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Advances in Brief

Crucial Role of Phospholipase Cϵ in Chemical Carcinogen-Induced Skin Tumor Development

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Abstract

Mutational activation of the *ras* proto-oncogenes is frequently found in skin cancers. However, the nature of downstream signaling pathways from Ras involved in skin carcinogenesis remains poorly understood. Recently, we and others identified phospholipase C (PLC) ϵ as an effector of Ras. Here we have examined the role of PLCϵ in *de novo* skin chemical carcinogenesis by using mice whose PLCϵ is genetically inactivated. *PLCϵ*$^{-/-}$ mice exhibit delayed onset and markedly reduced incidence of skin squamous tumors induced by initiation with 7,12-dimethylbenz(*a*)anthracene followed by promotion with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Furthermore, the papillomas formed in *PLCϵ*$^{-/-}$ mice fail to undergo malignant progression into carcinomas, in contrast to a malignant conversion rate of approximately 20% observed with papillomas in *PLCϵ*$^{+/+}$ mice. In all of the tumors analyzed, the Ha-*ras* gene is mutationally activated irrespective of the *PLCϵ* background. The skin of *PLCϵ*$^{-/-}$ mice fails to exhibit basal layer cell proliferation and epidermal hyperplasia in response to TPA treatment. These results indicate a crucial role of PLCϵ in *ras* oncogene-induced *de novo* carcinogenesis and downstream signaling from TPA, introducing PLCϵ as a candidate molecular target for the development of anticancer drugs.

Introduction

The *ras* proto-oncogenes are mutationally activated in about 15% of human neoplasms (1). Their products, Ras small GTPases, control cell proliferation and differentiation through interaction with multiple effector proteins, among which Raf kinases have been implicated in carcinogenesis from studies on *in vitro* transformation of fibroblast cell lines (2) and on genomic mutations in malignant melanoma (3). However, downstream signaling pathways from Ras involved in epithelial cell carcinogenesis remain poorly understood, despite the fact that *ras* mutations are more frequently found in epithelial cell-derived neoplasms (1). Likewise, the role of phosphoinositide-specific phospholipase C (PLC) in carcinogenesis remains obscure (4). PLC produces two vital intracellular second messengers, diacylglycerol and inositol 1,4,5-trisphosphate, which induce activation of protein kinase C and mobilization of Ca^{2+} from intracellular stores, respectively. Among 12 mammalian PLC isoforms classified into 5 classes (β, γ, δ, ϵ, and ζ), PLCϵ is characterized by possession of the Ras-associating domains, which are responsible for PLCϵ activation through direct association with the GTP-bound active forms of the small GTPases Ras (5, 6), Rap1 (7), and Rap2 (8). PLCϵ was also reported to be regulated by α_{12}, α_{13}, and $\beta_1\gamma_2$ subunits of heterotrimeric G proteins and Rho small GTPase (9). Identification of PLCϵ as a Ras effector has prompted us to examine the role of PLCϵ in carcinogenesis. Here we show that PLCϵ-deficient mice are resistant to chemical carcinogen-induced skin tumor formation, suggesting a crucial role of PLCϵ in tumor development downstream of Ras signaling.

Materials and Methods

***PLCϵ*$^{-/-}$ Mice.** Targeted inactivation of the *PLCϵ* gene was performed by a standard embryonic stem cell-based method.[4] The targeted allele (*PLCϵ*$^{-}$) expresses a mutant PLCϵ with an in-frame deletion of amino acids 1333 to 1408 corresponding to the NH_2-terminal part of the catalytic X domain. This mutant completely lost its PLC catalytic activity. *PLCϵ*$^{-/-}$ mice were maintained on a mixed 129/Sv × C57BL/6 background.

Reverse Transcription-Polymerase Chain Reaction Analysis. Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously (10). Primers used for amplification of *PLCϵ* were 5′-TCAGTGCCTGGAGCAGCAG-3′ and 5′-CTTGAAGGGGATCTTGGTTG-3′.

