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The SH3 domain, but not the catalytic domain, is required for phospholipase C-γ1 to mediate epidermal growth factor-induced mitogenesis

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ABSTRACT

Phospholipase C-γ1 (PLC-γ1) is a multiple-domain protein and plays an important role in epidermal growth factor (EGF)-induced cell mitogenesis, but the underlying mechanism is unclear. We have previously demonstrated that PLC-γ1 is required for EGF-induced mitogenesis of squamous cell carcinoma (SCC) cells, but the mitogenic function of PLC-γ1 is independent of its lipase activity. Earlier studies suggest that the Src homology 3 (SH3) domain of PLC-γ1 possesses mitogenic activity. In the present study, we sought to determine the role of the SH3 domain of PLC-γ1 in EGF-induced SCC cell mitogenesis. We examined the effect of overexpression of PLC-γ1, a catalytically active PLC-γ1 mutant lacking the SH3 domain or a catalytically inactive PLC-γ1 mutant lacking the X domain on EGF-induced SCC4 (tongue squamous cell carcinoma) cell mitogenesis. We found that overexpression of PLC-γ1 enhanced EGF-induced SCC4 cell mitogenesis. This enhancement was abolished by deletion of the SH3 domain but not by deletion of the X catalytic domain. These data suggest that the SH3 domain, but not the catalytic domain, is required for PLC-γ1 to mediate EGF-induced SCC4 cell mitogenesis.

1. Introduction

The epidermal growth factor receptor (EGFR) plays a key role in cancer cell proliferation. Therefore, elucidation of the pathway for EGFR-stimulated proliferation is important and might lead to development of new targets for cancer therapy. Activation of EGFR by its ligand such as epidermal growth factor (EGF) or transforming growth factor α (TGF-α) initiates multiple downstream signaling pathways [1]. One of the pathways involves phospholipase C-γ1 (PLC-γ1) [1]. Unlike other phospholipase C (PLC) isozymes, PLC-γ1 contains two Src homology 2 (SH2) domains and one Src homology 3 (SH3) domain between X and Y catalytic domains [2]. In response to EGFR ligands, the SH2 domains of PLC-γ1 interact with EGFR. This interaction allows tyrosine phosphorylation and activation of the catalytic domain of PLC-γ1 by the tyrosine kinase EGFR [3,4]. The catalytic activation of PLC-γ1 leads to the phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis which generates two intracellular second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). DAG is a physiologic activator of protein kinase C (PKC), and IP_3 induces the release of calcium from intracellular stores. The intracellular calcium release and PKC activation are essential for regulation of many cellular activities [5–7]. The SH3 domain is a protein-interaction module which has been found to play an important role in a variety of biological processes by binding with proline-rich ligands [8]. Although PLC-γ1 has been shown to mediate EGF-induced cell mitogenesis, the underlying mechanism is unclear. The PLC-γ1 SH3 domain has been reported to possess mitogenic activity [9,10]. However, microinjection of the PLC-γ1 SH3 domain fails to enhance EGF-induced mitogenesis [11]. We have previously shown that EGF-induced mitogenesis of squamous cell carcinoma (SCC) cells requires PLC-γ1 but not the catalytic activity of PLC [12], suggesting that regions other than the catalytic domain of PLC-γ1 may be responsible for EGF-induced SCC mitogenesis. In the present study, we examined the effect of overexpression of PLC-γ1 or PLC-γ1 mutant lacking the SH3 or X domain on EGF-induced SCC cell mitogenesis. We found that overexpression of PLC-γ1 enhanced EGF-induced mitogenesis, but this enhancement was abolished by deletion of the SH3 domain but not by deletion of the catalytic domain. These observations provide direct evidence for requirement of the SH3 domain, but not the catalytic domain, for PLC-γ1 to mediate EGF-induced SCC cell mitogenesis.

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; SCC, squamous cell carcinoma; PLC, phospholipase C; PLC-γ1, phospholipase C-γ1; PI3K, phosphoinositide 3-kinases; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; SH2, Src homology 2; SH3, Src homology 3.

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2. Materials and methods

2.1. Cell culture

Human tongue squamous cell carcinoma SCC4 cells obtained from ATCC were grown in the SCC medium composed of 1:1 mixture of Ham's F12 and Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum. Cells were serum-starved for 24 h before treatment with EGF.

