Phospholipase C δ_1 is required for skin stem cell lineage commitment

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Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in phosphoinositide turnover and is involved in a variety of physiological functions. Here we report that PLC δ_1 -deficient mice undergo progressive hair loss in the first postnatal hair cycle. Epidermal hyperplasia was observed, and many hairs in the skin of PLC δ_1 -deficient mice failed to penetrate the epidermis and became zigzagged owing to occlusion of the hair canal. Two major downstream signals of PLC, calcium elevation and protein kinase C activation, were impaired in the keratinocytes and skin of PLC δ_1 -deficient mice. In addition, many cysts that had remarkable similarities to interfollicular epidermis, as well as hyperplasia of sebaceous glands, were observed. Furthermore, PLC δ_1 -deficient mice developed spontaneous skin tumors that had characteristics of both interfollicular epidermis and sebaceous glands. From these results, we conclude that PLC δ_1 is required for skin stem cell lineage commitment.

Keywords: differentiation/PLC/skin stem cell

Introduction

Phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate two second messengers: diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP₃). DG mediates the activation of protein kinase C (PKC), and IP₃ releases calcium from intracellular stores (Berridge and Irvine, 1984; Nishizuka, 1988). PLC can be categorized into four types, β , γ , δ and ϵ , on the basis of sequence homology and activation mechanism (Rhee and Bae, 1997). The δ -type PLCs, δ_1 – δ_4 , are thought to be the primary forms expressed in mammals. Among these, PLC δ_1 is expressed abundantly in most tissues (Suh *et al.*, 1988; Lee *et al.*, 1999) and has been studied most intensively. PLC δ_1 is the most calcium-sensitive PLC

(Allen *et al.*, 1997) and is thought to be activated in response to calcium mobilization induced by β - or γ -type PLCs (Rhee and Bae, 1997; Kim *et al.*, 1999). PLC δ_1 activity is also regulated by sphingomyelin and spermine (Scarlata *et al.*, 1996; Pawelczyk and Lowenstein, 1997). Other factors, such as transglutaminase II and the GTPase-activating protein for Rho, are also reported to play a role in PLC δ_1 activation (Nakaoka *et al.*, 1994; Homma and Emori, 1995). It has been suggested that PLC δ_1 is involved in Alzheimer's disease (Shimohama *et al.*, 1991) and essential hypertension (Yagisawa *et al.*, 1991; Kato *et al.*, 1992); however, despite some progress in the structural understanding of PLC δ_1 (Essen *et al.*, 1996), its expression levels, regulation mechanisms, and biological and physiological functions are still poorly understood.

Mammalian skin is composed of at least three differentiated epithelial compartments: interfollicular epidermis (IFE), hair follicles and associated glands. It contains pluripotent stem cells that reside in the bulge and generate these compartments. The IFE is characterized by a polarized pattern of epithelial growth and differentiation, with a single basal layer of proliferating keratinocytes and multiple overlying differentiated layers. During differentiation, basal keratinocytes detach from the basement membrane, migrate to the suprabasal layers and are eventually shed. The hair follicle is a more complex structure composed of an outer root sheath (ORS), an inner root sheath (IRS) and a hair shaft, and it undergoes cycles of growth and regression. It has been reported that keratinocytes, the major component of IFE and hair follicles, possess a functionally active inositol lipid signaling system that plays an essential role in keratinocyte differentiation (Tang *et al.*, 1988; Punnonen *et al.*, 1993; Xie and Bikle, 1999).

In the present study, to elucidate the physiological roles of PLC δ_1 we generated PLC δ_1 -deficient mice and found that they undergo progressive hair loss that is attributable to epidermal hyperplasia, occlusion of the hair canal, sebaceous gland hyperplasia and formation of abnormal cysts. These observations indicate that PLC δ_1 is required for normal regulation of interfollicular epidermal keratinocyte proliferation and differentiation, and for determination of skin stem cell fate *in vivo*.

Results

Generation of PLC δ_1 -deficient mice

To inactivate the PLC δ_1 gene, we engineered a targeting vector in which the DNA encoding the catalytic domains of PLC was replaced with a neomycin-resistance cassette (Figure 1A). This vector yielded targeted recombination events in two independent embryonic stem (ES) cell clones. These ES cell clones were injected into blastocysts to generate chimeric germline heterozygous mice.

Fig. 1. Targeted disruption of the *PLCδ₁* gene in mice. (A) Gene targeting strategy. Exons encoding the X and Y domains were replaced with a neomycin-resistance cassette (*neo*). *gpt*, xanthine/guanine phosphoribosyl transferase gene; *DTA*, diphtheria toxin A gene; PH, pleckstrin homology domain; X, X domain; Y, Y domain; D, *Dra*I. (B) Southern blot analysis of mouse genomic DNA. *Dra*I-digested genomic DNAs isolated from the tails of wild-type (+/+), heterozygous (+/-) and *PLCδ₁*-deficient (-/-) mice were hybridized with the 3' probe. *Dra*I digestion yields a 12.5 kb fragment for the wild-type allele and a 6.5 kb fragment for the mutant allele. (C) Western blotting of protein extracts from the testes of wild-type (+/+), heterozygous (+/-) and *PLCδ₁*-deficient (-/-) mice. (D) Dorsal and (E) ventral views of 3.5-month-old wild-type (+/+) and *PLCδ₁*-deficient (-/-) mice.

