animals express markers of more differentiated epidermal layers, such as filaggrin, whereas the epidermis of IKKα$^{-/-}$ mice does not. IKKα$^{-/-}$ and Sfn$^{Er/Er}$ mice also have dramatic alterations in limb development that are not observed in RIP4$^{-/-}$ mice. Interestingly, epidermal-specific expression of IKKα from the human keratin-14 promoter is sufficient to restore both epidermal differentiation and limb development in IKKα$^{-/-}$ mice (Sil *et al.*, 2004).

The epidermal phenotypes of these mutants suggest that RIP4, IKKα, and Sfn function in either shared or independent pathways to promote epidermal differentiation. Stratifin protein has been biochemically purified as an enhancer of PKC activity *in vitro*, but it can bind many different proteins and has a wide variety of other functions attributed to it (Dellambra *et al.*, 1995; Laronga *et al.*, 2000; Benzinger *et al.*, 2005; Yang *et al.*, 2006; Wilker *et al.*, 2007). The function of IKKα in epidermal differentiation is independent of its role in NF-κB regulation (Hu *et al.*, 2001; Descargues *et al.*, 2008). Because blocking NF-κB signaling results in hyperplastic epidermal development without preventing differentiation (Seitz *et al.*, 1998; Zhang *et al.*, 2004), it is likely that RIP4 has NF-κB-independent functions as well.

Because RIP4 is necessary for normal epidermal differentiation, we focused our studies on the function of RIP4 in the skin. Using a K14-RIP4 transgene, we showed that RIP4 functions autonomously in the epidermis to regulate differentiation. Interestingly, K14-RIP4 mice are hypersensitive to inflammation induced by topical application of TPA, but have a normal inflammatory response in a TPA-independent model of contact hypersensitivity. In a 7,12-dimethylbenz[a]anthracene (DMBA)/TPA model of skin cancer, K14-RIP4 mice develop elevated inflammation and increased epidermal hyperplasia, yet do not show altered rates of tumor formation. In total, these studies suggest that RIP4 functions in PKC-dependent signaling pathways to regulate epidermal differentiation and cutaneous inflammation.

RESULTS

RIP4 expression is required within the epidermis for normal differentiation

To examine the functions of RIP4 in the epidermis, we generated transgenic mice using an RIP4 expression construct driven by the human keratin-14 promoter (K14-RIP4). Two independent lines were established, and the relative expression of the transgenes was determined from the adult tail skin cDNA using TaqMan PCR of a transgene-specific tag. Compared with endogenous RIP4 mRNA levels in the skin, the higher expressing line produced 15-fold more RIP4 encoding mRNA than did wild-type mice and was used in further experiments. The lower expressing line produced RIP4 encoding mRNA similar to wild-type levels (data not shown). On the C57BL/6 background, neither line exhibited any overt phenotype, and the mice were viable and fertile (data not shown). Expression of the transgene in the epidermis was confirmed using immunohistochemistry on sectioned E18.5 mouse embryos. Although the expression of endogenous RIP4 protein was undetectable, transgenic animals had clear expression of RIP4 primarily in the spinous layer of the epidermis (Figure 1a). No expression of RIP4 was observed in non-epidermal tissues (data not shown).

We next tested whether RIP4 expression was required only within the epidermis for differentiation by crossing the K14-RIP4 transgenic mice onto the RIP4$^{-/-}$ background (designated as K14-RIP4; RIP4$^{-/-}$). In contrast to RIP4$^{-/-}$ animals that died immediately after birth with fusions of all external orifices, K14-RIP4; RIP4$^{-/-}$ animals derived from either transgenic line developed with open mouths, nostrils, and anuses (Figure 1b). Furthermore, the epidermis from these mice was cornified and expressed differentiation markers in normal domains (Figure 1c–g). Postnatal K14-RIP4; RIP4$^{-/-}$ mice died within 2 days of birth, apparently due to a failure of the K14-RIP4 transgene to rescue the esophageal fusion present in RIP4$^{-/-}$ animals (Figure 1i). This correlated with a $>1{,}000$-fold less transgene expression in the adult esophagus compared with that in skin (data not shown), in agreement with previous data using a similar K-14 promoter (Sil *et al.*, 2004). These results show that RIP4 is required autonomously in the epidermis for differentiation.

K14-RIP4 does not alter epidermal differentiation in IKKα$^{-/-}$ or Sfn$^{Er/Er}$ mice

IKKα$^{-/-}$ and Sfn$^{Er/Er}$ mice form a poorly differentiated epidermis that resembles the epidermis of RIP4$^{-/-}$ animals, but it is unclear whether RIP4, IKKα, and stratifin function in the same or different pathways to regulate epidermal differentiation (Hu *et al.*, 1999, 2001; Li *et al.*, 1999, 2005; Takeda *et al.*, 1999; Holland *et al.*, 2002; Herron *et al.*, 2005). To determine whether RIP4 regulated the expression of IKKα or stratifin, we examined the expression of these proteins in RIP4$^{-/-}$ mice by immunohistochemistry. In control and RIP4$^{-/-}$ skin, stratifin protein was detected throughout the epidermis (Figure 1h). IKKα protein was not detected strongly enough to characterize its expression pattern in the skin, but by microarray analysis, IKKα and stratifin mRNA expression levels were found to change <3 fold in the skin from control and RIP4$^{-/-}$ mice (data not shown; Holland *et al.*, 2002). These analyses indicate that RIP4 is not required for the expression of IKKα or stratifin gene products.

In an attempt to alleviate the block in the epidermal differentiation of IKKα$^{-/-}$ or Sfn$^{Er/Er}$ mutants, we crossed the higher expressing line of K14-RIP4 mice onto these mutant backgrounds (designated K14-RIP4; IKKα$^{-/-}$ or K14-RIP4; Sfn$^{Er/Er}$). At E18.5, K14-RIP4; IKKα$^{-/-}$ and K14-RIP4; Sfn$^{Er/Er}$ mice developed oral, limb, and tail epithelial fusions and appeared phenotypically indistinguishable from mutants

lacking the K14-RIP4 transgene (Figure 2a and h, and data not shown). The epidermis of K14-RIP4; IKKα$^{-/-}$ animals was thickened, lacked granular and cornified layers, and had no indication of altered differentiation in comparison with the IKKα$^{-/-}$ epidermis (Figure 2b–g). Similarly, the epidermis of K14-RIP4; Sfn$^{Er/Er}$ embryos was phenotypically comparable with that of Sfn$^{Er/Er}$ mutants (Figure 2i–n). In both mutant backgrounds, immunohistochemical staining verified that exogenous RIP4 protein was dramatically upregulated in K14-RIP4; IKKα$^{-/-}$ and K14-RIP4; Sfn$^{Er/Er}$ mice compared with that in IKKα$^{-/-}$ and Sfn$^{Er/Er}$ mice (Figure 2c and j). These data show that elevating RIP4 expression is not sufficient to promote epidermal differentiation in IKKα$^{-/-}$ or Sfn$^{Er/Er}$ mice.