Skin Tumor Formation. A dorsal area of skin of 8-week–old mice was shaved and treated with a single application of 7,12-dimethylbenz(*a*)anthracene [DMBA (25 μg in 100 μL of acetone; Sigma, St. Louis, MO] and subsequently treated with 12-*O*-tetradecanoyl-phorbor-13-acetate [TPA (0.2 mmol/L in 100 μL of acetone; Sigma] twice a week for 20 weeks (11). Tumors were assessed weekly for up to 30 weeks and defined as raised lesions with a minimum diameter of 1 mm. *P* values were determined by unpaired Student's *t* test using GraphPad InStat software (GraphPad Software, Inc., San Diego, CA).

Histologic Analysis. Paraffin-embedded sections were prepared and stained with hematoxylin and eosin or with a specific antibody against mouse PLCϵ (10), keratin 14 (PRB-155P; BAbCO, Berkeley, CA), or keratin 1 (PRB-165P; BAbCO). Detection of immunoreactive signals was performed with Histo-Mouse Plus kit (Zymed Laboratories, South San Francisco, CA) or with a fluorescein isothiocyanate-conjugated secondary antibody (AP182F; Chemicon, Temecula, CA).

12-*O*-Tetradecanoylphorbol-13-acetate–Induced Skin Hyperplasia. A dorsal area of skin of 10-week–old mice was treated with TPA (0.2 mmol/L in 100 μL of acetone). The mouse skin was analyzed by staining with an anti-proliferating cell nuclear antigen (PCNA) antibody (M0879; Dako Cytomation, Copenhagen, Denmark) or hematoxylin and eosin. The thickness of the epidermis was measured at a minimum of five different points on the specimens and averaged.

Analysis of Ha-*ras* Gene Mutations. Ha-*ras* gene mutations at the 61st codon of the tumors were analyzed as described previously (12).

Results and Discussion

RT-PCR analysis of skin RNA detected two amplified products whose sizes were identical to those predicted from the wild-type and mutant *PLCϵ* mRNAs (Fig. 1*A*). Immunohistochemical analysis

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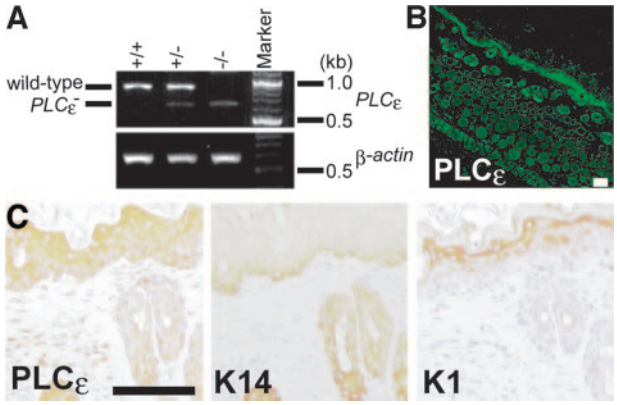

Fig. 1. Analysis of PLCε expression. *A*, RT-PCR analysis of *PLCε* mRNA in the skin. *β-Actin* mRNA was used as an internal control. *B* and *C*, immunohistochemical analysis of the expression of PLCε, keratin 14 (*K14*), and keratin 1 (*K1*) in the $PLC\epsilon^{+/+}$ mouse skin. Detection was performed with a fluorescein isothiocyanate-conjugated secondary antibody (*B*) or the HistoMouse Plus kit (*C*). *Scale bars*, 100 μm.