Selective breeding of heterozygous knockout mice yielded homozygous knockout mice. Correct gene targeting was verified by Southern blot analysis (Figure 1B). Western blot analysis of testes (which normally express *PLCδ₁* abundantly) from knockout mice lacked the 85 kDa *PLCδ₁* protein (Figure 1C). Because our targeting strategy left the possibility that N-terminal fragments containing the pleckstrin homology (PH) domain could be expressed, we generated a rabbit polyclonal antibody directed against the N-terminal 60 amino acids of *PLCδ₁* and performed western blot analysis with this antibody to confirm that N-terminal fragments of *PLCδ₁* protein were not expressed. No such fragments were detected (data not shown).

Heterozygous knockout mice of *PLCδ₁* appeared phenotypically normal and fertile, and intercrosses between heterozygous knockout mice produced pups

with the expected Mendelian distribution (+/+ : +/- : -/- = 45:74:39), indicating that disruption of *PLCδ₁* did not cause embryonic lethality. Homozygous knockout mice (*PLCδ₁*-deficient mice) appeared fertile, although runting was evident. However, ~90% of *PLCδ₁*-deficient mice showed marked defects in ventral body hair, and ~60% of them showed defects in dorsal body hair at 3.5 months of age (Figure 1D and E).

Hair canal occlusion and epidermal hyperplasia resulting from *PLCδ₁* disruption

Hair loss in *PLCδ₁*-deficient mice was first observed 8 days after birth and was followed by progressive alopecia. Therefore, we analyzed morphological changes in hair follicles from wild-type and *PLCδ₁*-deficient mice by hematoxylin and eosin (HE) staining. The number of hair follicles was normal, but many hairs in the skin of *PLCδ₁*-deficient mice at 8 days of age failed to penetrate the epidermis and became zigzagged (Figure 2D), whereas hairs in wild-type mice penetrated the epidermis. Hair canals were occluded by differentiated keratinocytes including keratohyalin granules in mutant hair follicles (Figure 2D, arrow). In addition, the IFE of *PLCδ₁*-deficient mice was thicker than that of wild-type mice (Figure 2A–D). The number of viable suprabasal cell layers was increased by one to two layers in comparison with that of the IFE of wild-type mice.

Because interfollicular epidermal hyperplasia was observed, we investigated the proliferative activity of epidermal keratinocytes by bromodeoxyuridine (BrdU) incorporation. The number of interfollicular and upper-follicular cells that incorporated BrdU was ~8 times greater in *PLCδ₁*-deficient mice than in wild-type mice (30 ± 3.8 and 3.6 ± 1.9 cells/field, respectively) (Figure 2E–G). These data indicate that *PLCδ₁* plays a critical role in the regulation of keratinocyte proliferation and that disruption of this gene results in interfollicular epidermal hyperplasia.

To further understand the physiological role of *PLCδ₁* in skin, we determined the localization of *PLCδ₁* in wild-type mice. In wild-type skin, *PLCδ₁* was localized predominantly in the upper region of the hair follicle ORS and in all of the IFE except the cornified layers (Figure 2H). *PLCδ₁* immunoreactivity was not detected in skin from *PLCδ₁*-deficient mice (Figure 2I) or in skin from wild-type mice stained with antigen-absorbed *PLCδ₁* antibody (Figure 2J). These expression patterns of *PLCδ₁* in skin imply that it plays an important role in regulating keratinocyte growth and differentiation in IFE and upper ORS.

Abnormal differentiation of epidermis and hair follicles in *PLCδ₁*-deficient mice

Because abnormal epidermal and hair follicle morphologies were observed in skin from *PLCδ₁*-deficient mice, we investigated how the absence of *PLCδ₁* influences differentiation of keratinocytes in these structures. To do this, we examined the expression of molecular markers in skin from wild-type and *PLCδ₁*-deficient mice at 8 days of age by immunofluorescence. Cytokeratin 1 (K1) and loricrin, markers of terminal differentiation in normal IFE, were also detected in the upper region of mutant hair follicle ORS (Figure 3B and H, arrows), where occlusion of hair canals was observed on HE-stained sections, whereas

Fig. 2. Histological abnormalities in the skin of PLC δ_1 -deficient mice at 8 days of age. HE staining of dorsal skin sections from (A and C) wild-type (+/+) and (B and D) PLC δ_1 -deficient (-/-) mice at 8 days of age. The arrow in (D) indicates hair canal occlusion by differentiated keratinocytes. The length of both heads and arrows (C and D) indicates the thickness of the epidermis except for the cornified layers. Analysis of keratinocyte proliferation in dorsal skin sections from (E) wild-type and (F) PLC δ_1 -deficient mice at 8 days of age by BrdU incorporation. Sections were counterstained with hematoxylin. (G) Number of BrdU-positive cells per field. Values represent the average number of BrdU-positive cells in 30 fields from three sections from different mice. The statistical significance was determined by Student's *t*-test ($p < 0.05$). (H-J) Immunohistochemical detection of PLC δ_1 in dorsal skin sections. Immunohistochemical staining of (H) wild-type and (I) PLC δ_1 -deficient mice at 8 days of age with a polyclonal anti-PLC δ_1 antibody. (J) Immunohistochemical staining with antigen-absorbed antibody on wild-type skin was also carried out. Bars: (A and B), shown in (A), 100 μ m; (C and D), shown in (C), 50 μ m; (E and F), shown in (E), 50 μ m; (H-J), shown in (H), 50 μ m.

these markers were detected only in IFE in wild-type mice (Figure 3A and G). These observations suggest that the upper region of mutant hair follicle ORS differentiated in a manner similar to that of normal IFE. In addition, cytokeratin 6 (K6), a hair follicle ORS marker that is normally absent from keratinocytes in the IFE (Figure 3E), was also expressed in mutant IFE (Figure 3F). Aberrant expression of K6 was reported to be associated with epidermal hyperproliferation (Porter *et al.*, 1998), and PLC δ_1 -deficient mice displayed this phenomenon. Expression of cytokeratin 5 (K5), a marker of interfollicular basal keratinocytes and the hair follicle ORS, was slightly enhanced in PLC δ_1 -deficient mice in comparison with wild-type mice (Figure 3C and D). Thus, differen-