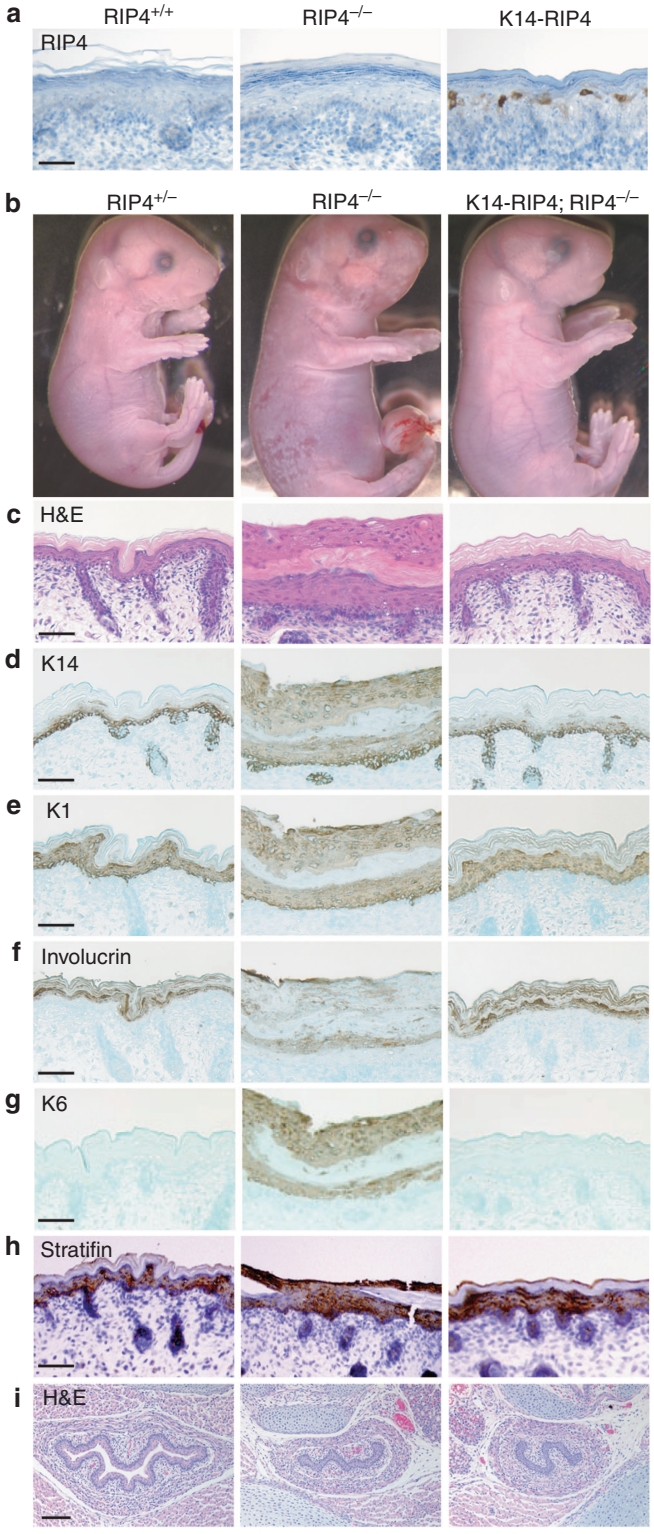

Figure 1. A K14-RIP4 transgene rescues the epithelial defects in RIP4$^{-/-}$ animals. (**a**) Immunohistochemistry of RIP4 protein in sectioned E18.5 embryos detected RIP4 only in the epidermis of K14-RIP4 transgenic mice (right panel). (**b**) The taut skin and oral fusions present in E18.5 RIP4$^{-/-}$ mice (center panel) are rescued in K14-RIP4; RIP4$^{-/-}$ animals (right panel). Sections of E18.5 skin stained with (**c**) H&E, or immunohistochemistry detecting (**d**) keratin-14 (K14), (**e**) keratin-1 (K1), (**f**) involucrin, (**g**) keratin-6 (K6), and (**h**) stratifin show a normal epidermal architecture and protein expression in K14-RIP4; RIP4$^{-/-}$ skin (right column) compared with that in RIP4$^{-/-}$ (center column) or control RIP4$^{+/-}$ skin (left column). (**i**) H&E-stained sections reveal that esophageal fusions typical of RIP4$^{-/-}$ embryos (center panel) are also present in K14-RIP4; RIP4$^{-/-}$ embryos. An animal with the K14-RIP4 transgene from the higher expressing line is shown in panel a, and animals from a cross with the lower expressing line are shown in panels b–i. Bars = 100 μm (**a** and **c**–**h**); or 50 μm (**i**).

K14-RIP4 mice are specifically sensitive to TPA-driven, TNFR1-independent inflammation

Other research suggests that RIP4 functions to transduce TPA-initiated signaling cascades (Meylan *et al.*, 2002; Muto *et al.*, 2002; Moran *et al.*, 2003). This raised the question of whether K14-RIP4 mice would show altered sensitivity in a model of TPA-induced inflammation. TPA was applied to the ears of K14-RIP4 mice from the higher expressing line and wild-type littermates for 3 consecutive days, and ear thickness was measured. Strikingly, the transgenic mice showed a marked increase in inflammation characterized by erythema, edema, and surface crusting (Figure 3a and b). This correlated with a dramatic and prolonged increase in the ear thickness of transgenic mice that was clearly distinct from that of control animals (Figure 3e, $P<0.0001$). Vehicle-treated ears of either genotype had no detectable inflammation (Figure 3c and d and data not shown).

