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RIP4 Is an Ankyrin Repeat-Containing Kinase Essential for Keratinocyte Differentiation

Pamela M. Holland,[1,3] Cynthia R. Willis,[1]
Suzanne Kanaly,[1] Moira B. Glaccum,[1]
Annjanette S. Warren,[1] Keith Charrier,[1]
J. Greg Murison,[2] Jonathan M. J. Derry,[1]
G. Duke Virca,[1] Timothy A. Bird,[1]
and Jacques J. Peschon[1]
[1]Immunex Corporation
51 University Street
Seattle, Washington 98101
[2]Genesis Research and Development Corporation
P.O. Box 50
Auckland
New Zealand

Summary

The epidermis is a stratified, continually renewing epithelium dependent on a balance among cell proliferation, differentiation, and death for homeostasis. In normal epidermis, a mitotically active basal layer gives rise to terminally differentiating keratinocytes that migrate outward and are ultimately sloughed from the skin surface as enucleated squames. Although many proteins are known to function in maintaining epidermal homeostasis, the molecular coordination of these events is poorly understood [1, 2]. RIP4 is a novel RIP (receptor-interacting protein) family kinase with ankyrin repeats cloned from a keratinocyte cDNA library. RIP4 deficiency in mice results in perinatal lethality associated with abnormal epidermal differentiation. The phenotype of RIP4$^{-/-}$ mice in part resembles that of mice lacking IKKα, a component of a complex that regulates NF-kappaB [3–5]. Despite the similar keratinocyte defects in RIP4- and IKKα-deficient mice, these kinases function in distinct pathways. RIP4 functions cell autonomously within the keratinocyte lineage. Unlike IKKα, RIP4-deficient skin fails to fully differentiate when grafted onto a normal host [6]. Instead, abnormal hair follicle development and epidermal dysplasia, indicative of progression into a more pathologic state, are observed. Thus, RIP4 is a critical component of a novel pathway that controls keratinocyte differentiation.

Results and Discussion

RIP4 was identified in a screen for novel genes in a mouse keratinocyte cDNA library. RIP4 contains an N-terminal serine/threonine kinase domain with roughly 40% identity to RIP family kinases [7–9] and a C-terminal domain with nine ankyrin repeats. More recently, RIP4 was independently identified by two groups as a novel PKC-interacting protein in yeast two-hybrid screens [10, 11].

To assess the function of RIP4 in vivo, we generated a targeted disruption of RIP4 in mice (see Supplementary Material available with this article online). Heterozygous animals appeared phenotypically normal and were intercrossed to generate homozygous mutant mice (RIP4$^{-/-}$). No viable RIP4$^{-/-}$ mice were recovered within several hours of birth. Examination of RIP4$^{-/-}$ fetuses at E17.5 and E18.5 revealed marked skin defects (Figures 1A and 1B). The skin of RIP4$^{-/-}$ fetuses was significantly reduced in skin folds, and the hind limbs and tail were consistently shorter and partially fused to the body cavity. Most striking was the fusion of all external orifices, including the nose, mouth, and anus, in RIP4$^{-/-}$ fetuses. As a consequence of oral fusion, RIP4$^{-/-}$ fetuses most likely die at birth due to suffocation. Histological examination of the oral cavity and esophagus of RIP4$^{-/-}$ fetuses confirmed the oral fusion and indicated that RIP4$^{-/-}$ vibrissae were poorly developed (Figures 1C–1F). This atresia continued through the esophagus into the squamous portion of the stomach (Figures 1G and 1H). In the paws of E18.5 RIP4$^{-/-}$ fetuses, the epithelium between the digits was also fused (our unpublished data). To determine if defective apoptosis was responsible for the epithelial defects, we evaluated interdigital epithelium and dorsal skin by TUNEL staining. No differences in apoptosis were noted between wild-type and RIP4$^{-/-}$ fetuses (our unpublished data). These data indicate that tissues composed of keratinized stratified epithelial cells depend upon RIP4 for normal differentiation. No other defects were revealed in complete histopathological analyses (our unpublished data).