2.2. DNA constructs and transfection

SCC4 cells were transiently transfected in suspension with pcDNA 3.1 containing the full-length human PLC-γ1 cDNA (a gift from Dr. John Imboden, San Francisco General Hospital) or the various mutants tagged with a FLAG epitope (Fig. 1) using the polybrene/glycerol method [13]. The pcDNA3.1 vector contains the neomycin (G418) resistant gene to allow selection. The transfected cells were selected by 4-day incubation in 300 μM G418 starting 2 days after transfection to enrich transfected cells as we previously described [14,15].

2.3. Immunoblotting

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 protein concentration of the lysate was measured by bicinchoninic acid protein assay kits (Thermo Fisher Scientific, Rockford, IL). Equal amounts of protein were electrophoresed by the reducing SDS–polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride microporous membranes (Immobilon-P, 0.45 μm; 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 h at room temperature, blots were then incubated overnight at 4 °C with a monoclonal antibody against the FLAG peptide at a dilution of 1:1000 (Agilent Technologies, Santa Clara, CA).

2.4. PLC-1 activity assay

PLC-γ1 activity was determined by measuring accumulation of IP_3 according to the experimental procedure described [16]. SCC4 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, Indianapolis, IN) 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 h. Sixty micrograms of protein from the supernatant collected after centrifugation was incubated with 2 μg polyclonal antibody against the C-terminus of PLC-γ1 (BD Biosciences, San Jose, CA) at 4 °C overnight, and then with 20 μl UltraLink Immobilized Protein G (Thermo Fisher Scientific) at 4 °C for 1 h. After 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 μg/ml bovine serum albumin and 10 μM $CaCl_2$) and resuspended in 200 μl reaction buffer. In triplicates, 50 μl of the suspension was incubated with sonicated vesicles containing [^{3}H]-PIP_2 (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 μl reaction buffer. The reaction was ended by adding 200 μl of 10% trichloroacetic acid at 5 min and 200 μl 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 [17–19].

2.5. Mitogenesis assay

Cell mitogenesis was measured by [^{3}H]-thymidine incorporation as previously described [12]. Briefly, keratinocytes were incubated with 1 μCi/well of [^{3}H]-thymidine (PerkinElmer Life Science) for 8 h. 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 0.5 N 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.

3. Results and discussion

We have previously demonstrated that PLC-γ1 is required for EGF-induced SCC cell mitogenesis and the mitogenic function of PLC-γ1 is independent of its lipase activity [12], suggesting that regions other than the catalytic domain mediate EGF-induced SCC mitogenesis. Earlier studies have shown that microinjection of the PLC-γ1 SH3 domain into fibroblasts induces cell mitogenesis [9,10]. The present study was undertaken to determine whether

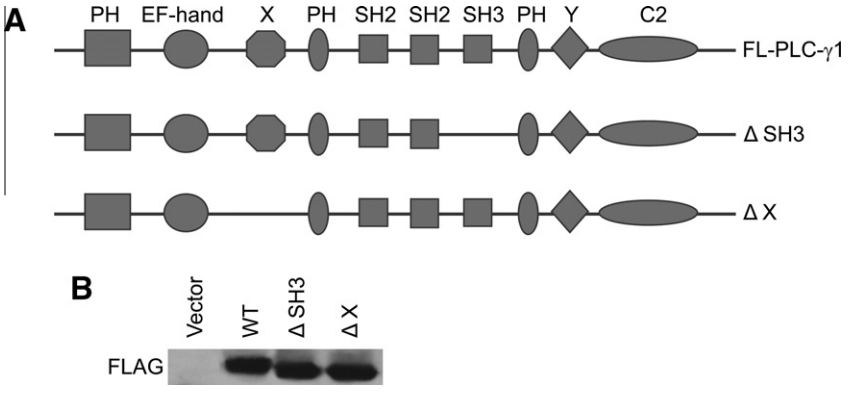

Fig. 1. Schematic diagram of full-length and truncated PLC-γ1-FLAG-tagged fusion constructs. (A) The constructs of the full-length PLC-γ1 cDNA (FL-PLC-γ1), PLC-γ1 mutant lacking the SH3 domain (ΔSH3) or PLC-γ1 mutant lacking the X catalytic domain (ΔX). (B) SCC4 cells were transfected with various constructs as indicated. Transfected cells were selected by 4-day incubation in 300 μM G418. The total cell lysates were isolated. The level of the FLAG fusion protein was determined by immunoblotting with anti-FLAG antibody.