Histological examination of the TPA-treated ears in transgenic mice showed that the inflammation consisted predominantly of neutrophils. By day 3, neutrophils infiltrated the epidermis and ranged from intracorneal pustules to full-thickness involvement of the epidermis and the superficial dermis. On day 7, the inflammation was more extensive, with corresponding necrosis and ulceration of the epidermis. After 24 days, inflammation consisted of a mixed population of mononuclear cells in the dermis with associated fibrosis and reactive hyperplasia of the overlying, intact epidermis (Figure 3g). At 17 weeks post treatment, the examination of ears from K14-RIP4 mice showed no indication of inflammation, although dermal fibrosis and epidermal thickening typical of scarred tissue was present (data not shown). In contrast, inflammation in the control ears on days 3 and 7 was mild, and was characterized by a mixture of neutrophils and mononuclear cells. By day 24, the

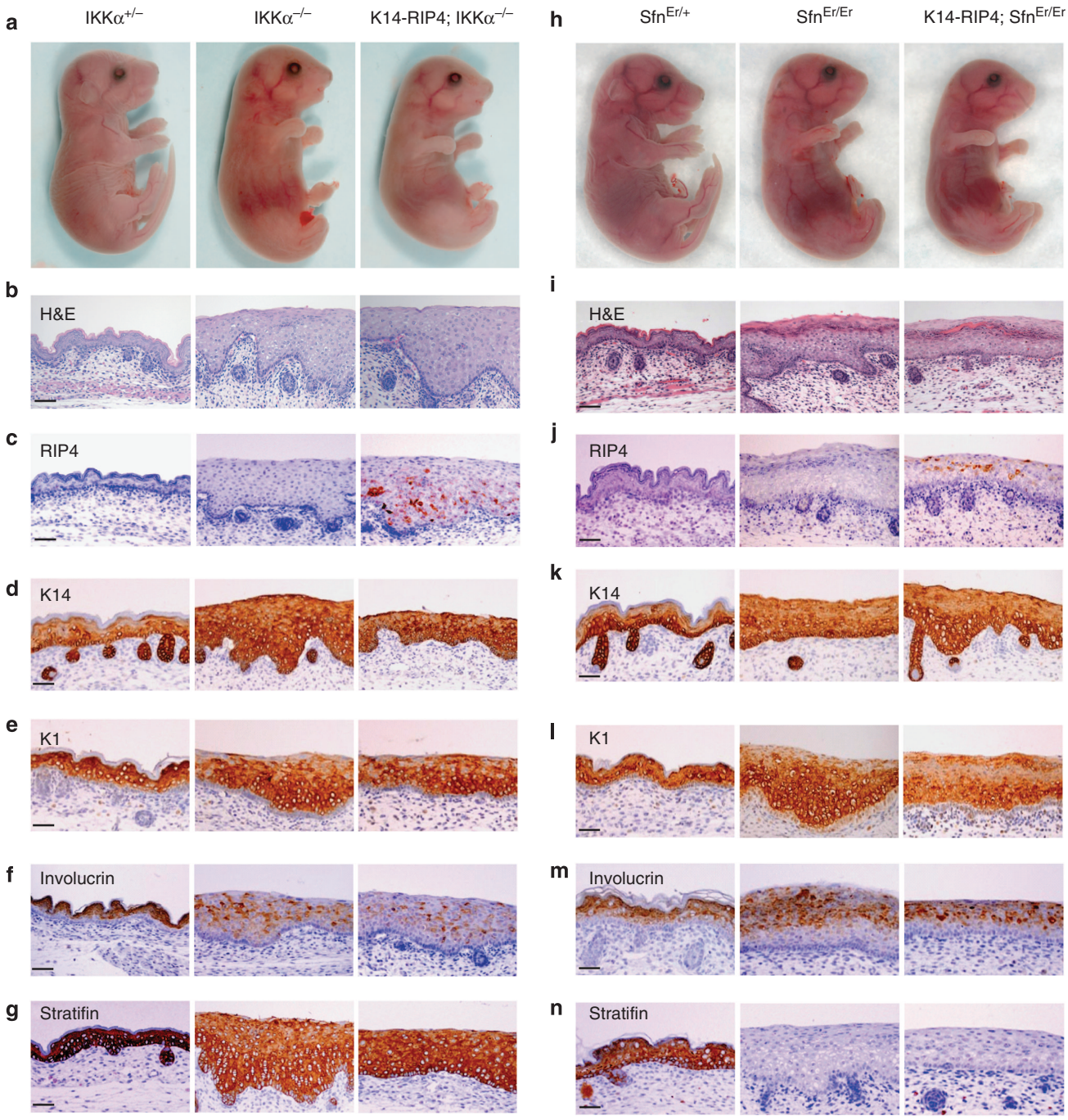

Figure 2. Overexpression of RIP4 fails to rescue epidermal differentiation in IKKα$^{-/-}$ or Sfn$^{Er/Er}$ animals. (**a**) E18.5 whole mount or sectioned embryos showing that the taut skin, oral fusions, truncated limbs, and undifferentiated epidermis characteristic of IKKα$^{-/-}$ (**a**–**g**, center panel) or Sfn$^{Er/Er}$ mice (**h**–**n**, center panel) are unaltered in K14-RIP4; IKKα$^{-/-}$ (**a**–**g**, right panel) or K14-RIP4; Sfn$^{Er/Er}$ mice (**h**–**n**, right panel). Sections were stained with H&E (**b** and **i**), or immunohistochemistry to detect the following proteins: (**c** and **j**) RIP4, (**d** and **k**) keratin-14 (K14), (**e** and **l**) keratin-1 (K1), (**f** and **m**) involucrin, and (**g** and **n**) stratifin. RIP4 expression is present throughout the epidermis of (**c**) K14-RIP4; IKKα$^{-/-}$ and (**j**) K14-RIP4: Sfn$^{Er/Er}$ embryos. Bars = 50 μm.

inflammation was resolved and the ears appeared normal (Figure 3f).

Skin inflammation, whether induced by phorbol esters or spontaneously occurring in transgenic or null mutant mice, is often dependent on the activity of tumor necrosis factor type 1 receptor (TNFR1)/p55 (Kondo and Sauder, 1997; Pasparakis *et al.*, 2002; Lind *et al.*, 2004). In addition, TNF signaling through TNFR1/p55 has a critical role in the pathogenesis of human skin inflammatory disease (Bradley, 2008). We therefore crossed the higher expressing line of K14-RIP4 mice onto the p55$^{-/-}$ background and tested them in the TPA-driven model of inflammation. As the TNFR1/p55