Fig. 3. Abnormal differentiation of keratinocytes in the epidermis and hair follicles of PLC δ_1 -deficient mice at 8 days of age. Green corresponds to antibody staining, and red corresponds to PI (BO-PRO-3) staining. Dorsal skin sections from wild-type (left, +/+) and PLC δ_1 -deficient (right, -/-) mice at 8 days of age were stained with antibody against (A and B) cytokeratin 1 (K1), (C and D) cytokeratin 5 (K5), (E and F) cytokeratin 6 (K6) and (G and H) loricrin (lor). Arrows indicate aberrant K1 (B) and loricrin (H) expression in the upper portion of mutant hair follicles. Bars: (A-H), shown in (A), 100 μ m.

tiation of keratinocytes in the IFE and hair follicle was disturbed in the absence of PLC δ_1 .

Characterization of PLC δ_1 -deficient primary keratinocytes

Keratinocytes possess a functionally active inositol lipid signaling system that plays an essential role in their differentiation. PLC γ_1 is expressed abundantly in keratinocytes and regulates this process (Punnonen *et al.*, 1993; Xie and Bikle, 1999). Therefore, we examined expression of PLC γ_1 , PLC δ_1 and the terminal differentiation markers involucrin and loricrin in primary keratinocytes.

Expression of PLC δ_1 protein was increased >10-fold after calcium treatment, whereas PLC γ_1 protein expression was increased only slightly (Figure 4A). However, there were no apparent differences in levels of involucrin and loricrin between wild-type and PLC δ_1 -deficient keratinocytes (data not shown).

Because PLC δ_1 is the most calcium-sensitive PLC, it is presumed to be activated by calcium mobilization induced by β - and γ -type PLCs, and it boosts and sustains calcium elevation (Allen *et al.*, 1997; Rhee and Bae, 1997; Kim *et al.*, 1999). Thus, it may play a specific role in calcium mobilization which cannot be compensated by other PLCs such as PLC γ_1 . To test this hypothesis, we compared the change in the intracellular calcium concentration, $[Ca^{2+}]_i$, in response to the extracellular calcium concentration, $[Ca^{2+}]_o$, in primary keratinocytes from wild-type and PLC δ_1 -deficient mice. In this case we used keratinocytes differentiated for 72 h because PLC δ_1 is expressed most abundantly (Figure 4A). The basal $[Ca^{2+}]_i$ of keratinocytes from wild-type mice (158 nM) was slightly higher than that of keratinocytes from PLC δ_1 -deficient mice (93 nM). In keratinocytes from PLC δ_1 -deficient mice, $[Ca^{2+}]_i$ peaked (834 nM) 50 s after calcium stimulation, whereas that of keratinocytes from wild-type mice peaked (1004 nM) at 140 s. At that time point, the $[Ca^{2+}]_i$ of keratinocytes from PLC δ_1 -deficient mice had already decreased to 597 nM. At 240 s, the $[Ca^{2+}]_i$ of keratinocytes from PLC δ_1 -deficient mice had decreased to 453 nM, although that of keratinocytes from wild-type mice remained high (917 nM). Thus, elevation of $[Ca^{2+}]_i$ in response to $[Ca^{2+}]_o$ was more transient in keratinocytes from PLC δ_1 -deficient mice than in those from wild-type mice (Figure 4B). However, when these experiments were carried out with non-differentiated keratinocytes in which PLC δ_1 is expressed at very low levels, the mobilization of $[Ca^{2+}]_i$ in response to $[Ca^{2+}]_o$ was the same in keratino-

Fig. 4. Changes in calcium signaling in PLC δ_1 -deficient primary cells. (A) PLC δ_1 and PLC γ_1 contents during calcium-induced keratinocyte differentiation. Primary keratinocytes were treated with 1 mM $CaCl_2$ for 0, 24, 48, 72 and 96 h, and 15 μ g of total protein were subjected to SDS-PAGE and western blot analysis was performed. Actin was used as the loading control. δ_1 , PLC δ_1 ; γ_1 , PLC γ_1 ; inv, involucrin; lor, loricrin. (B) Keratinocytes from wild-type and PLC δ_1 -deficient mice differentiated for 72 h were stimulated with 1.6 mM $CaCl_2$. Fluorescence ratio (F_{340}/F_{380}) images of wild-type (+/+) and PLC δ_1 -deficient (-/-) keratinocytes at the indicated times are shown in the upper panels. Time courses of $[Ca^{2+}]_i$ mobilization in wild-type (+/+) and PLC δ_1 -deficient (-/-) keratinocytes are shown in the lower panel. Data were collected in independent experiments ($n = 40$). (C) Adenoviral infection of PLC δ_1 or GFP was performed in PLC δ_1 -deficient keratinocytes. PLC δ_1 - and GFP-infected keratinocytes from PLC δ_1 -deficient mice differentiated for 72 h were stimulated with 1.6 mM $CaCl_2$. Time courses of $[Ca^{2+}]_i$ mobilization in PLC δ_1 -infected (Ad-GFP-PLC δ_1) and GFP-infected (Ad-GFP) keratinocytes are shown. Values were calculated by comparison with the 340/380 ratio of cells without Ca^{2+} stimulation. (D) The change in NFAT transcriptional activity in keratinocytes. Adenoviral infection of PLC δ_1 or GFP was performed in PLC δ_1 -deficient keratinocytes. Luciferase activity was measured 76 h after transfection of pNFAT-Luc in wild-type (+/+), PLC δ_1 -deficient (-/-), PLC δ_1 -infected PLC δ_1 -deficient (-/- Ad-GFP-PLC δ_1) and GFP-infected PLC δ_1 -deficient (-/- Ad-GFP) cells untreated or treated with high Ca^{2+} (1.0 mM) for the last 24, 48 and 72 h of the experiment (0 h, 24 h, 48 h, 72 h). Values were calculated by comparison with the activity of cells untreated with high Ca^{2+} (0 h). Statistical significance between +/+ and -/- at 48 and 72 h was determined by Student's *t*-test ($p < 0.05$).

