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Phospholipase C- γ 1 is Required for the Epidermal Growth Factor Receptor-induced Squamous Cell Carcinoma Cell Mitogenesis

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Abstract

The epidermal growth factor receptor (EGFR) is a key driver in the process of squamous cell carcinoma (SCC) cell mitogenesis. Phospholipase C- γ 1 (PLC- γ 1) is a downstream target of EGFR signaling, but the role and necessity of PLC- γ 1 in EGFR-induced cell mitogenesis remain unclear. In the present study, we report an elevated expression of PLC- γ 1 in human SCC biopsies relative to adjacent normal epidermis, and in human SCC cell lines compared to normal human keratinocytes. EGFR-induced SCC cell mitogenesis was blocked by small interfering RNA knockdown of PLC- γ 1. However, inhibition of the catalytic activity of phospholipase C had no effect on EGFR-induced SCC cell mitogenesis. In response to the EGFR ligand epidermal growth factor (EGF), PLC- γ 1 was translocated not only to the plasma membrane but also to the nucleus. These data suggest that PLC- γ 1 is required for EGFR-induced SCC cell mitogenesis and the mitogenic function of PLC- γ 1 is independent of its lipase activity.

Keywords

Epidermal growth factor receptor; phospholipase C- γ 1; mitogenesis; squamous cell carcinoma

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Introduction

Keratinocyte mitogenesis is controlled by a delicate balance between mitogenesis-promoting and mitogenesis-inhibiting factors. In normal squamous epithelium, the production and activity of these factors result in growth of differentiated keratinocytes in a controlled and regulated manner that maintains normal integrity and function of the epithelial tissue. Squamous cell carcinoma (SCC) cells derived from keratinocytes have evaded this control. Their normal balances are disturbed and unregulated, and thus aberrant cell mitogenesis occurs. A key driver for SCC cell mitogenesis is the activation of the epidermal growth factor receptor (EGFR). This mechanism has been implicated in the development and progression of SCC. Overexpression of EGFR occurs in the majority of SCC, which is an adverse prognostic factor [1-3]. EGFR has been selected as a rational strategic target in the present era of anticancer drug development. Therefore, understanding the mechanism by which EGFR-mediated signaling leads to the rapid and uncontrolled cell division characteristic of SCC cells will bring the possibility of novel therapeutic options for the control of the disease.

EGFR is a transmembrane protein receptor with intrinsic tyrosine kinase activity [4]. Upon activation by its specific ligands, including epidermal growth factor (EGF), transforming growth factor- α , amphiregulin, heparin-binding EGF-like growth factor, betacellulin, epiregulin, and epigen [5], the activated EGFR tyrosine kinase phosphorylates the receptor itself and numerous downstream molecules to initiate multiple signaling pathways, including stimulation of Ras/mitogen-activated protein kinase, phosphoinositide 3-kinases, signal transducers and activators of transcription proteins, and phospholipase C- γ 1 (PLC- γ 1) [4].

PLC- γ 1 is a critical molecule in growth factor-dependent signal transduction. Unlike other phospholipase C (PLC) isozymes, the linker region between the X and Y catalytic domains in PLC- γ 1 is extended and contains two SH₂ domains and one SH₃ domain. PLC- γ 1 is phosphorylated by the EGFR tyrosine kinase after physical association with EGFR via its SH₂ domains [6]. The phosphorylated PLC- γ 1 is translocated to the plasma membrane. This phosphorylation allows phospholipase domains to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃), which releases calcium from intracellular stores, and diacylglycerol, which activates protein kinase C. These second messengers trigger a series of molecular interactions that alter the physiologic state of the cell [7,8]. Although PLC- γ 1 is implicated in the EGFR signaling, the necessity of PLC- γ 1 in the mitogenic signaling of EGFR remains unclear. In the present study, we demonstrated that PLC- γ 1 expression is elevated in human SCC biopsies and SCC cell lines. EGF-induced SCC cell mitogenesis was blocked by knockdown of PLC- γ 1, but not inhibition of the lipase activity of PLC. These data suggest that PLC- γ 1 is required for EGF-induced SCC mitogenesis and the mitogenic function of PLC- γ 1 is independent of its lipase activity.