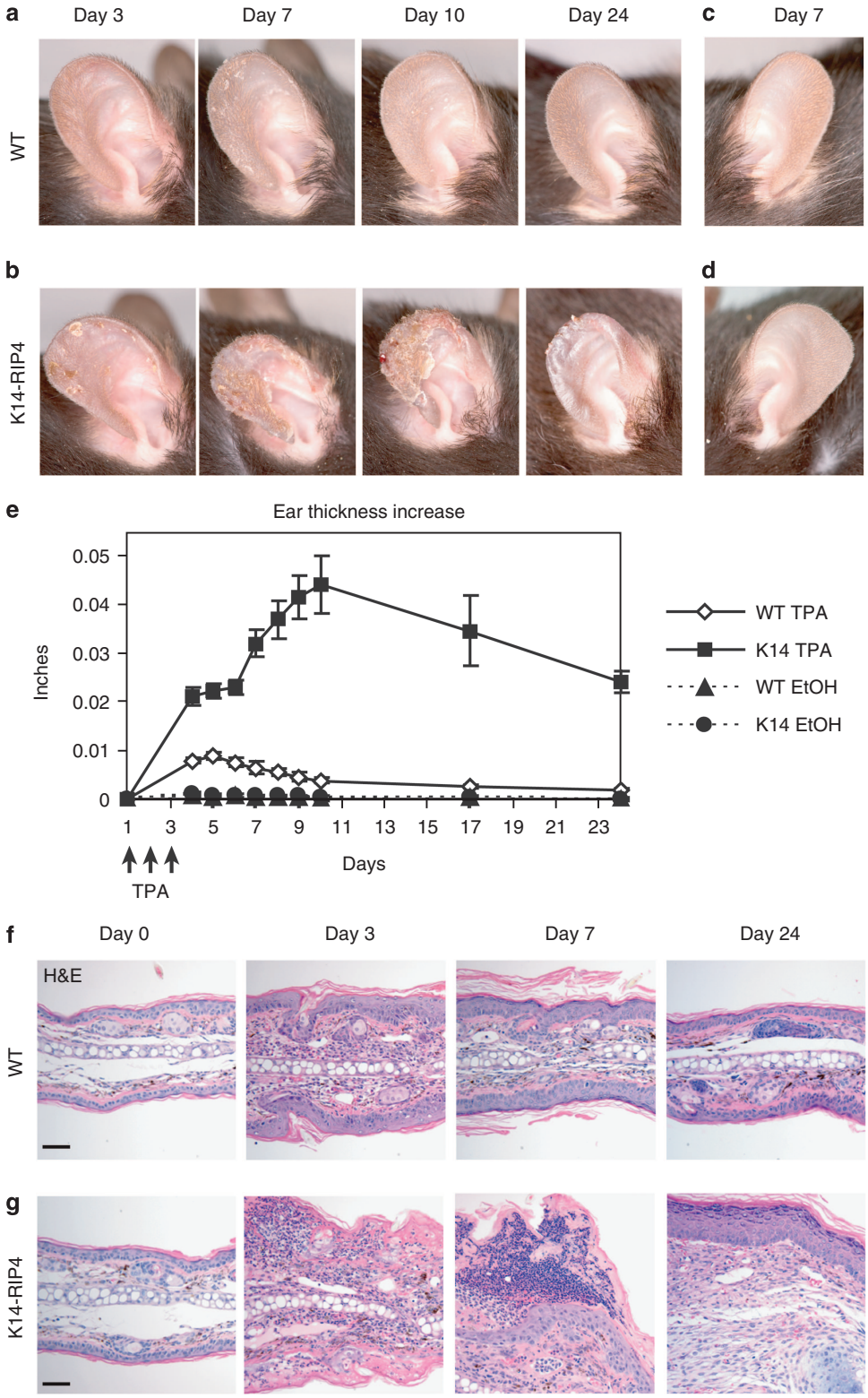

Figure 3. TPA induces neutrophilic inflammation in K14-RIP4 transgenic mice. Right ears from (**a**) wild-type (WT) or (**b**) K14-RIP4 mice over 24 days after topical TPA application (days 1–3) in a model of inflammation. The ears of K14-RIP4 mice show increased edema, surface crusting, and scarring compared with those of wild-type mice. (**c** and **d**) The left, vehicle control ears of mice shown in panels a and b, respectively. (**e**) Thickness of either TPA- or ethanol (vehicle control)-treated ears measured over 24 days ($n = 5$ animals per genotype; thickness is plotted as mean ± SEM). (**f**) H&E-stained sections of TPA-treated ears from WT mice show a mild infiltration of cells at day 3, which declines by day 7 and is completely resolved by day 24. (**g**) H&E-stained sections of TPA-treated ears from K14-RIP4 transgenic mice showing full-thickness epidermal infiltration of neutrophils on day 3, an intracorneal pustule of neutrophils on day 7, and a mild dermal infiltrate and hyperplastic epidermis on day 24. Bars = 50 μm.