showed that PLCε is expressed in the epidermis (Fig. 1*B*), including keratin 14-positive proliferative keratinocytes and keratin 1-positive differentiating keratinocytes, but not in the dermis, except for hair follicles (Fig. 1*C*). To address the role of PLCε in *de novo* skin carcinogenesis, we applied the skin two-stage chemical carcinogenesis protocol (11) on $PLC\epsilon^{-/-}$ mice. Initiation was carried out with a single application of DMBA, which almost invariably introduced oncogenic mutations on the Ha-*ras* gene (11, 12). Subsequent promotion by repeated treatment with TPA for 20 weeks caused the selective clonal outgrowth of the initiated cells to produce benign squamous tumors (Fig. 2*A*). $PLC\epsilon^{-/-}$ mice showed significant delay in the average time of tumor onset compared with $PLC\epsilon^{+/+}$ mice [average ± SE: 12.63 ± 0.42 weeks ($PLC\epsilon^{-/-}$; 21 mice analyzed) *versus* 10.14 ± 0.47 weeks ($PLC\epsilon^{+/+}$; 14 mice); $P < 0.001$; Fig. 2*B*]. $PLC\epsilon^{+/-}$ mice showed an intermediate phenotype (11.79 ± 0.31 weeks; 23 mice; $P < 0.01$), indicating the existence of an apparent gene-dosage effect. The time to develop the first tumor also showed a significant difference [$PLC\epsilon^{+/+}$, 6.06 ± 0.36 weeks; $PLC\epsilon^{+/-}$, 7.87 ± 0.30 weeks ($P < 0.001$); $PLC\epsilon^{-/-}$, 9.86 ± 0.43 weeks ($P < 0.0001$)]. The number of tumors reached a maximum at 15 weeks. At this point, the average number of tumors per mouse was reduced by approximately 70% in $PLC\epsilon^{-/-}$ mice (4.14 ± 0.40; $P < 0.0001$) compared with $PLC\epsilon^{+/+}$ mice (14.36 ± 1.25). Again, $PLC\epsilon^{+/-}$ mice showed an intermediate phenotype (10.22 ± 0.65; $P < 0.0001$; Fig. 2*B*). In $PLC\epsilon^{-/-}$ mice, no tumor greater than 6 mm in diameter was observed at 20 weeks (Fig. 2*C*). In the two-stage protocol, a population of papillomas undergo progression into squamous cell carcinoma (SCC) (11). At 30 weeks after initiation, tumors of at least 2 mm in diameter were isolated and subjected to histologic analysis (Table 1; Fig. 2*D*). In $PLC\epsilon^{+/+}$ mice, approximately 20% of the tumors were carcinomas. In contrast, essentially no carcinoma was found in $PLC\epsilon^{-/-}$ mice. $PLC\epsilon^{+/-}$ mice showed a partial resistance to malignant progression. Thus, PLCε deficiency strongly suppressed malignant progression. All of the tumors tested carried the activating mutations at the 61st codon of the Ha-*ras* gene, irrespective of the *PLCε* genetic background (data not shown).

We next investigated the effect of PLCε deficiency on TPA-induced proliferation of the skin epidermis. Before or after treatment

Table 1 *Histological analysis of tumors*

PLCε genotypes	+/+ (n = 6)	+/− (n = 14)	−/− (n = 9)
Hyperplasias	6	8	6
Papillomas	32	60	20
Carcinomas	10	2	0
Carcinomas/tumors (%)	20.8	2.9	0
Total no. of tumors analyzed	48	70	26

NOTE. n represents the number of mice analyzed.