Materials and Methods

Histology

Human SCC tissue was collected at the Second Xiangya Hospital of Central South University in China from 14 patients undergoing primary resection of SCC of the tongue or skin. Half of these cases were SCC involving the tongue and the other half were SCC involving the skin. All these patients were diagnosed with well to moderately differentiated SCC. Paraffin-embedded tissue was cut into 5 μ m sections. Routine hematoxylin and eosin (H&E) staining was repeated to confirm the presence of SCC. Immunohistochemistry staining was performed by using the Rabbit ImmunoCruz™ Staining System (Santa Cruz Biotechnology Inc., Santa Cruz, CA) to examine the expression and distribution of PLC- γ 1 in SCC.

Cell Culture

Normal human keratinocytes were isolated from neonatal human foreskins as described previously [9], and cells were cultured in serum-free medium (medium 154CF with human keratinocyte growth supplement, Cascade Biologics, Portland, OR) containing 0.07 mM calcium. Human SCC cell lines including SCC4 (tongue squamous cell carcinoma) and SCC 12B2 (epidermal squamous cell carcinoma) obtained from American Type Culture Collection were cultured under the same conditions as were normal human keratinocytes.

siRNA Transfection

SCC4 and SCC12B2 cells cultured in the SCC medium at 5% confluency were transfected with siRNA for PLC- γ 1, or negative control (ON-TARGETplus™ siRNA, Thermo Scientific, Lafayette, CO) at a concentration of 100 nM in accordance with manufacturer's recommendations using TransIT-siQUEST transfection reagent (Mirus, PanVera Corp., Madison, WI) at a dilution of 1:750 according to the manufacturer's protocol.

Cell Lysate Preparation and Western Analysis

The total cell lysates were isolated using radioimmunoprecipitation assay lysis buffer containing 50 mM HEPES, pH 7.4, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and Complete Protease Inhibitor Cocktail Tablets (Roche Applied Science, Indianapolis, IN). The nuclear lysates were extracted using the NE-PER kit (Thermo Fisher Scientific, Rockford, IL). The plasma membrane lysates were extracted using the Mem-PER kit (Thermo Fisher Scientific). The protein concentration of the lysate was measured by a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Equal amounts of protein were electrophoresed by the reducing SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride microporous membrane (Immobilon-P, 0.45 μ m; Millipore Billerica, MA). After incubation in blocking buffer (100 mM Tris base, 150 mM NaCl, 5% nonfat milk, and 0.5% Tween 20) for 1 hour at room temperature, the blot was then incubated overnight at 4°C with appropriate primary antibodies: polyclonal antibodies against human PLC- γ 1, PLC- β 1 and PLC- δ 1 at a dilution of 1:200, monoclonal antibodies against human involucrin at a dilution of 1:2000, monoclonal antibodies against human integrin α 2 (plasma membrane marker), BIP (endoplasmic reticulum marker), GM-130 (*cis*-Golgi marker) or histone H3 (nuclear marker) at a dilution of 1:250 (BD Biosciences, San Jose, CA).

PLC- γ 1 Activity Assay

PLC- γ 1 activity was determined by measuring accumulation of IP₃ according to the experimental procedure described [10]. SCC4 and SCC12B2 cells in 150 mm dishes were washed with PBS containing 0.1% sodium orthovanadate and 0.1% sodium fluoride, and then incubated with 1% NP-40 containing Phosphatase Inhibitor Cocktail (Roche Applied Science) and Complete Protease Inhibitor Cocktail Tablets (Roche Applied Science) for 5 min. Cells were scraped into microfuge tubes and incubated at 4°C on a rotator for 1 hour. Sixty micrograms of protein from the supernatant collected after centrifugation was incubated with 2 μ g polyclonal PLC- γ 1 antibody (BD Biosciences, San Jose, CA) at 4°C overnight, and then with 20 μ l UltraLink Immobilized Protein G (Thermo Fisher Scientific) at 4°C for 1 hour. After the centrifugation, the pellet was washed with the reaction buffer (10 mM HEPES, pH 7.0, 10 mM NaCl, 120 mM KCl, 2 mM EGTA, 0.05% deoxycholate, 5 μ g/ml bovine serum albumin and 10 μ M CaCl₂) and resuspended in 200 μ l reaction buffer. In triplicates, 50 μ l of the suspension was incubated with sonicated vesicles containing [³H]-PIP₂ (PerkinElmer Life Science, Waltham, MA), phosphatidylcholine (Sigma Aldrich Corporation, St. Louis, MO) and phosphatidylserine (Sigma Aldrich Corporation) in a molar ratio of 1:3:3 in 100 μ l reaction buffer. The reaction was ended by adding 200 μ l of 10%