To further analyze the epidermal defects, we histologically examined dorsal skin sections from E18.5 wild-type and RIP4$^{-/-}$ fetuses (Figure 2). Compared to skin from wild-type littermates, the skin of RIP4$^{-/-}$ fetuses was significantly thicker and had a smooth outer surface (Figures 2A and 2B). The mean dorsal-skin thickness of RIP4$^{-/-}$ fetuses was 112.9 μm ± S.D. 13.12, compared to 41.11 μm ± S.D. 7.8 for skin from wild-type fetuses ($p < 0.0001$). The outermost cornified layers were absent in RIP4$^{-/-}$ skin and were replaced by a thick layer of flattened, parakeratotic cells (Figures 2A and 2B). In addition, there was marked hyperplasia of the spinous and granular layers. Some areas of orthokeratosis, which appeared as a thin anuclear layer beneath the parakeratotic layer, were also present in RIP4$^{-/-}$ skin. We found no significant differences in the number of bromodeoxyuridine (BrdU)-positive cells from wild-type skin (33.3 ± S.D. 3.2) as compared to RIP4$^{-/-}$ skin (35.3 ± S.D. 2.5, $p = 0.4455$), suggesting that the increased thickness of the suprabasal layers of RIP4$^{-/-}$ skin was not a consequence of increased proliferation.

Progressive differentiation in epidermis can be followed by the unique expression of different keratins in specific epidermal layers. RIP4$^{-/-}$ skin sections were examined for the presence of early and late differentiation markers, including K1, K14, and filaggrin, by immunocytochemistry. In normal skin, K14 is expressed exclusively in the innermost mitotically active basal cell layer (Figure 2C; [12]). In addition to expression in the

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[3] Correspondence: hollandp@amgen.com

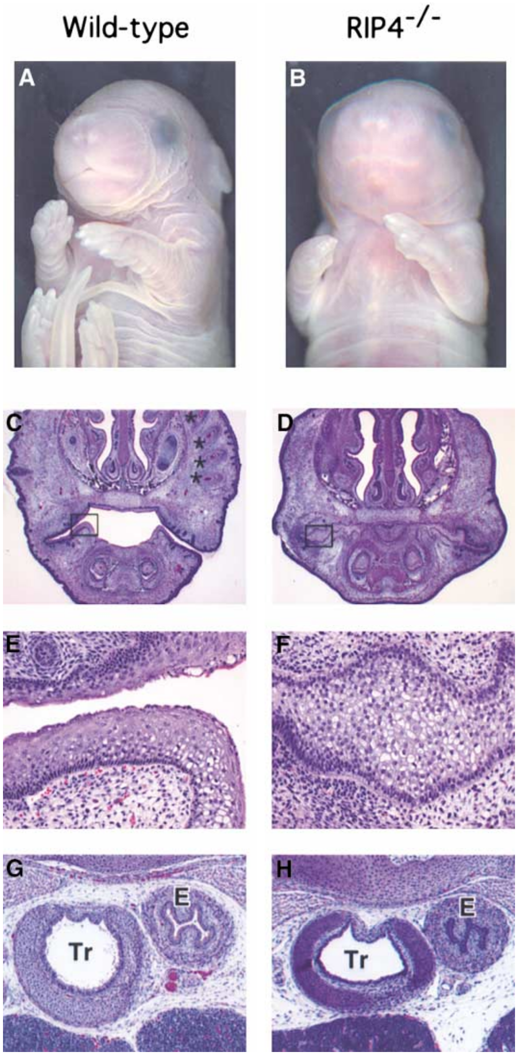

Figure 1. Phenotypic Analysis of RIP4$^{-/-}$ Mice

(A and B) Gross appearance of wild-type and RIP4$^{-/-}$ fetuses. Frontal views of wild-type (A) and RIP4$^{-/-}$ (B) E18.5 fetuses. The RIP4$^{-/-}$ fetus has a fused mouth and lacks whiskers.

(C and D) Hematoxylin-and-eosin (H&E)-stained coronal sections of E17.5 wild-type (C) and RIP4$^{-/-}$ (D) heads, 20×. Asterisks in (C) denote vibrissae. The RIP4$^{-/-}$ mouth is fused, and the vibrissae are poorly developed. Boxed regions are magnified in (E) and (F).