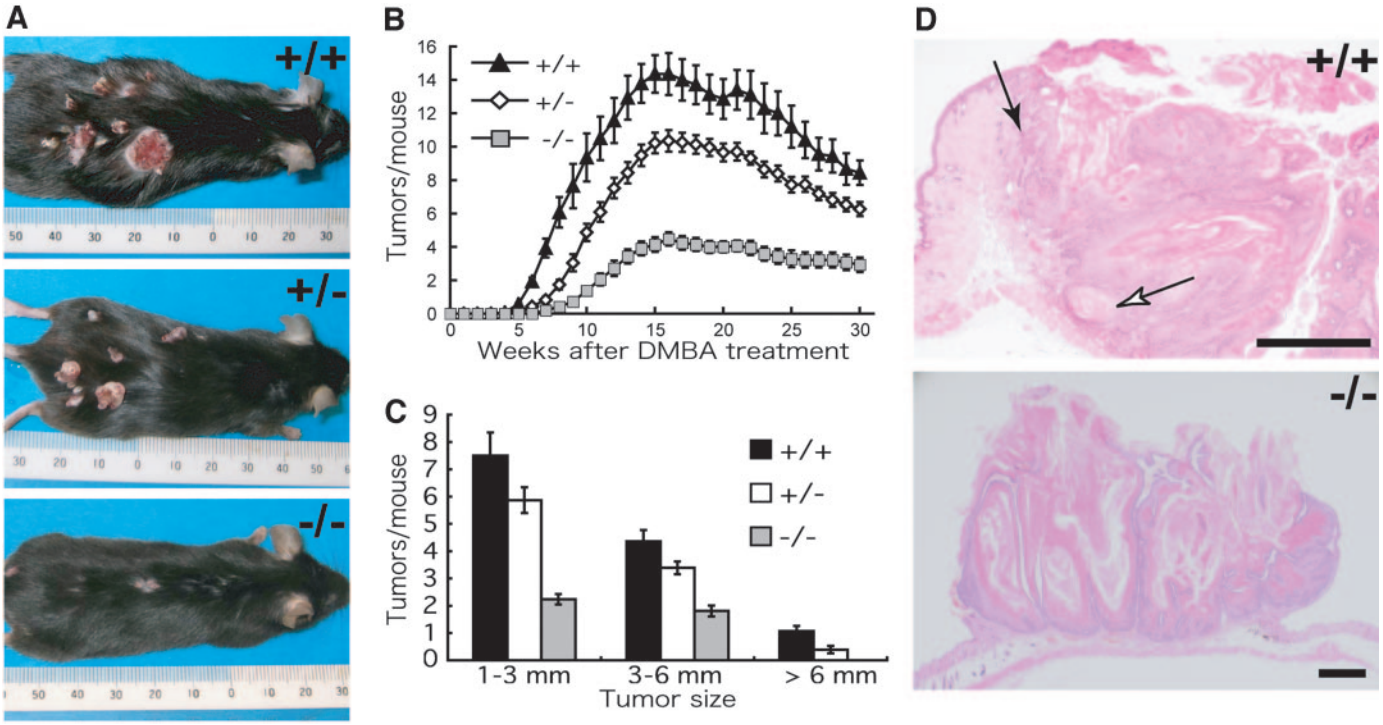

Fig. 2. Skin tumor formation. *A*, representative tumors developed in $PLC\epsilon^{+/+}$ (+/+), $PLC\epsilon^{+/-}$ (+/−), and $PLC\epsilon^{-/-}$ (−/−) mice at 30 weeks after initiation. *B*, time course of tumor formation. The average number of tumors per mouse (average ± SE) is shown. *C*, size distribution of tumors at 20 weeks after initiation. *D*, photomicrographs of hematoxylin and eosin-stained sections of a representative SCC in a $PLC\epsilon^{+/+}$ mouse (+/+) and a papilloma in a $PLC\epsilon^{-/-}$ mouse (−/−) at 30 weeks. The SCC exhibits tumor invasion (*black arrow*) and a cancer pearl with parakeratosis (*white arrow*). *Scale bars*, 1 mm.