cytes from both wild-type and PLC δ_1 -deficient mice (data not shown). These data suggest that PLC δ_1 is important for sustaining high $[Ca^{2+}]_i$. In fact, adenoviral infection of the PLC δ_1 gene in PLC δ_1 -deficient keratinocytes rescued this defect (Figure 4C). Calcium-dependent nuclear translocation of calcineurin β , one event subsequent to calcium elevation, was also lower in PLC δ_1 -deficient keratinocytes (22.4% of cells, $n = 514$) than in wild-type keratinocytes (35.7% of cells, $n = 487$). Because sustained elevation of $[Ca^{2+}]_i$ is required for activation of the nuclear factor of activated T cells (NFAT) and because calcineurin regulates NFAT activity (Dolmetsch *et al.*, 1997), we next examined NFAT activity using a luciferase assay. The transcriptional activity of NFAT was increased 15.2-fold at 48 h and 12.7-fold at 72 h after exposure to high $[Ca^{2+}]_o$ in wild-type cells, whereas this elevation of transcriptional activity was lower in PLC δ_1 -deficient cells (6.7-fold at 48 h and 4.9-fold at 72 h) (Figure 4D). Furthermore, we confirmed that adenoviral infection of PLC δ_1 into PLC δ_1 -deficient keratinocytes recovered the impaired NFAT promoter activity of PLC δ_1 -deficient keratinocytes

(Figure 4D). Expression of terminal differentiation markers was not altered by infection of PLC δ_1 (data not shown). These results suggest that suppression of NFAT activity is not caused by altered differentiation, but rather is a primary consequence of loss of PLC δ_1 . Thus, calcium mobilization and activation of calcineurin and NFAT were impaired in PLC δ_1 -deficient primary keratinocytes.

Change in downstream effectors of PLC in skin

We then examined activation of PKC, the downstream effector of PLC in skin. PKC activity is controlled by phosphorylation of conserved sites (Keranen *et al.*, 1995). We performed immunohistochemical staining of skin sections with an antibody that discriminates between phosphorylated and non-phosphorylated PKCs. Phosphorylated PKCs were clearly detected in the suprabasal layers of the IFE and the upper regions of hair follicles in the skin of wild-type mice (Figure 5A), whereas they were barely visible in the skin of PLC δ_1 -deficient mice (Figure 5B). We also analyzed the levels of phosphorylated PKCs in epidermal extracts. Levels of phosphorylated PKCs were markedly decreased in PLC δ_1 -deficient epidermis extracts (Figure 5C). We then examined levels of some PKC isozymes (α , δ , η and ζ). To our surprise, levels of PKC isozymes were also decreased and possible degradation products of PKC isozymes were detected within the lower molecular weight range (Figure 5C). These degradation products were not observed in the heart and testis, in which PLC δ_1 is also abundantly expressed, suggesting that PLC δ_1 plays an important role in the stability and degradation of PKCs in the suprabasal layers of the IFE and the upper region of the hair follicles, although other factors may contribute to the instability of PKC in skin. Because we observed marked changes in levels of PKC isozymes between wild-type and PLC δ_1 -deficient epidermis, we further examined activation of nuclear factor- κ B (NF- κ B), which has been reported to be regulated by PKC and calcium signaling (Hughes *et al.*, 1998; Jang *et al.*, 2001; Kanke *et al.*, 2001; Vancurova *et al.*, 2001). NF- κ B plays an important role in balancing growth and differentiation in the IFE and hair follicles, and inhibition of NF- κ B signaling in these regions leads to epidermal hyperplasia (van Hogerlinden *et al.*, 1999; Kaufman and Fuchs, 2000; Schmidt-Ullrich *et al.*, 2001) similar to that observed in PLC δ_1 -deficient mice. NF- κ B activity is regulated primarily by translocation from the cytoplasm, where it is bound to the inhibitor of κ B (I κ B), to the nucleus, where it induces expression of target genes (Verma *et al.*, 1995; Beg and Baltimore, 1996). Therefore, we investigated the intracellular localization of NF- κ B with an antibody directed against the p50 subunit. NF- κ B was present predominantly in nuclei in wild-type IFE, whereas nuclear localization of NF- κ B was not prominent in IFE from PLC δ_1 -deficient mice (Figure 5D and E). In addition, we ascertained whether suppression of NF- κ B activity was an effect of decreased PKC signaling. To examine this, we investigated the localization of NF- κ B after treatment with PMA, a PKC activator, in the skin of wild-type and PLC δ_1 -deficient mice. If suppression of NF- κ B activity was caused by insufficient PKC activation, PKC activation by PMA should rescue impaired NF- κ B activity in the skin of PLC δ_1 -deficient mice. In fact, strong nuclear localization

