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 rate of approximately 20% observed with papillomas in PLCε+/+ mice. In all of the tumors analyzed, the Ha-ras gene is mutational 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 mutational 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 oncogenesis 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 Ca2+ 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.

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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<sup>−/−</sup>) expresses a mutant PLCε with an in-frame deletion of amino acids 1333 to 1408 corresponding to the NH2-terminal part of the catalytic X domain. This mutant completely lost its PLC catalytic activity. PLCε<sup>−/−</sup> 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'-TCAATGTC-CTGGAGCAAGCGAG-3' and 5'-CTTGAAGGGATCTTGTTG-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-tetradecanoylphorbol-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 keratin 14 (PRB-155P; BAbCO, Berkeley, CA), or keratin 1 (PRB-165P; BAbCO). Detection of immunoreactive signals was performed with HistoMouse 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 Cytoation, 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 61<sup>st</sup> 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. 1A). Immunohistochemical analysis

showed that PLCε is expressed in the epidermis (Fig. 1B), including keratin 14-positive proliferative keratinocytes and keratin 1-positive differentiating keratinocytes, but not in the dermis, except for hair follicles (Fig. 1C). To address the role of PLCε in de novo skin carcinogenesis, we applied the skin two-stage chemical carcinogenesis protocol (11) on PLCε−/− 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. 2A). PLCε−/− mice showed significant delay in the average time of tumor onset compared with PLCε+/+ mice [average ± SE: 12.63 ± 0.42 weeks (PLCε−/−; 21 mice analyzed) versus 10.14 ± 0.47 weeks (PLCε+/+; 14 mice); P < 0.001; Fig. 2B].

**Table 1**  
<table>
<thead>
<tr>
<th>PLCε genotypes</th>
<th>+/+ (n = 6)</th>
<th>+/- (n = 14)</th>
<th>−/− (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperplasias</td>
<td>6</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Papillomas</td>
<td>32</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>Carcinomas</td>
<td>10</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Carcinomas/tumors (%)</td>
<td>20.8</td>
<td>2.9</td>
<td>0</td>
</tr>
<tr>
<td>Total no. of tumors analyzed</td>
<td>48</td>
<td>70</td>
<td>26</td>
</tr>
</tbody>
</table>

NOTE. n represents the number of mice analyzed.

**Fig. 2.** Skin tumor formation. A, representative tumors developed in PLCε+/+ (+/+), PLCε+/− (+/-), and PLCε−/− (−/−) 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 PLCε+/+ mouse (+/+), and a papilloma in a PLCε−/− mouse (−/−) at 30 weeks. The SCC exhibits tumor invasion (black arrow) and a cancer pearl with parakeratosis (white arrow). Scale bars, 1 mm.
with acetone, there was no apparent difference between \( \text{PLC}^+/+ \) and \( \text{PLC}^{-/-} \) mice in the skin architecture and the number of proliferating cells positive for PCNA (Fig. 3). On TPA treatment, \( \text{PLC}^{-/-} \) mouse skin showed a marked increase in the number of PCNA-positive cells in the basal layer cells (Fig. 3A). In striking contrast, \( \text{PLC}^{-/-} \) mouse skin showed only a moderate increase (Fig. 3A). TPA-induced epidermal hyperplasia was also suppressed in \( \text{PLC}^{-/-} \) mice (Fig. 3B). The average thickness of the epidermis after 48 hours of TPA treatment was 98.4, 66.3, and 31.3 \( \mu \text{m} \) in \( \text{PLC}^+/+, \text{PLC}^{-/-} , \) and \( \text{PLC}^{-/-} \) mice, respectively, whereas that after acetone treatment was 27.7, 25.4, and 24.6 \( \mu \text{m} \), respectively.

We have shown here that \( \text{PLC} \) plays a crucial role in skin papilloma formation and malignant progression, which are induced by \( \text{ras} \) activation followed by TPA treatment. Furthermore, \( \text{PLC} \) is shown to function downstream of TPA to induce hyperproliferation of the basal layer cells and skin hyperplasia. Thus, it is likely that \( \text{PLC} \) functions in TPA-induced tumor promotion of the initiated cells carrying the activated \( \text{ras} \) genes. There are two possible mechanisms linking TPA to \( \text{PLC} \) activation. TPA may activate \( \text{PLC} \) 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 \( \text{PLC} \) activation. Alternatively, TPA may activate \( \text{PLC} \) through secretion of tumor necrosis factor (TNF)-\( \alpha \) from keratinocytes (16) and subsequent TNF-\( \alpha \)-mediated induction of Rac activation (17). TNF-\( \alpha \) has been implicated in both two-stage skin carcinogenesis and TPA-induced skin hyperplasia (16).

Because targeted inactivation of protein kinase C (PKC) \( \eta \) resulted in enhancement of both papilloma formation and TPA-induced skin hyperplasia, TPA-induced down-regulation of PKC\( \eta \) 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 \( \text{PLC}^{-/-} \) mice, although TPA is known to mimic diacylglycerol, a product of \( \text{PLC} \), in regulating PKC\( \gamma \). The result indicates that the \( \text{PLC} \) pathway has an intrinsic role in skin hyperplasia and carcinogenesis, which is independent of the PKC\( \gamma \) pathway. This intrinsic function may be mediated by another of its products, inositol 1,4,5-trisphosphate. On the other hand, activation of \( \text{PLC} \) 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 \( \text{PLC}^{-/-} \) 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 \( \text{PLC} \) may be mediated through prostaglandin signaling.

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

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