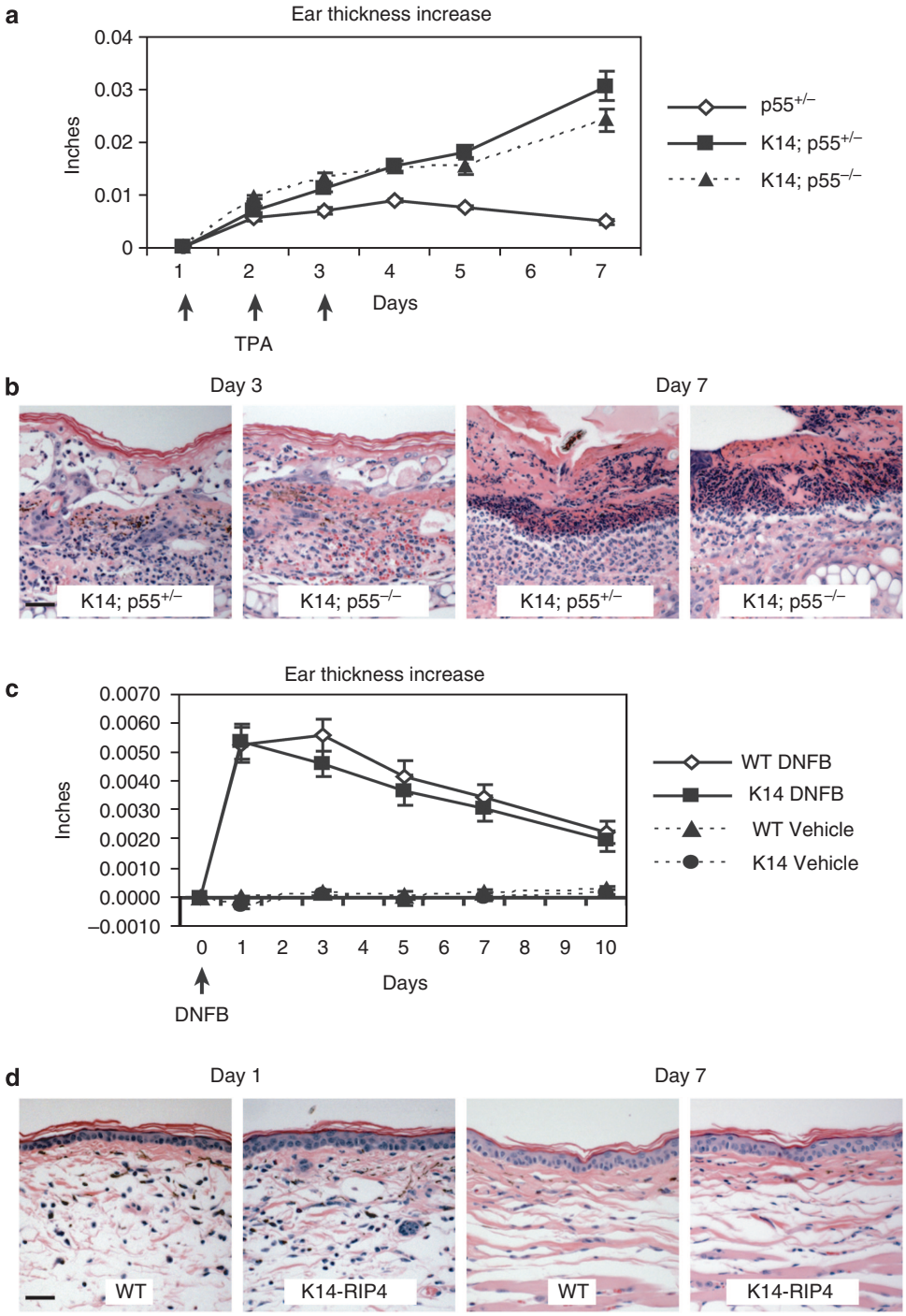

Figure 4. K14-RIP4 mice are specifically sensitive to TPA-driven, TNFR1/p55-independent inflammation. (**a**) Thickness of TPA-treated ears from p55$^{+/-}$, K14-RIP4; p55$^{+/-}$, or K14-RIP4; p55$^{-/-}$ mice as measured over 10 days (days 1–3, $n=7$ animals per genotype; days 4–7, $n=5$; thickness is plotted as mean ± SEM). (**b**) H&E-stained sections of TPA-treated ears from K14-RIP4; p55$^{+/-}$ or K14-RIP4; p55$^{-/-}$ mice. Neutrophils can be seen infiltrating the epidermis on day 3, and by day 7, regions of the skin are ulcerated with overlying neutrophils. (**c**) Thickness of DNFB- or vehicle-treated ears from mice in a model of contact hypersensitivity ($n=$ at least 11 animals per genotype for each time point; overall, $P=0.624$ for wild-type versus K14-RIP4 DNFB-treated ears). (**d**) H&E-stained sections of ears on 1 or 7 days after challenge with DNFB. Note similar amounts of infiltrating cells in wild-type and K14-RIP4 transgenic ears, and lack of epidermal involvement. Bars = 50 μm.

receptor is the dominant mediator of TNFα-induced skin inflammation, we did not generate additional crosses with either TNFα$^{-/-}$ or TNFR2/p75$^{-/-}$ mice (Kondo and Sauder, 1997). The ears of K14-RIP4; p55$^{-/-}$ mice were thickened and contained infiltrating neutrophils, similar to the ears of K14-RIP4; p55$^{+/-}$ littermates (Figure 4a and b). Similarly, the average ear thickness of K14-RIP4; p55$^{-/-}$ mice was not significantly different from that of K14-RIP4; p55$^{+/-}$ mice

($P=0.632$), although both groups were significantly different from p55$^{+/-}$ mice ($P<0.0001$). These data indicate that the excessive inflammation induced by TPA in K14-RIP4 mice is not dependent on TNFR1 function.

We next tested the higher expressing line of K14-RIP4 mice in a DNFB-induced model of contact hypersensitivity. This model produces a T_H1-dependent reaction in response to the production of pro-inflammatory cytokines by immune cells and keratinocytes (Wang *et al.*, 2003). Interestingly, K14-RIP4 transgenic mice were no different from control mice with regard to the magnitude of inflammation in this model, and showed no bias toward a neutrophilic infiltrate (Figure 4c and d). In total, these data show that the K14-RIP4 mice are specifically sensitive to TPA-triggered inflammation, and that this inflammation occurs independent of TNFR1 function.

Transgenic mice overexpressing PKCα in the skin also develop neutrophilic inflammation that is TNFR1-independent (Cataisson *et al.*, 2005). To assess whether RIP4 regulates the expression or activity of PKC isoforms, we stimulated primary keratinocytes derived from wild-type, RIP4$^{-/-}$, or K14-RIP4 animals with TPA and examined extracts of these cells by western blot analysis. Multiple PKC and phospho-PKC isoforms were tested, including PKCα and PKCδ. No difference was detected in the expression level or phosphorylation kinetics of PKC isoforms in TPA-stimulated mutant, transgenic, or control keratinocytes (data not shown). Similarly, TPA-induced phosphorylation of the MARCKS protein, a general target of multiple PKC isoforms, was unaffected by the genotype of RIP4 (data not shown). Conditioned media from TPA-treated wild-type and K14-RIP4 keratinocytes were also collected and analyzed for cytokine/chemokine profiling (Rules Based Medicine, Charles River Laboratories), but no reproducible differences were found between the two genotypes (data not shown). These findings are still consistent with RIP4 functioning downstream of PKC during signal transduction, but further studies are required to determine this definitively.

K14-RIP4 mice spontaneously develop inflammation, but do not have an altered rate of skin cancer with treatment of DMBA and TPA

TPA application and inflammation are factors known to promote tumorigenesis in the skin (Mueller, 2006). As the K14-RIP4 mice were more sensitive to TPA-induced inflammation, we tested whether they would have an altered incidence of papilloma or squamous cell carcinoma formation in a DMBA/TPA skin cancer model. Because the C57BL/6 background of the K14-RIP4 mice used above is resistant to carcinogenesis in this model, we first bred the higher expressing K14-RIP4 line onto the more sensitive FVB/N background (Hennings *et al.*, 1993). It can be noted that K14-RIP4 mice on the FVB/N background spontaneously developed ringtail or inflamed tails (23 of 43, or 54%, of mice aged 4–13 months old) and/or inflamed ears (11 of 43, or 26%, Figure 5a) beginning at ~3 months of age. These phenotypes were associated with a mixed lymphocytic and neutrophilic infiltrate, epidermal hyperplasia, and mild hyperkeratosis in some affected tails (data not shown). These responses are another indication that elevated RIP4 expression promotes inflammation in the skin and further suggest the presence of strain-specific genetic modifiers of RIP4 activity.