Fig. 5. Downstream effectors of PLC δ_1 . Green corresponds to antibody staining (A, B, D, E), and red corresponds to PI (BO-PRO-3) staining (A and B). Immunohistochemical detection of active PKC in dorsal skin sections from (A) wild-type (+/+) and (B) PLC δ_1 -deficient (-/-) mice at 8 days of age. (C) Protein levels of phosphorylated PKCs and PKC isozymes. Western blot analysis of protein extracts from the epidermis of wild-type (+/+) and PLC δ_1 -deficient (-/-) mice was carried out. Arrowheads indicate the molecular size of full-length phosphorylated PKCs and PKC isozymes. Arrows indicate possible degraded PKC isozymes. Actin was used as the loading control. Localization of NF- κ B (p50) in dorsal skin sections from (D) wild-type (+/+) and (E) PLC δ_1 -deficient (-/-) mice at 8 days of age. Arrows in (D) indicate nuclear localization of p50 in normal skin. Back skins of (F) wild-type (+/+) and (G) PLC δ_1 -deficient mice (-/-) were treated with 1 μ g/ml PMA in acetone for 2 h and harvested. Immunohistochemical detection of NF- κ B p50 subunit was performed on frozen sections. Bars: (A and B), shown in (A), 50 μ m; (D and E), shown in (D), 50 μ m; (F and G), shown in (F), 50 μ m.

Fig. 6. Abnormal cyst formation and sebaceous gland hyperplasia in PLCδ₁-deficient mice. HE staining of dorsal skin sections from (A, C and E) wild-type (+/+) and (B, D and F) PLCδ₁-deficient (-/-) mice at 15 days of age. Arrows in (C) and (D) indicate sebaceous glands. Arrow in (E) indicates granular layers of wild-type IFE. Arrow in (F) indicates cells of abnormal cysts, which resemble those of the granular layers of wild-type IFE. HE staining of dorsal skin sections from (G) wild-type and (H) PLCδ₁-deficient mice at 25 days of age. Comparison of ultrastructure of (I) normal epidermis and (J) hair follicle-derived cysts. Arrows in (I) and (J) indicate desmosomes. Arrowheads in (I) and (J) indicate keratohyalin granules. Bars: (A, B, G and H), shown in (A), 100 μm; (C, D and F), shown in (C), 50 μm; (E), 50 μm; (I and J), shown in (I), 2 μm.

of NF-κB was observed in the skin of both wild-type and PLCδ₁-deficient mice (Figure 5F and G). These data indicate that PKC-mediated NF-κB activation plays an important role downstream of PLCδ₁ in skin.

Sebaceous gland hyperplasia and cyst formation in PLCδ₁-deficient mice

Hair follicle morphology was severely disrupted in the skin of PLCδ₁-deficient mice at 15 days of age, although

the interfollicular epidermal hyperplasia was less remarkable than that at 8 days of age (Figure 6A and B). Sebaceous glands were enlarged, and the number of sebocytes was greatly increased (Figure 6C and D). In addition, many hair follicle-derived cysts were identified within the dermis of PLCδ₁-deficient skin (Figure 6B). These cysts were filled with horny cells and were surrounded by multilayered epithelium containing cells that resembled the wild-type epidermal granular layers (Figure 6E and F, arrows). Such cysts were never observed in skin from wild-type mice (Figure 6A). At 25 days of age, when hair follicles in wild-type skin enter a new anagen phase and grow down into the subcutis (Figure 6G), the cysts enlarged somewhat and the hair follicles showed more severe disorganization in PLCδ₁-deficient mice (Figure 6H).

Similarity of cysts to IFE

We also performed ultrastructural analysis of normal IFE and hair follicle-derived cysts (Figure 6I and J). The cysts were surrounded by several viable cell layers containing prominent desmosomes (Figure 6J, arrows). These layers corresponded to the spinous layers of normal IFE. Further within the inner layer, there was a layer containing keratohyalin granules (Figure 6J, arrowheads), which corresponded to the granular layer of normal IFE. In the innermost layers, electron-dense squames that were equivalent to the cornified layers of normal IFE were identified.

Next, we examined the expression of molecular markers in the cysts observed in PLCδ₁-deficient mice at 15 days of age. Keratinocytes in the central layers of the cysts expressed K6 (Figure 7E and F). K5, K1 and loricrin were detected in the peripheral cell layer, all viable cell layers except the most peripheral and the innermost cell layer of the cysts, respectively. Thus, moving inward from the peripheral cell layer in mutant hair follicle-derived cysts, an expression pattern, first K5, then K1 and finally loricrin, was observed (Figure 7B, D, H and N). Such an expression pattern is usually present only in the basal to cornified layers of wild-type IFE (Figure 7A, C, G and M), indicating that the cysts undergo a program of terminal differentiation similar to that of IFE. Thus, the cysts detected in PLCδ₁-deficient mice showed remarkable similarities to the IFE of wild-type mice at the ultrastructural and molecular marker levels. Furthermore, the expressions of AE13 and AE15, pan-hair keratin marker and IRS marker, respectively, were not detected in the cysts (Figure 7J and L), whereas expressions of these markers were detected in hair follicles of wild-type mice (Figure 7I and K). These observations indicate that, in the absence of PLCδ₁, cells of hair follicle-derived cysts have lost their follicular characteristics and exhibit interfollicular epidermal differentiation. Thus, these data strongly suggest that PLCδ₁ is involved in lineage determination of keratinocytes in IFE and hair follicles.

Spontaneous skin tumors observed in PLCδ₁-deficient mice

PLCδ₁-deficient mice developed spontaneous skin tumors in ventral skin. The incidence of tumors in PLCδ₁-deficient mice was ~20% (18/88) of mice over 3 months old. Such tumors were never observed in wild-type mice.