trichloroacetic acid at 5 min and 200 μ l of 10% bovine serum albumin. The radioactivity of supernatant after centrifugation was determined by a scintillation counter and normalized to the protein content in the immunoprecipitates. We have previously shown that activity of PLC- γ 1 is detectable using PLC- γ 1 immunoprecipitated from cell lysates solubilized by the low stringency detergent NP-40 [11-13].

Mitogenesis Assay

Cell mitogenesis was assayed by [3 H]-thymidine incorporation. Briefly, SCC4 and SCC12B2 cells were seeded in 6-well plates at 5% confluence and grown in SCC medium with 10% fetal calf serum. Cells were transfected by siRNA as described above. After 72 hours of incubation in 5% CO₂ at 37°C, EGF (200 ng/ml) was added and cells were incubated for 24 hours. During the last 8 hours of incubation, 1 μ Ci/well of [3 H]-thymidine (PerkinElmer Life Science, Waltham, MA) was added. Cells were then washed with refrigerated PBS, treated with 5% trichloroacetic acid (ice cold) for 30 min, washed again with PBS, and solubilized in 0.5 ml 0.5N NaOH/0.5% SDS. The cell associated radioactivity was then measured by liquid scintillation counting and normalized to DNA content. Six wells were harvested for each treatment; three wells were used for [3 H]-thymidine incorporation, and the remaining three wells were used to determine the DNA content.

Results and Discussion

Elevated expression of PLC- γ 1 in human SCC

To determine the expression level of PLC- γ 1 in SCC, we analyzed human SCC cell lines and SCC tissue slides. The expression level of PLC- γ 1 in SCC4, SCC12B2 cell lines and normal human keratinocytes were examined by western analysis. Our results showed that the expression of PLC- γ 1 was substantially higher in SCC4 and SCC12B2 cell lines than in normal human keratinocytes (Figure 1a). As expected, the expression of involucrin, a keratinocyte differentiation marker, was markedly lower in SCC4 and SCC12B2 cells than in normal human keratinocytes (Figure 1a). These results are consistent with our previous observations of elevated mRNA expression of PLC- γ 1 in SCC4 and SCC12B2 cells [14]. Immunohistochemistry analysis showed elevated expression of PLC- γ 1 in SCC in 12 out of 14 (86%) patients compared to the adjacent normal epithelial tissue. A representative field of these sections showed that the expression of PLC- γ 1 (brown) was mainly seen in the basal layer of the epidermis. The expression of PLC- γ 1 was elevated, and the high expression was not only limited to the basal layer in the well-differentiated SCC, compared to that in the adjacent normal epidermis (Figure 1b, 1c). The punctuate staining of PLC- γ 1 (brown) was present in the nucleus of some SCC cells although the predominant staining was in the membrane and cytoplasm (Figure 1b, 1c).

Overexpression of PLC- γ 1 has previously been reported in both benign and malignant hyperproliferative tissues, including benign hyperproliferative epidermal diseases [15], familial adenomatous polyposis [16], breast carcinoma [17], and colorectal cancer [18]. Our present data show that PLC- γ 1 is overexpressed in human SCC. The elevated expression of PLC- γ 1 may be associated with increased mitogenesis of these tissues. Given that PLC- γ 1 is an important downstream molecule in EGFR signaling pathway and EGFR is overexpressed in SCC [19,20], PLC- γ 1 overexpression may contribute to the amplification of EGFR signaling in SCC.