(E and F) High magnification of oral mucosa in (C) and (D), 200×.

(G and H) H&E-stained sagittal sections of E17.5 wild-type (G) and RIP4$^{-/-}$ (H) torso, 40×. Tr, trachea; E, esophagus. The esophagus from the RIP4$^{-/-}$ fetus contains no lumen.

basal layer, RIP4$^{-/-}$ skin showed a dramatic upregulation of K14 expression in the granular and outermost parakeratotic layer (Figure 2D). The spinous layer of healthy skin normally expresses K1, and expression was limited to the suprabasal layers in wild-type mice (Figure 2E; [12]). In contrast, K1 was strongly expressed in all the hyperplastic layers of RIP4$^{-/-}$ skin (Figure 2F). Filaggrin, a component of keratohyalin granules and a marker of late differentiation, was also abnormally expressed in RIP4$^{-/-}$ skin. Compared to wild-type skin, which only expressed filaggrin in the granular and cornified layers (Figure 2G), RIP4$^{-/-}$ skin expressed filaggrin in the spinous and granular layers but not in the parakeratotic layer (Figure 2H). Although filaggrin was expressed in the inappropriate cell layers of RIP4$^{-/-}$ skin, some fraction of it was processed into its mature form, based on immunoblot analysis (our unpublished data) [13]. In the layers of RIP4$^{-/-}$ skin where filaggrin was expressed, we also observed inappropriate expression of two additional late differentiation markers, loricrin and involucrin (our unpublished data). Collectively, the expression of keratins and differentiation markers in RIP4$^{-/-}$ skin suggests that the outermost parakeratotic layer is largely undifferentiated and displays characteristics of more basal cells. Furthermore, electron microscopy revealed that in addition to the marked increase in thickness of the spinous and granular layers, the outermost stratum corneum of RIP4$^{-/-}$ skin was absent and was replaced by several layers of nucleated parakeratotic cells (Figures 2I and 2J).

To further investigate gene expression changes resulting from loss of RIP4 in skin, we performed microarray analyses of wild-type and RIP4$^{-/-}$ skin mRNAs. This revealed elevated expression of a number of genes, including those for K19, several S100 proteins, and the Sprr family of cornified envelope proteins (Table S1, see the Supplementary Material). These proteins are similarly dysregulated in inflammatory dermatoses and various epithelial cancers [14–18].

Features of RIP4$^{-/-}$ skin resemble those recently reported in mice lacking IKKα, and in the mouse mutants *pupoid fetus (pf)* and *repeated epilation (Er)* [3–5, 19]. All of these phenotypes are typefied by a failure of epidermal keratinocytes to differentiate properly, resulting in hyperplasia of the epidermis and the absence of a stratum corneum. However, several lines of evidence indicate that RIP4 does not lie on a linear signaling pathway that includes IKKα. First, filaggrin expression is absent in IKKα-deficient skin and present, albeit in the inappropriate cell layer, in RIP4$^{-/-}$ skin [4, 5]. Second, IKKα, *pf*, and *Er* do not function cell autonomously within the epidermal compartment; in each case, the phenotype of mutant skin is rescued when engrafted onto a wild-type recipient [19]. In marked contrast, the phenotype of RIP4-deficient skin is not fully rescued in similar engraftment studies (Figures 3A and 3B). Ten weeks post-engraftment, RIP4$^{-/-}$ grafts were devoid of hair and displayed varying degrees of epidermal and hair follicle dysplasia as well as sebaceous-gland hyperplasia (Figures 3C and 3D). Although some of the cornified layer was present in the mutant grafts, the epidermis remained thickened, and there were increased mitotic figures in the basal layer (Figures 3E and 3F). We also observed hair follicles that were unevenly spaced or in abnormal positions, delayed hair follicle differentiation, and keratin-filled invaginations (Figures 3G and 3H). Whereas hair follicles in the wild-type grafts were predominantly in the telogen or resting phase, those in the mutants were primarily blocked in the anagen or growth phase [20]. Although no hair follicle defects other than poorly developed vibrissae were observed in the E18.5 RIP4$^{-/-}$ fetuses, these results raise the possibility that RIP4 may contribute to proper hair follicle growth and/or development. Third, IKKα is essential for normal B cell development, whereas RIP4 appears dispensable