Next, we characterized these tumors at the histological level. They arose from underneath the IFE, and large cysts and many sebocyte-like cells surrounded by basaloid keratinocytes were observed in them (Figure 8C and D). The sebocyte-like cells were positively stained with Oil Red O, a histochemical dye specific for lipid-containing cells (Figure 8E and F). K1 was also expressed in the sebocyte-like cells in addition to the inner layer of large cysts (Figure 8G and H). K5 was expressed in peripheral

Fig. 8. Development of spontaneous skin tumors in the skin of PLC δ_1 -deficient mice. (A and B) Skin tumors developed in PLC δ_1 -deficient mice. Arrows indicate tumors. (C and D) HE staining of tumor sections from PLC δ_1 -deficient mice at 8 days of age. Arrows in (C) indicate large cysts and the arrow in (D) indicates sebocyte-like cells observed in these tumors. Sections were stained with (E and F) Oil Red O, (G and H) cytochrome 1 and (I and J) cytochrome 5. (K) Summary of disturbed lineage commitment in PLC δ_1 -deficient mice. Bars: (C), 200 μ m; (D and H), shown in (D), 25 μ m; (E), 100 μ m; (F and J), shown in (F), 50 μ m; (G and I), shown in (G), 50 μ m.

Fig. 7. Similarities in expression of molecular markers between cysts and IFE. In (A–H), green corresponds to antibody staining, and red corresponds to PI (BO-PRO-3) staining. Dorsal skin sections from wild-type (left, +/+) and PLC δ_1 -deficient (right, -/-) mice at 15 days of age were stained with antibody against (A and B) cytochrome 1 (K1), (C and D) cytochrome 5 (K5), (E and F) cytochrome 6 (K6) and (G and H) loricin (lor). Arrows indicate aberrant K1 (B) and loricin (H) expression in hair follicle-derived cysts. In (I–L), green corresponds to antibody staining, and red corresponds to K5 staining. Dorsal skin sections from wild-type (left, +/+) and PLC δ_1 -deficient (right, -/-) mice at 15 days of age were stained with antibody against (I and J) AE13 and (K and L) AE15. (M and N) The similarity between hair follicle-derived cyst in (M) PLC δ_1 -deficient mice and (N) normal epidermis. Bars: (A–H), shown in (A), 100 μ m; (I–L), shown in (I), 50 μ m.

cell layers surrounding the sebocyte-like cells and in large cysts (Figure 8I and J). Thus, these tumors had the characteristics of both IFE and the sebaceous glands. Since it has been reported that impaired lineage commitment of skin stem cells induces development of tumors that have the properties of sebaceous glands in the skin (Niemann *et al.*, 2002), these results strongly support the idea that PLC δ_1 is involved in skin stem cell lineage determination.

Discussion

In the present study, we show that interfollicular epidermal hyperplasia, which reflects an increase in the number of differentiated cell layers, is observed in PLC δ_1 -deficient mice at 8 days of age. The IFE is maintained by a finely tuned balance between keratinocyte proliferation and differentiation that results in a multilayer structure consisting of basal, spinous, granular and cornified layers. Therefore, interfollicular epidermal hyperplasia of PLC δ_1 -deficient mice suggests that PLC δ_1 is required for regulation of balance between keratinocyte proliferation and differentiation.

PKC is one downstream effector of PLC activation. At least six PKC isoenzymes (PKC- α , δ , ϵ , η , ζ and μ) are known to be expressed in keratinocytes, and they are thought to be central regulators of keratinocyte proliferation and differentiation (Dlugosz and Yuspa, 1993). In the suprabasal layers of the IFE and the upper region of the hair follicle ORS, where PLC δ_1 is expressed at fairly high levels in wild-type mice, phosphorylated forms of PKCs were barely detected in PLC δ_1 -deficient mice. This was caused by enhanced PKC degradation in PLC δ_1 -deficient epidermis, although the mechanism is still unclear. This degradation was not observed in testis and heart, where PLC δ_1 is abundantly expressed, suggesting that this enhanced PKC degradation is the skin-specific event. Thus, insufficient PKC activity may be the cause of the epidermal hyperplasia and abnormal morphologies of hair follicles in PLC δ_1 -deficient skin. Furthermore, translocation of NF- κ B to the nucleus, which occurs during keratinocyte differentiation, was also inhibited in the IFE of PLC δ_1 -deficient mice. Because it has been reported that NF- κ B plays a role in balancing keratinocyte growth and differentiation (Seitz *et al.*, 1998), these skin abnormalities of PLC δ_1 -deficient mice may be attributable to impaired NF- κ B signaling. In addition, this impaired NF- κ B activity can be rescued by treatment with the PKC activator PMA, which strongly stimulates PKC and induces sufficient PKC activity to result in the activation of NF- κ B, even though the protein levels of PKC are decreased in the skin of PLC δ_1 -deficient mice.

We also found that expression of PLC δ_1 is induced transiently after calcium treatment and plays critical roles in calcium mobilization and activation of its downstream effectors, calcineurin and NFAT, in primary cells. After calcium treatment, intracellular PLC activation occurs through a calcium-sensing receptor (Hebert and Brown, 1995; Chattopadhyay *et al.*, 1996) or by an undefined mechanism (Jaken and Yuspa, 1988). It was reported recently that PLC γ_1 is critical for mobilization of intracellular calcium in response to calcium treatment (Xie and Bikle, 1999). Despite the presence of PLC γ_1 , defective calcium mobilization was observed in PLC δ_1 -deficient