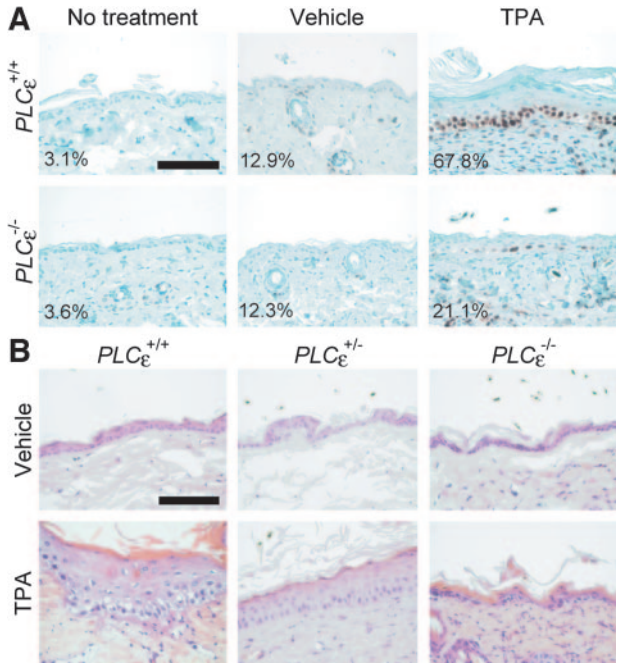

Fig. 3. Suppression of TPA-induced epidermal cell proliferation in $PLC\epsilon^{-/-}$ mouse. Mouse skin was treated with acetone alone (*Vehicle*) or with TPA in acetone (*TPA*), or left untreated (*No treatment*). The sections were examined by staining with the anti-PCNA antibody at 24 hours (*A*) or by staining with hematoxylin and eosin at 48 hours (*B*). Representative photomicrographs of at least three independent experiments are shown. The frequency of PCNA-positive basal layer cells is shown as percentage in *A*. *Scale bars*, 100 μm.

with acetone, there was no apparent difference between $PLC\epsilon^{+/+}$ and $PLC\epsilon^{-/-}$ mice in the skin architecture and the number of proliferating cells positive for PCNA (Fig. 3). On TPA treatment, $PLC\epsilon^{+/+}$ mouse skin showed a marked increase in the number of PCNA-positive cells in the basal layer cells (Fig. 3*A*). In striking contrast, $PLC\epsilon^{-/-}$ mouse skin showed only a moderate increase (Fig. 3*A*). TPA-induced epidermal hyperplasia was also suppressed in $PLC\epsilon^{-/-}$ mice (Fig. 3*B*). The average thickness of the epidermis after 48 hours of TPA treatment was 98.4, 66.3, and 31.3 μm in $PLC\epsilon^{+/+}$, $PLC\epsilon^{+/-}$, and $PLC\epsilon^{-/-}$ mice, respectively, whereas that after acetone treatment was 27.7, 25.4, and 24.6 μm, respectively.

We have shown here that PLCε plays a crucial role in skin papilloma formation and malignant progression, which are induced by *ras* activation followed by TPA treatment. Furthermore, PLCε is shown to function downstream of TPA to induce hyperproliferation of the basal layer cells and skin hyperplasia. Thus, it is likely that PLCε functions in TPA-induced tumor promotion of the initiated cells carrying the activated *ras* genes. There are two possible mechanisms linking TPA to PLCε activation. TPA may activate PLCε through Ras activation, which is mediated by RasGRP1, a TPA-regulated Ras-specific guanine nucleotide exchange factor (GEF) expressed in keratinocytes (13). Rap1, whose activation is mediated by TPA-responsive Rap GEFs including CalDAG-GEFI (14) and RasGRP2 (15), may also be responsible for PLCε activation. Alternatively, TPA may activate PLCε through secretion of tumor necrosis factor (TNF)-α from keratinocytes (16) and subsequent TNF-α–induced Ras activation (17). TNF-α has been implicated in both two-stage skin carcinogenesis and TPA-induced skin hyperplasia (16).

Because targeted inactivation of protein kinase C (PKC) η resulted in enhancement of both papilloma formation and TPA-induced skin hyperplasia, TPA-induced down-regulation of PKCη is thought to play a crucial role in induction of these phenomena (18). In the present study, TPA treatment failed to compensate for the deficiency in papilloma formation and skin hyperplasia of $PLC\epsilon^{-/-}$ mice, although TPA is known to mimic diacylglycerol, a product of PLCε, in regulating PKCη. The result indicates that the PLCε pathway has an intrinsic role in skin hyperplasia and carcinogenesis, which is independent of the PKCη pathway. This intrinsic function may be mediated by another of its products, inositol 1,4,5-trisphosphate. On the other hand, activation of PLCε in DMBA-initiated cells, which must be induced by constitutively active Ras and produce diacylglycerol, could not substitute for TPA treatment in promoting papilloma formation. This suggests that TPA possesses another target that is also required for tumor promotion. In addition, papillomas developed in $PLC\epsilon^{-/-}$ mice failed to undergo malignant conversion. It was reported that prostaglandins are involved in skin tumor progression in addition to promotion (19) and play a key role in intestinal polyposis (20). Considering that arachidonic acid, a precursor of prostaglandins, can be produced from diacylglycerol, it is possible that the role of PLCε may be mediated through prostaglandin signaling.