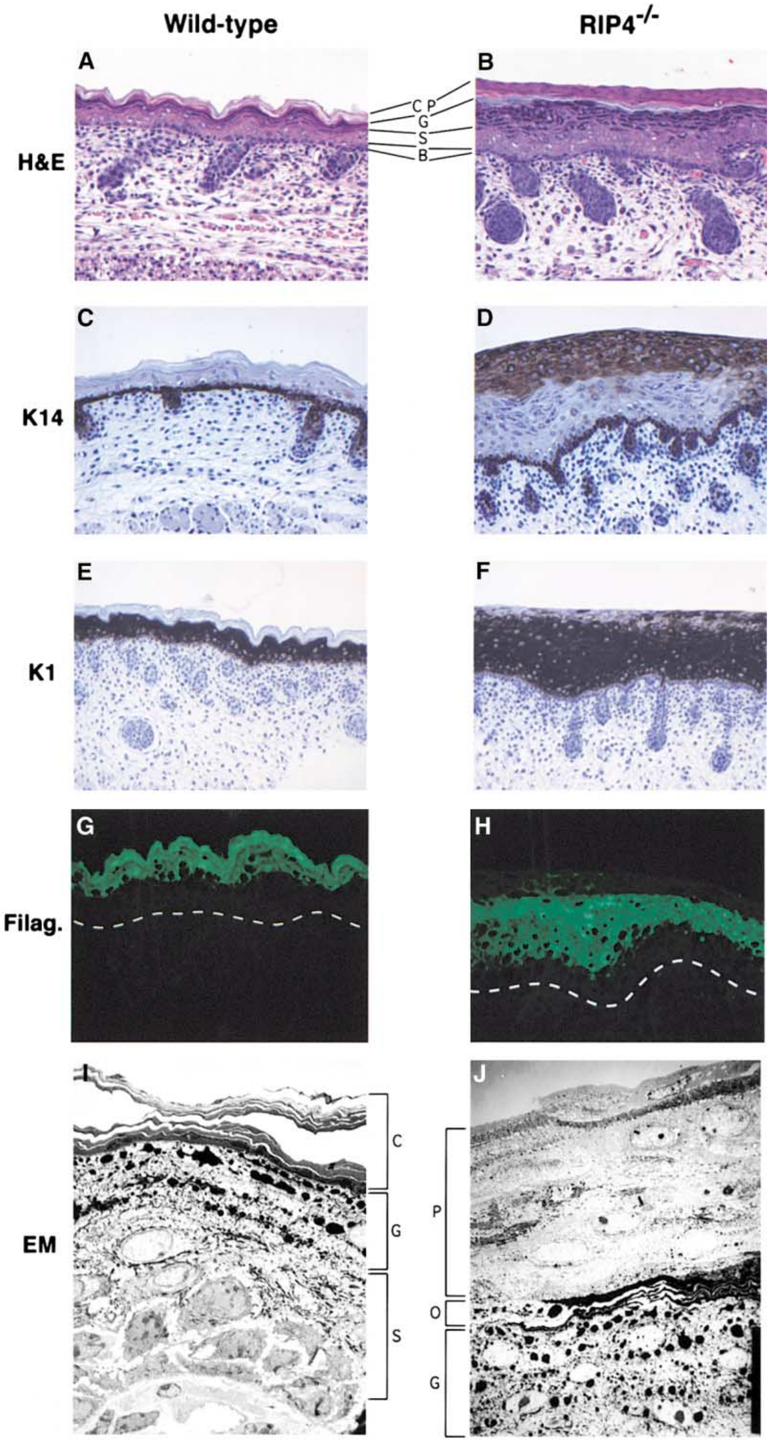

Figure 2. Abnormal Keratinocyte Differentiation in RIP4$^{-/-}$ Skin

Dorsal skin from the backs of E18.5 wild-type (A, C, E, and G) and RIP4$^{-/-}$ (B, D, F, and H) fetuses were sectioned and stained with the following: (A and B) H&E; (C and D) Keratin K14; (E and F) Keratin K1; and (G and H) Filaggrin. The white dashed line denotes the location of the basal layer. In (A) and (B), epidermal layers are B, basal; S, spinous; G, granular; and C, cornified. In (B), the RIP4$^{-/-}$ skin lacks a cornified layer that is replaced by a thick parakeratotic outer layer, P. (I and J) Electron micrographs of wild-type (I) and RIP4$^{-/-}$ (J) epidermis. Magnification is 1500×. Complete absence of the superficial corneal layer, C, is apparent in the mutant. The parakeratotic layer, P, and orthoparakeratotic layer, O, are visible above the granular layer, G, in RIP4$^{-/-}$ skin.

for B cell development (see Supplementary Material; [21, 22]).