mice. One possible explanation is that PLC γ_1 is first activated by calcium treatment and triggers IP $_3$ production and intracellular calcium elevation. This transient calcium elevation by PLC γ_1 stimulates PLC δ_1 , the most calcium-sensitive PLC, which prolongs the intracellular calcium elevation and the activation of its downstream effectors. In fact, in keratinocytes from PLC δ_1 -deficient mice, elevation of [Ca $^{2+}$] $_i$ in response to [Ca $^{2+}$] $_o$ was more transient, and activation of NFAT, which is activated by sustained elevation of calcium, was inhibited. A direct influx of extracellular calcium through non-specific cation channels and cGMP-gated channels has been reported in keratinocytes (Galletta *et al.*, 1991; Mauro *et al.*, 1993; Oda *et al.*, 1997), and elevation of calcium levels by this influx may also activate PLC δ_1 . It has been reported that calcineurin and NFAT activities were induced during calcium-induced differentiation of keratinocytes, and suppression of calcineurin activity by cyclosporin A (CsA) results in impaired expression of terminal differentiation markers (Santini *et al.*, 2001). However, expression of terminal differentiation markers induced by exposure to high calcium was not altered in primary keratinocytes from wild-type and PLC δ_1 -deficient mice. One possible explanation is that suppression of NFAT activity caused by the loss of PLC δ_1 was insufficient to interfere with induction of terminal differentiation markers. In fact, a low concentration of CsA failed to suppress expression of terminal differentiation markers, although NFAT promoter activity was suppressed approximately one-third at this CsA concentration (Santini *et al.*, 2001).

In addition, we show that sebaceous gland hyperplasia and the formation of cysts, which show remarkable similarities to IFE and lose the characteristics of hair follicle, occur in the skin of PLC δ_1 -deficient mice. The epidermis is maintained throughout life by pluripotent stem cells producing, via daughter cells of high differentiation probability, IFE, hair follicles and sebaceous glands (Taylor *et al.*, 2000; Watt and Hogan, 2000; Oshima *et al.*, 2001). Therefore, sebaceous gland hyperplasia and the formation of such cysts reflect suppression of the hair follicle differentiation pathway and promotion of IFE and sebaceous gland differentiation. Furthermore, spontaneous skin tumor development was observed in PLC δ_1 -deficient mice. Single oncogenic mutations in keratinocytes that are undergoing terminal differentiation will be innocuous, as the cells are rapidly shed from the epidermis. In contrast, stem cells have the potential to accumulate multiple mutations and undergo neoplastic conversion (Jensen *et al.*, 1999; Taipale and Beachy, 2001). Therefore, the cells that generate tumors in the skin of PLC δ_1 -deficient mice are likely to be stem cells. In addition, these tumors have characters of sebocytes and IFE keratinocytes, indicating that in the absence of PLC δ_1 , stem cells preferentially differentiate into sebocytes and IFE. Thus, these changes indicate that PLC δ_1 is required for lineage commitment of skin stem cells (Figure 8K).

Taken together, these results show that PLC δ_1 regulates skin stem cell lineage commitment and is involved in interfollicular epidermal keratinocyte proliferation and differentiation *in vivo*. However, in comparison with the dramatic change in hair follicle morphology, that of IFE was comparatively small although interfollicular epidermal hyperplasia and abnormal K6 expression were

observed. Thus, the phenotype of PLC δ_1 -deficient mice appears to reflect the disturbance of skin stem cell lineage commitment more. Similar cysts are observed upon overproduction of Zippro1, a zinc-finger transcription factor (Yang *et al.*, 1999), after disruption of RXR α (Li *et al.*, 2000) or the vitamin D receptor (Li *et al.*, 1997) and in mice with mutation of the *Hairless* (h) gene, which encodes a putative transcription factor (Panteleyev *et al.*, 1998). In addition, inhibition of Wnt signaling by ablation of the β -catenin gene (Huelsken *et al.*, 2001) or by expressing the Lef1 transgene lacking the β -catenin binding site (Merrill *et al.*, 2001; Niemann *et al.*, 2002) results in production of cysts. However, the apparent molecular relationship between these genes and PLC δ_1 is unknown. In PLC δ_1 -deficient mice, hair loss was first observed 8 days after birth owing to occlusion of the hair, and such a phenotype was never observed at this stage in these mice. Thus, the phenotype of these mice was different from that of PLC δ_1 -deficient mice in some points. This suggests that PLC δ_1 may control skin stem cell lineage commitment via another novel pathway. Two major downstream effectors of PLC δ_1 , which also have broad signaling functions in cells, are PKC activation and calcium mobilization. Since calcium influx and activation of PKC are altered in the skin of mutant mice, it is possible that PLC δ_1 may control the lineage commitment of skin stem cells by altering these effectors, thereby modifying many downstream targets.

We show here for the first time that PLC δ_1 is required for the determination of skin stem cell lineage. Clarifying the mechanisms underlying the determination of skin stem cell fate may permit engineering of the epidermis to produce more hair follicles as a treatment for balding or the development of therapies for excess sebum production in teenagers, for example. Thus, understanding the way that PLC δ_1 regulates skin stem cell lineage commitment is very important. However, further investigation is needed to clarify this, and it is a subject for the future.

Materials and methods

Generation of PLC δ_1 -deficient mice

Phage clones containing the exons that encode the X and Y domains of PLC δ_1 were isolated from a FIXII mouse genomic library (strain 129SV/J). In the targeting vector, the 5.7 kb DNA fragment encompassing the X and Y domains of the PLC δ_1 coding sequence was deleted and replaced with a neomycin-resistance cassette. The *NotI*-linearized targeting vector was electroporated into 129/Ola embryonic stem cells (ES cells). Two independent, heterozygous ES cell clones were used to generate chimeric mice by blastocyst injection, and mutant animals were bred on a mixed 129/Ola $\times$ C57BL/6J background. Genotyping was carried out by Southern blot and polymerase chain reaction (PCR) assays. For Southern blot analysis, genomic DNA was digested with *DraI* and hybridized with a 3' probe. The 3' probe corresponded to a 0.2 kb *HindIII*-*PstI* DNA fragment isolated from the PLC δ_1 genomic clone. Primers for PCR analysis were 5'-CAAGGAGGTGAAGGACTTCGTG-3', 5'-CTGGGT-CAGCATCCTGTAGAAG-3' and 5'-CCTGTGCTCTAGTAGCTTACG-3'.