Suppression of EGF-induced SCC cell mitogenesis by knockdown of PLC- γ 1 but not by inhibition of the catalytic activity of PLC

To determine the role of PLC- γ 1 in EGF-induced SCC mitogenesis, SCC4 and SCC12B2 cells were treated with siRNA for PLC- γ 1 for three days and then with EGF for 24 hours.

The level of PLC- γ 1 and the mitogenesis were determined. The results showed that siRNA for PLC- γ 1 reduced the expression and activity of PLC- γ 1 in these cell lines over 90% without affecting other isoforms of PLC such as PLC- β 1 and - δ 1. In contrast, control siRNA did not affect the expression of the PLC isoforms. Treatment of cells with EGF induced the mitogenesis of SCC4 and SCC12B2 cells. Knockdown of PLC- γ 1 markedly reduced EGF-induced mitogenesis of SCC4 and SCC12B2 cells (Figure 2a, 2b). However, complete inhibition of the catalytic activity of PLC using the PLC inhibitor U73122 did not have any effect on EGF-induced mitogenesis although the PLC- γ 1 activity was completely inhibited (Figure 3a, 3b). These data suggest that PLC- γ 1 is required for EGF-induced SCC cell mitogenesis, and the mitogenic function of PLC- γ 1 is independent of its lipase activity.

The role and necessity of PLC- γ 1 in EGF-induced proliferation remain controversial. Study results varied in different cell types. Wang et al demonstrated that microinjection into Madin-Darby canine kidney epithelial cells and NIH3T3 fibroblasts (Mouse embryonic fibroblast cell line) of a polypeptide corresponding to the noncatalytic SH2-SH2-SH3 domains of PLC- γ 1 (PLC- γ 1 SH2-SH2-SH3) blocks EGF-induced S-phase entry [21]. Treatment of cells with DAG or the combination of DAG and microinjected IP₃, the products of activated PLC- γ 1, attenuates the inhibitory effects of the PLC- γ 1 SH2-SH2-SH3 polypeptide [21]. These observations suggest that the lipase activity of PLC- γ 1 is required for EGF-induced mitogenesis. Other studies demonstrated that the SH3 domain of PLC- γ 1 mediates the mitogenic activity of PLC- γ 1 in NIH3T3 fibroblasts [22-24]. However, fibroblasts isolated from PLC- γ 1 knockout mice display normal mitogenic responses to EGF [25], suggesting that EGF-induced fibroblast mitogenesis does not require PLC- γ 1. Inhibition of PLC- γ 1 expression or the catalytic activity using PLC inhibitors or antisense PLC- γ 1 cDNA constructs in NR6 fibroblasts, DU-145 prostate tumor cells or squamous cell carcinoma cells does not affect mitogenesis [26-28]. Our present results showed that EGF-induced SCC cell mitogenesis was blocked by knockdown of PLC- γ 1. This finding is inconsistent with previous studies which have found that the knockdown of PLC- γ 1 using antisense technology does not affect EGF-induced SCC cell mitogenesis [28]. The reason for this inconsistency is unclear but might be explained by the difference in the degree of inhibition of PLC- γ 1 expression between studies. The low level of PLC- γ 1 in the cell may also be sufficient to mediate EGF-induced mitogenesis. A similar discrepancy was observed in our studies showing that EGF-induced SCC cell mitogenesis was inhibited by 90% knockdown (Figure 2a, 2b), but not by 50% knockdown of PLC- γ 1 (data not shown).