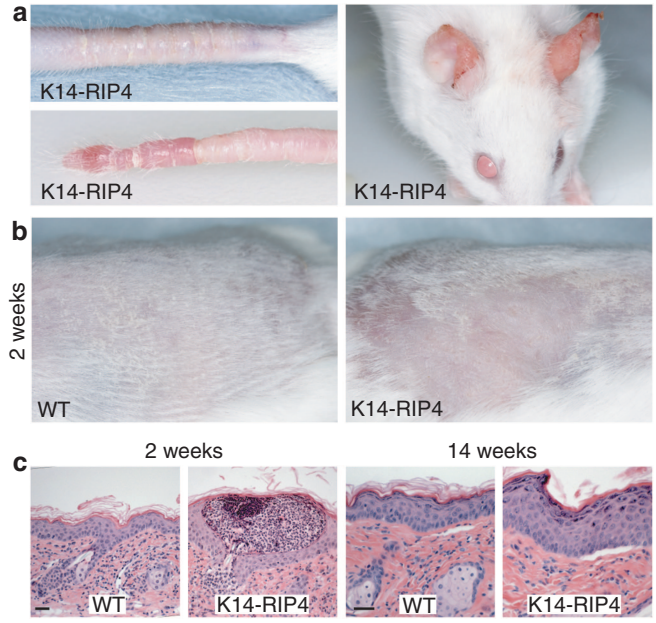

Figure 5. Spontaneous inflammation and increased sensitivity to TPA in FVB/N K14-RIP4 mice. (**a**) Examples of adult K14-RIP4 mice on the FVB/N genetic background that spontaneously developed tail or ear inflammation (mice aged 4 months (top left), 10 months (bottom left), and 7 months (right). (**b**) Shaved backs of wild-type (WT) or K14-RIP4 mice in the DMBA/TPA skin cancer model after 2 weeks of twice weekly TPA application. K14-RIP4 animals have reddened skin and patchy hair loss. (**c**) H&E-stained sections from WT and transgenic mice showing intracorneal pustules in K14-RIP4 mice after 2 weeks of TPA treatment and the hyperplastic epidermis in K14-RIP4 mice after 14 weeks of TPA treatment. Bars = 50 μm.

We next enrolled 7- to 8-week-old K14-RIP4 and control mice into a DMBA/TPA skin cancer model using a single application of 25 μg of DMBA, followed by 5 μg of TPA twice per week for 20 weeks. No difference in the development of papillomas, squamous cell carcinomas, or metastases was found between transgenic and control mice (data not shown). However, 2 weeks after TPA treatment, the skin of transgenic mice became reddened and showed signs of hair loss (Figure 5b). To further characterize this phenotype, a separate group of mice was treated bi-weekly with TPA for 2 weeks and skin samples were collected 18 hours after the last treatment. Histology of sectioned skin showed that the K14-RIP4 mice developed an increased number of intracorneal pustules, primarily associated with hair follicles (Figure 5c, wild-type: 0.59 ± 0.12 pustules per cm, $n=6$; K14-RIP4: 2.61 ± 0.34 pustules per cm, $n=6$; $P=0.0022$). Mice in the cancer model were also examined after 14 weeks of TPA treatment, with or without initiation by DMBA. At this time point, inflammation in the epidermis, dermis, and associated with the papillomas was comparable between K14-RIP4 and control mice, and the epidermis from both groups contained occasional pustules. However, the transgenic mice showed a slightly more pronounced epidermal hyperplasia with a

prominent granular layer (Figure 5c, average hyperplasia score for wild-type: 2.0 ± 0.0, $n = 8$; K14-RIP4: 2.9 ± 0.1, $n = 8$; $P = 0.0014$). These data indicate that chronic TPA application to the skin of K14-RIP4 mice leads to enhanced epidermal inflammation and hyperplasia. However, elevated RIP4 expression in the epidermis does not alter the onset, growth, or progression of skin cancer in this model.

DISCUSSION

Here, we show that RIP4 expression in the epidermis is sufficient to promote epidermal differentiation in RIP4-deficient mice. Furthermore, to the best of our knowledge, we provide the first evidence for a role of RIP4 in cutaneous inflammation. These findings extend previous work that identified skin as the primary tissue requiring RIP4 for normal development (Holland *et al.*, 2002). The specific sensitivity of K14-RIP4 transgenic mice to TPA-driven inflammation also supports previous studies, suggesting that RIP4 functions primarily in PKC-dependent signaling pathways (Meylan *et al.*, 2002; Muto *et al.*, 2002; Moran *et al.*, 2003).

PKC activation in keratinocytes leads to many events critical for differentiation, such as cell-cycle exit, expression of components found in the granular layer and cornified envelope, and apoptosis. Several PKC isoforms are expressed in the epidermis, including the classical isoform PKCα, the novel isoforms PKCδ, PKCε, PKCη, and the atypical isoforms PKCζ and PKCι (Denning, 2004; Helfrich *et al.*, 2007). However, none of the reported null mutant mice lacking individual PKC isoforms develop defects in epidermal differentiation (Castrillo *et al.*, 2001; Leitges *et al.*, 2001a, b, 2002; Chida *et al.*, 2003; Hara *et al.*, 2005). The reason for this may be functional redundancy between isoforms that compensate when only one is lacking. Our finding that a K14-RIP4 transgene can rescue epidermal differentiation in RIP4-deficient mice indicates that RIP4 functions within keratinocytes to promote differentiation. Furthermore, RIP4 has been shown to interact with classical (PKCβI) and novel (PKCδ) isoforms of PKC (Bahr *et al.*, 2000; Chen *et al.*, 2001). Therefore, an intriguing possibility is that RIP4 interacts with multiple PKC isoforms in keratinocytes to integrate or promote PKC signaling. A critical test of this model will be to identify proteins that interact with RIP4 *in vivo*. Also, the generation of compound mutants that lack multiple PKC isoforms in the epidermis may lead to phenotypic abnormalities that resemble RIP4$^{-/-}$ animals.

Endogenous RIP4 protein is undetectable by our immunohistochemistry protocol in wild-type embryos, but expression is clearly visible in the skin of K14-RIP4 transgenics. Surprisingly, the human K14 promoter we used typically targets gene expression to the basal layer of the epidermis (Vassar *et al.*, 1989; Sil *et al.*, 2004; Hirakawa *et al.*, 2005), but K14-RIP4 expression is found primarily in the spinous layer (Figures 1a, 2c and j). This finding may imply that RIP4 protein functions most actively in the spinous layer during epidermal differentiation, where it is regulated by a mechanism that increases its translation rate or stability in the spinous layer. This layer has been noted for its expression of PKCα (the only classical isoform expressed in the epidermis) and PKCδ (Denning, 2004), and highlights these two isoforms as potential RIP4 interaction partners *in vivo*.