RIP4 was recently cloned as both a PKCδ- and a PKCβ-interacting protein [10, 11]. PKC is known to function in keratinocyte differentiation; however, no defects in skin development are reported in mice lacking either PKCδ or PKCβ [23–27]. This suggests either that PKC isoforms may compensate for one another in skin development or that RIP4 function in skin differentiation is independent of PKC.

In conclusion, loss of RIP4 in mice results in a phenotype similar to that observed for IKKα, *pf*, and *Er*, although genetic evidence clearly indicates that RIP4 is an essential component of a unique pathway controlling keratinocyte differentiation. RIP4 deficiency may also affect hair follicle morphogenesis. Recent studies have established that follicular stem cells are bipotent and can give rise to keratinocytes of both the the hair follicle and the epidermis [28, 29]. Our results suggest that RIP4 may function early in the differentiation program at a

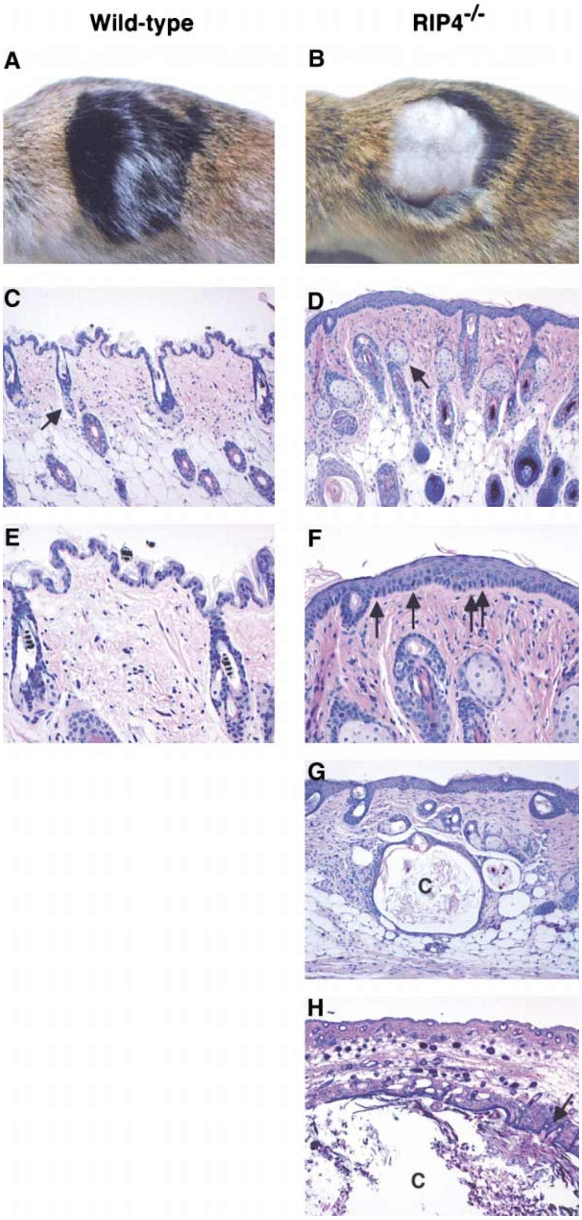

Figure 3. RIP4$^{-/-}$ Skin Is Not Rescued When Grafted onto a Normal Host

(A and B) Gross appearance of (A) wild-type and (B) RIP4$^{-/-}$ skin grafts 10 weeks after engraftment onto Rag1$^{-/-}$ mice. Photos are representative of at least four wild-type and RIP4$^{-/-}$ grafts with similar phenotypes.