EGF-induced translocation of PLC- γ 1 to the plasma membrane and nucleus

To determine the cellular localization of PLC- γ 1 in response to EGF, the total cell lysates, plasma membrane and nuclear lysates were isolated from SCC4 cells treated with EGF. The level of PLC- γ 1 in these fractions was determined by western analysis. The results showed that 2 minutes of EGF treatment increased not only the level of PLC- γ 1 in the plasma membrane but also in the nucleus, although no changes in the levels of these proteins in the total lysates were found (Figure 4). Western analysis with antibody against the plasma membrane marker integrin α 2, endoplasmic reticulum marker BIP, *cis*-Golgi marker GM-130, or nuclear marker histone H3 confirmed the purity of the plasma membrane and nuclear lysate preparations (Figure 4). These data suggest that EGF stimulates translocation of PLC- γ 1 to plasma membrane and the nucleus. Translocation of PLC- γ 1 to the plasma membrane is a key step during the activation of PLC- γ 1 by EGF [29]. However, the biological significance of EGF-induced nuclear translocation of PLC- γ 1 is unclear. Nuclear translocation of PLC- γ 1 has been found previously in neuronal cells induced by nerve growth factor receptor [30]. The nuclear pool of PLC- γ 1 has been shown to be crucial for nerve growth factor receptor-induced neuronal cell mitogenesis [30]. Whether PLC- γ 1 in the nucleus plays a role in EGF-induced mitogenesis needs further investigation.

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Abbreviations

SCC	squamous cell carcinoma
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
PLC	phospholipase C
PLC-β1	phospholipase C- β 1
PLC-γ1	phospholipase C- γ 1
PLC-δ1	phospholipase C- δ 1
IP₃	inositol 1,4,5-trisphosphate
DAG	diacylglycerol

siRNA small interfering RNA

Figure 1. Elevated expression of PLC- γ 1 in human SCC

Cultured normal human keratinocytes, SCC4 and SCC12B2 cells were grown to 90% confluence and total protein was isolated. Protein levels for PLC- γ 1 and involucrin were determined by western analysis (a). Sections of paraffin-embedded human skin SCC from 14 different patients were processed for hematoxylin and eosin (H&E) staining and immunohistochemical staining with antibody for PLC- γ 1. The figure shows a representative field of sections stained red and blue with H&E (b) or immunostained brown with PLC- γ 1 antibody and counterstained blue with hematoxylin (c).

Figure 2. EGF-induced SCC cell mitogenesis was blocked by knockdown of PLC-γ1

Cultured SCC4 (a) and SCC12B2 (b) cells were treated with PLC-γ1 siRNA (100 nM) for 72 hours and then with EGF (200 ng/ml) for 24 hours. Cells were harvested and total cell lysates were isolated. The protein levels for PLC-γ1, β1 and δ1 were determined by western analysis. Cell mitogenesis was determined by [³H]-thymidine incorporation assay. The results of [³H]-thymidine incorporation are expressed as percentages of the control values. Data are mean ± SD of triplicates within a single representative experiments, *p< 0.01 (highly significantly different from the vehicle and control siRNA treated cells). Results shown are representative of three independent experiments.

Figure 3. EGF-induced SCC cell mitogenesis was not affected by inhibition of the catalytic activity of PLC

Cultured SCC4 (a) and SCC12B2 (b) cells were treated with the PLC inhibitor U73122 (10 μ M) which inhibits the catalytic activity of PLC for 1 hour and then with EGF (200 nM) for 2 min. Cells were harvested and total cell lysates were isolated. PLC- γ 1 in the total cell lysates were immunoprecipitated with a PLC- γ 1 antibody, and the PLC- γ 1 activity were assayed. For cell mitogenesis assay, SCC4 (a) and SCC12B2 (b) cells were treated with PLC- γ 1 siRNA (100 nM) for 72 hours and then with EGF (200 ng/ml) for 24 hours. Cell mitogenesis was determined by [³H]-thymidine incorporation assay. The results of PLC- γ 1 activity and [³H]-thymidine incorporation are expressed as percentages of the control values. Data are mean $\pm$ SD of triplicates within a single representative experiments, *P< 0.01 (highly significantly different from the vehicle and control siRNA treated cells). Results shown are representative of three independent experiments.

Figure 4. EGF-induced nuclear translocation of PLC-γ1 in SCC4 cells

SCC4 cells were treated with EGF (200 ng/ml) for 2 minutes. The nuclear, plasma membrane and total lysates were isolated and analyzed by western analysis with antibodies against PLC-γ1, integrin α2 (plasma membrane marker), BIP (endoplasmic reticulum marker), GM-130 (cis-Golgi marker), or histone H3 (nuclear marker). The results are from a representative experiment that was repeated three times.