Although the K14-RIP4 transgene produces high levels of RIP4 protein and can rescue epidermal differentiation in RIP4$^{-/-}$ animals, it is unable to promote epidermal differentiation in stratifin- or IKKα-deficient mice. Progress is being made on identifying the signaling pathways involving these proteins, but much is still unknown (Wynshaw-Boris, 2007; Descargues *et al.*, 2008). If RIP4, stratifin, and IKKα function in the same pathway to promote skin differentiation, then this could indicate that RIP4 functions upstream of IKKα and stratifin. This interpretation is supported by the observation that RIP4 activation of NF-κB requires IKKβ function (Meylan *et al.*, 2002). Alternatively, RIP4, stratifin, and/or IKKα could function in independent pathways. This would explain the phenotypic differences between the respective mutants (Fisher, 2000; Holland *et al.*, 2002). It is also consistent with the findings that epidermal differentiation can be rescued by grafting IKKα$^{-/-}$ skin onto IKKα$^{+/+}$ hosts, but not by grafting RIP4$^{-/-}$ skin onto RIP4$^{+/+}$ hosts (Hu *et al.*, 2001; Holland *et al.*, 2002). Finally, it is possible that RIP4 can drive epidermal differentiation in IKKα$^{-/-}$ or Sfn$^{Er/Er}$ animals, but simply elevating RIP4 expression is not sufficient to activate the necessary signaling events. Therefore, our experiments do not exclude the possibilities that forced stimulation of RIP4-specific signaling pathways, or the expression of a constitutively active version RIP4, could promote differentiation on these mutant backgrounds.

Challenging K14-RIP4 mice with topically applied TPA leads to a striking infiltration of neutrophils. Similar studies have also been carried out in transgenic mice overexpressing specific PKC isoforms in the epidermis with keratin-14 or keratin-5 promoters. Of the isoforms that have been tested, only PKCα transgenic mice develop a neutrophilic inflammatory response after TPA application (Reddig *et al.*, 1999, 2000; Wang and Smart, 1999; Jansen *et al.*, 2001; Cataisson *et al.*, 2003). This treatment leads to the formation of intracorneal pustules at hair follicles that appear remarkably similar to those of K14-RIP4 animals (Figure 5c). Furthermore, the neutrophilic inflammation of both K14-RIP4 and K5-PKCα animals is TNFR1-independent (Cataisson *et al.*, 2005). These results may indicate that RIP4 functions downstream of PKCα to promote epidermal inflammation. We found that the expression level and phosphorylation of PKCα and other PKC isoforms were similar between keratinocytes derived from C57BL/6 wild-type, RIP4$^{-/-}$ or K14-RIP4 mice (data not shown). This result is consistent with a model in which RIP4 signals downstream of PKCα. Inhibition of PKCα reduces expression of differentiation markers in keratinocytes stimulated to differentiate in response to calcium (Lee *et al.*, 1997; Yang *et al.*, 2003). Therefore, it is also a strong candidate for an interacting partner with RIP4 during epidermal differentiation. PKCα$^{-/-}$ mice have been reported (Leitges *et al.*, 2002; Hara *et al.*, 2005), and it would be interesting to test how K14-RIP4 transgenic mice respond to TPA on a PKCα-deficient background.

In PKCα transgenic mice, a single topical treatment with TPA is sufficient to mediate epidermal inflammation. In

addition, TPA stimulation of keratinocytes from PKCα transgenic mice leads to excessive elevation of mRNA or protein levels of several cytokines and chemotactic factors, including TNF-α and macrophage inflammatory protein-2 (Wang and Smart, 1999; Cataisson *et al.*, 2003, 2005, 2006). In contrast, it requires at least two TPA applications for K14-RIP4 mice on the C57BL/6 background to develop a neutrophilic infiltrate similar to that seen with one TPA application in PKCα transgenic mice (data not shown). Furthermore, no consistent changes in cytokines or chemotactic factors were detected in conditioned media from TPA-treated C57BL/6 K14-RIP4 keratinocytes compared with wild-type (data not shown). The differences between these transgenic strains could suggest that other factors downstream of PKCα in addition to RIP4 contribute to epidermal inflammation. Alternatively, because PKCα is directly bound and activated by TPA, perhaps increasing the levels of PKCα is more effective at initiating a TPA-driven signal than elevating RIP4 expression in the presence of normal levels of PKCα. It is possible that on the FVB/N background, which is known to have an enhanced sensitivity to TPA over C57BL/6 mice (Hennings *et al.*, 1993) and on which K14-RIP4 mice showed spontaneous inflammation (Figure 5a and b), TPA stimulation might result in a clearer enhancement of cytokine production in K14-RIP4 keratinocytes.

Inflammation is believed to be an important promoting factor in the DMBA/TPA skin cancer model and other cancers (Balkwill *et al.*, 2005; Mueller, 2006). Therefore, we were surprised to find that K14-RIP4 transgenic mice in a DMBA/TPA model developed exacerbated inflammation without an increased rate of carcinogenesis. This discrepancy may be due to the relatively high dose of DMBA and TPA used in our experiments. Although similar regimens have shown strong differences in transgenic mouse strains overexpressing PKC isoforms in the skin, they did not alter carcinogenesis in K14-PKCα or K5-PKCα transgenic mice (Reddig *et al.*, 1999, 2000; Wang and Smart, 1999; Jansen *et al.*, 2001). However, later experiments showed that K5-PKCα transgenic mice were indeed more susceptible to DMBA/TPA cancer promotion when a suboptimal dose of TPA was used (Cataisson *et al.*, 2009). We have not attempted to characterize K14-RIP4 mice using a suboptimal cancer promotion model, but this could reveal an increased sensitivity that has been masked by our protocol.

In conclusion, our study shows that RIP4 functions in the epidermis to regulate differentiation, potentially through PKC-dependent signaling cascades. This work also identifies a previously unknown activity of RIP4 in promoting cutaneous inflammation. In the future, it will be important to gain a better understanding of the molecular mechanisms underlying these diverse RIP4 functions, and to uncover how RIP4 interacts with other pathways regulating epidermal differentiation or inflammation.