(C–F) H&E-stained cross-sections of wild-type (C and E) and RIP4$^{-/-}$ (D and F) grafts 10 weeks after engraftment at 100× (C and D) and 200× (E and F) magnification. Note that RIP4$^{-/-}$ epidermis remains thickened and hair follicles do not penetrate above the surface. Arrows in (C) and (D) indicate sebaceous glands. Arrows in (F) indicate mitotic figures in the basal layer.

(G) Keratin-filled cyst in the dermal layer of a RIP4$^{-/-}$ graft, 100×. C, cyst.

(H) Keratin-filled intraepidermal inclusion cyst (4 mm × 2 mm) in the subcutis of a RIP4$^{-/-}$ graft, 40×. Arrow in (H) indicates hair follicles growing into the body of the cyst. C, cyst.

level that impairs the development of both hair follicle and epidermal cells. Thus, it will be important to elucidate the RIP4-dependent mechanisms that link differentiation of hair follicle and epidermal cells. RIP4, or components of a pathway that includes RIP4, may represent targets for the treatment of inflammatory or neoplastic lesions that involve the keratinocyte lineage.

Experimental Procedures

Cloning of *RIP4*

Partial sequence of a novel, putative kinase corresponding to *RIP4* was identified among a series of ESTs obtained from a partnership with Genesis Research and Development Corporation (Auckland, NZ). These ESTs were derived from a transit-amplifying cell library generated at Genesis (J.G.M., unpublished data). The *RIP4* EST contained a complete kinase domain, a series of ankyrin repeats, and an in-frame stop codon but no initiator methionine. Subsequently, another EST that contained the missing *RIP4* initiation codon was deposited in GenBank (accession number AI317448). This sequence information was used for generating PCR primers and cloning full-length *RIP4* from a mouse stromal cell library. Our murine *RIP4* cDNA sequence is 99.8% identical to mouse *PKK* (accession number AF302127), a gene recently reported to be a novel PKC-interacting protein [11].

Immunohistochemistry

For H&E staining, skin from the backs of E18.5 fetuses was fixed in formalin, paraffin embedded, and cut into 4 μm sections prior to staining. For K1 and K14 (Covance) immunostaining, skin was fixed in Methyl Carnoy's fixative, paraffin embedded, and cut into 4 μm sections. Indirect immunostaining with K1 and K14 rabbit anti-mouse primary antibodies was visualized with peroxidase goat anti-rabbit secondary antibody (Vector Labs). Frozen sections embedded in OCT (Sakura Tissue-Tek) and cut into 5 μm sections were used for filaggrin (Covance) indirect immunostaining and were visualized with a fluoresceinated goat anti-rabbit secondary antibody (Vector Labs). For transmission electron microscopy, skin was fixed in Karnovsky's, stained with lead citrate, and embedded in plastic for analysis.

Skin Grafts

Skin (dermis and epidermis) from C57BL/6 × 129 random hybrid E18.5 wild-type and RIP4$^{-/-}$ fetuses was surgically grafted onto the sides of 8-week-old female Rag1$^{-/-}$ mice (Jackson Labs). Each fetus represented one graft of roughly 1 cm^2. Bandages were removed after 7 days, and animals were examined regularly 1–10 weeks post-engraftment until sacrifice. At 10 weeks, five wild-type and four mutant grafted animals with similar phenotypes were collected for histology.

Supplementary Material

Additional Experimental Procedures, results on RIP4 in B cell development, and a table showing the fold upregulation in gene expression in RIP4$^{-/-}$ (KO) versus WT skin may be found with this article online at http://images.cellpress.com/supmat/supmatin.htm.

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