Western blot analysis

Testes from wild-type, heterozygote and homozygote knockout mice were flash frozen, pulverized in liquid nitrogen and incubated for 10 min in SDS-PAGE sample buffer at 95°C. For primary keratinocytes, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed directly with SDS-PAGE sample buffer. For the analysis of phosphorylated PKCs, epidermis was separated from skin and the separated epidermis was washed with PBS, placed into RIPA buffer,

homogenized, sonicated and centrifuged for 15 min at 10 000 g to remove insoluble debris. SDS sample buffer was then added and incubated for 10 min at 95°C. The lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed with antibodies.

Histochemistry and immunohistochemistry

All skin biopsies were matched for age, sex and body site. For HE staining, skin samples were fixed in PBS containing 4% paraformaldehyde and embedded in paraffin. Immunofluorescence was performed on 10 μ m thick cryosections and on keratinocytes. Cryosections were incubated with primary antibodies to cytokeratin 1, cytokeratin 5, cytokeratin 6, loricrin (Babco), NF- κ Bp50, NF- κ Bp65 (Santa Cruz Biotechnology), AE13, AE15 (a gift from T.T.Sun, New York University Medical School) or phospho-PKC (pan) (Cell Signaling), which recognizes phosphorylated PKC α , β_1 , β_2 , δ , ϵ and η after fixation in acetone. Sections were then incubated with Alexa Fluor 488 goat anti-rabbit IgG antibody (Molecular Probes). Keratinocytes were fixed in 3.7% formaldehyde in PBS. Cells were then permeabilized with 0.2% Triton X-100 in PBS for 5 min and incubated with anti-calceineurin β antibody (Upstate Biotechnology) and then Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes). Counterstaining was performed with BO-PRO-3 (Molecular Probes). Stained sections and cells were examined and photographed with a confocal microscope (Radiance 2000; Bio-Rad). For PLC δ_1 immunohistochemistry, 5 μ m cryosections of skin were fixed in acetone at -80°C for 5 min and incubated with a rabbit polyclonal anti-PLC δ_1 antibody followed by the procedure described in the technical bulletin of the tyramide signal amplification (TSA) immunodetection kit (NEN).

BrdU labeling

BrdU labeling was performed with a 5-bromo-2'-deoxyuridine labeling and detection kit II (Roche) by intraperitoneal injection of BrdU into mice according to the manufacturer's recommendations.

Cell culture

Primary murine keratinocytes were isolated from newborn wild-type or PLC δ_1 -deficient mice (<1 day old) by overnight trypsin flotation. Isolated keratinocytes were plated at 6×10^6 cells per 60 mm dish in serum-free keratinocyte growth medium (KGM) (Clonetics) supplemented with 0.05 mM Ca $^{2+}$. To induce differentiation, the cells were grown to ~50% confluence, treated with culture medium supplemented with 1 mM Ca $^{2+}$ and cultured for various times before harvest.

Generation of adenoviruses

Recombinant adenoviruses were generated using the AdEasy System as described previously (He *et al.*, 1998).

Intracellular calcium measurement

Primary murine keratinocytes cultured for various times after exposure to high calcium (1 mM) were loaded with 7.5 μ M Fura-2 AM and 0.05 mM calcium in buffer A (20 mM HEPES, 120 mM NaCl, 5 mM KCl, 1 mM MgCl $_2$, 1 mg/ml sodium pyruvate, 1 mg/ml glucose). Cells were then exposed to 1.6 mM calcium. The fluorescence (ratio of $F_{340\text{ nm}}$ to $F_{380\text{ nm}}$) from single cells was recorded using an image analyzer. The signals from 10 single cells for each preparation were recorded and an average profile of 40 single cells from four preparations was calculated. Each sample was calibrated by the addition of ionomycin (20 μ M final concentration) (F_{max}) followed by 25 mM EGTA-Tris-HCl pH 8.3 (F_{min}). Intracellular calcium concentration was calculated from the following formula:

$$[\text{Ca}^{2+}] = K_d Q (R - R_{\text{min}}) / (R_{\text{max}} - R)$$

where $R = F_{340\text{ nm}}/F_{380\text{ nm}}$, $Q = F_{\text{min}}/F_{\text{max}}$ at 380 nm and K_d for Fura-2 for Ca $^{2+}$ is 224 nM.

Luciferase assay

For the luciferase assay, primary keratinocytes were seeded in 24-well plates. At ~70–80% confluence, cells were transfected with pNFAT-Luc plasmid (Stratagene). Transfection was performed using FuGENE6 reagent (Roche) according to the manufacturer's protocol. Luciferase activity was measured 76 h after transfection in untreated cells or cells treated with high Ca $^{2+}$ (1.0 mM) for the final 24, 48 or 72 h of the experiment. Preparation of cell lysates and measurement of luciferase activity were performed with a Luciferase Assay Kit (Stratagene) and a Fusion $^{\text{TM}}$ α (Packard) according to the manufacturer's protocol. Samples from triplicate wells were examined and the experiment was repeated

twice. Differences were analyzed for statistical significance using Student's *t*-test.

Ultrastructural analysis

Skin tissues were processed for conventional transmission electron microscopy by fixation in 2% glutaraldehyde in 0.1 M cacodylate buffer followed by 2% osmium tetroxide in water for 3 h on ice. Ultrathin sections (70–80 nm) on copper grids were treated with 2% uranyl acetate and examined with a JEOL JEM-200EX electron microscope at 100 kV.

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