Our present results have shown that PLCε plays a crucial role in *ras* oncogene-induced *de novo* carcinogenesis of skin epithelial cells. They also provide the first concrete evidence for the importance of the PLC signaling in carcinogenesis. This leads to the idea that specific inhibitors of PLCε may be useful for treatment and prevention of certain types of cancer.

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References

1. Bos JL. ras oncogenes in human cancer: a review. Cancer Res 1989;49:4682–9.
2. White MA, Nicolette C, Minden A, et al. Multiple Ras functions can contribute to mammalian cell transformation. Cell 1995;80:533–41.
3. Davies H, Bignell GR, Cox C, et al. Mutations of the *BRAF* gene in human cancer. Nature (Lond) 2002;417:949–54.
4. Noh DY, Shin SH, Rhee SG. Phosphoinositide-specific phospholipase C and mitogenic signaling. Biochim Biophys Acta 1995;1242:99–114.
5. Kelley GG, Reks SE, Ondrako JM, Smrcka AV. Phospholipase Cε: a novel Ras effector. EMBO J 2001;20:743–54.
6. Song C, Hu CD, Masago M, et al. Regulation of a novel human phospholipase C, PLCε, through membrane targeting by Ras. J Biol Chem 2001;276:2752–7.
7. Song C, Satoh T, Edamatsu H, et al. Differential roles of Ras and Rap1 in growth factor-dependent activation of phospholipase Cε. Oncogene 2002;21:8105–13.
8. Schmidt M, Evellin S, Weernink PA, et al. A new phospholipase-C-calcium signaling pathway mediated by cyclic AMP and a Rap GTPase. Nat Cell Biol 2001;3:1020–4.
9. Wing MR, Bourdon DM, Harden TK. PLC-ε: a shared effector protein in Ras-, Rho-, and Gαβγ-mediated signaling. Mol Interv 2003;3:273–80.
10. Wu D, Tadano M, Edamatsu H, et al. Neuronal lineage-specific induction of phospholipase Cε expression in the developing mouse brain. Eur J Neurosci 2003;17:1571–80.
11. Yuspa SH. The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis. Cancer Res 1994;54:1178–89.
12. Finch JS, Albino HE, Bowden GT. Quantitation of early clonal expansion of two mutant 61st codon c-Ha-ras alleles in DMBA/TPA treated mouse skin by nested PCR/RFLP. Carcinogenesis (Lond) 1996;17:2551–7.
13. Rambaratsingh RA, Stone JC, Blumberg PM, Lorenzo PS. RasGRP1 represents a novel non-protein kinase C phorbol ester signaling pathway in mouse epidermal keratinocytes. J Biol Chem 2003;278:52792–801.
14. Kawasaki H, Springett GM, Toki S, et al. A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. Proc Natl Acad Sci USA 1998;95:13278–83.
15. Clyde-Smith J, Silins G, Gartside M, et al. Characterization of RasGRP2, a plasma membrane-targeted, dual specificity Ras/Rap exchange factor. J Biol Chem 2000; 275:32260–7.
16. Moore RJ, Owens DM, Stamp G, et al. Mice deficient in tumor necrosis factor-α are resistant to skin carcinogenesis. Nat Med 1999;5:828–31.
17. Zhou L, Tan A, Iasvovskaia S, et al. Ras and mitogen-activated protein kinase kinase kinase-1 coregulate activator protein-1- and nuclear factor-κB-mediated gene expression in airway epithelial cells. Am J Respir Cell Mol Biol 2003;28:762–9.
18. Chida K, Hara T, Hirai T, et al. Disruption of protein kinase Cη results in impairment of wound healing and enhancement of tumor formation in mouse skin carcinogenesis. Cancer Res 2003;63:2404–8.
19. Muller-Decker K, Neufang G, Berger I, et al. Transgenic cyclooxygenase-2 overexpression sensitizes mouse skin for carcinogenesis. Proc Natl Acad Sci USA 2002; 99:12483–8.
20. Sonoshita M, Takaku K, Sasaki N, et al. Acceleration of intestinal polyposis through prostaglandin receptor EP2 in $Apc^{\Delta 716}$ knockout mice. Nat Med 2001;7:1048–51.

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