MATERIALS AND METHODS

Plasmid construction

Plasmid pHR2 containing the human keratin 14 (K14) promoter was a kind gift of Sabine Werner (Zurich, Switzerland) (Vassar *et al.*, 1989; Munz *et al.*, 1999). The unique *Sma*1 cloning site for cDNA insertion was converted to an *Fse*1/*Pme*1/*Asc*1 polylinker to create pHB137-9. Mouse RIP4 (GenBank accession number AF302127) was used as a template for the PCR to generate the open reading frame (50–2,410 in mouse RIP4) flanked by a 5′-*Fse*1 site and a consensus translation initiation sequence, and a 3′-*Asc*1 site directly after the stop codon. This fragment was inserted in pHB137-9 digested with *Fse*1/*Asc*1 to yield pHB151-6. Nucleotide sequencing confirmed that the PCR fragment was identical to the template sequence. The K14/mouse RIP4/human growth hormone (hGH) polyA expression cassette was excised from pHB151-6 with *Kpn*1, to eliminate bacterial sequences from the microinjection fragment.

Mouse strains

To generate K14-RIP4 mice, the *Kpn*1 fragment of pHB151-6 was microinjected at a concentration of 2 ng μl^{-1} in 10 mM of Tris and 0.1 mM of EDTA (pH 8.0) into the pronuclei of B6D2F2 mouse embryos (Taconic, Germantown, NY), and implanted into pseudo-pregnant Swiss Webster recipients (Taconic). Mice were genotyped by PCR with primers in the hGH polyA region to screen for founders. Transgenic expressers were determined using quantitative reverse transcription-PCR analysis of tail skin biopsies from founders. Total tail skin RNA was isolated using the RNeasy Midi Kit (Qiagen, Valencia, CA). Reverse transcription-PCR was carried out using a TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA). Expression levels were determined by the expression of an hGH probe (Amgen, Seattle, WA) or murine RIP4 probe (Amgen) relative to an hypoxanthine phosphoribosyl transferase housekeeper gene (Applied Biosystems). The highest expressers were developed into lines by backcrossing to C57BL/6 mice (Taconic).

Before using in the skin cancer model, K14-RIP4 mice were backcrossed to FVB/N mice (Taconic) using Marker-Assisted Accelerated Backcrossing (Charles River Laboratories, Wilmington, MA) and were found to be at least 99.5% FVB/N mice.

TNFR1-, IKKα-, and RIP4-deficient mice are maintained on C57BL/6 backgrounds and have been described earlier (Peschon *et al.*, 1998; Takeda *et al.*, 1999; Holland *et al.*, 2002). Sfn$^{Er/+}$ mice were maintained on a mixed C57BL/6 and CBA background (Jackson Laboratory, Bar Harbor, ME).

Immunohistochemistry

For histopathological examination, cross-sectioned embryos or tissues were fixed in zinc fixative (K14-RIP4; RIP4$^{-/-}$), zinc-buffered formalin (Z-fix, Anatech, Battle Creek, MI) (K14-RIP4; IKKα$^{-/-}$), or 10% neutral buffered formalin (K14-RIP4; Sfn$^{Er/Er}$, K14-RIP4 mice used in inflammation studies), and then paraffin embedded. Sections 4 μm thick were stained with hematoxylin and eosin (H&E) or with antibodies for immunohistochemical detection of proteins. Staining was primarily carried out by Phenopath Laboratories (Seattle, WA) using an autostainer (Dako, Carpinteria, CA). After antigen retrieval in 10 mM of citrate buffer (pH 6), primary antibodies were detected with horseradish peroxidase-polymer-conjugated secondary antibodies using Dako EnVision+ (RIP4 antibody), or biotinylated secondary antibody using Elite ABC peroxidase (all other antibodies, Vector Laboratories, Burlingame, CA). Signal was developed in Dako DAB+ and counterstained with Mayer's hematoxylin. Immunostaining of murine RIP4 used polyclonal antibodies from

rabbits immunized with a KLH-conjugated peptide of the sequence GCGQENIVRTLLRRGVDVGLQGKD. Specific antibodies were column-purified using protein A and then the RIP4 peptide. Other primary antibodies used were K1 (Covance, Princeton, NJ, AF109), K6 (Covance, cat. no. PRB-169P), K14 (Covance, AF64), involucrin (Covance, cat. no. PRB-142C), stratifin (Santa Cruz Biotechnology, Santa Cruz, CA, C-18), and IKKα (Cell Signaling Technology, Beverly, MA, #2682). Under the conditions used, no stratifin or IKKα protein was detectable in $Sfn^{Er/Er}$ or $IKK\alpha^{-/-}$ skin, respectively (data not shown).

Animal models

For TPA-mediated ear inflammation assays, 20 μl of 50 μg ml^{-1} TPA (Sigma-Aldrich, St Louis, MO) in ethanol was applied using a pipette to the right ears of anesthetized mice, and 20 μl of ethanol was applied to the left. Ear thickness was measured on anesthetized mice using a dial thickness gauge (Mitutoyo America Corporation, Aurora, IL). Increase in ear thickness was calculated by subtracting baseline thickness from subsequent measurements. A log transformation was applied to the data to ensure normality. Statistical significance was evaluated using a repeated measure model for the overall effect and analysis of variance for each time point.

To assay contact hypersensitivity, the back skin of anesthetized mice was shaved, and 25 μl of 0.5% DNFB (Sigma-Aldrich) in acetone/olive oil (4:1) was applied to the skin using a pipette. Five days later, ear thickness was measured, and 10 μl of 0.25% DNFB was applied to the inner surface of the right ear, or 10 μl of vehicle to the left ear. Increase in ear thickness and statistical significance was evaluated as above.

For the DMBA/TPA skin carcinogenesis model, tumors were initiated on the shaved back skin of 7- to 8-week-old mice with 25 μg of DMBA (Sigma-Aldrich) in 0.2 ml of acetone on week 0, followed by twice weekly promotion with 5 μg of TPA in 0.2 ml acetone for 20 weeks. Mice were scored weekly, and were killed 30 weeks after DMBA application or earlier if they showed signs of lethargy or distress. To quantify intracorneal pustules, animals were treated bi-weekly with TPA for 2 weeks, and three strips of skin were collected from each animal 18 hours after the final TPA treatment. The number of pustules observed in 4-μm-thick H&E-stained sections of skin was divided by the total length of skin sectioned to yield the number of pustules per centimeter. After 14 weeks of TPA treatment to animals enrolled in the DMBA/TPA model, three strips of skin were collected and sectioned from each animal, and epidermal hyperplasia was scored blindly on a 4-point graded scale. The Wilcoxon–Mann–Whitney test was used to evaluate statistical significance.

All protocols used in these studies were in compliance with federal guidelines and approved by the Amgen Institutional Animal Care and Use Committee (IACUC).

CONFLICT OF INTEREST

The authors declare that they are current or previous employees and shareholders of Amgen.

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