Mutations at residues Tyr^{771} and Tyr^{783} of phospholipase C-γ1 have different effects on cell actin-cytoskeleton organization and cell proliferation in CCL-39 cells

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Abstract Currently a central question that remains unresolved is the potential importance of phospholipase C (PLC)-γ1 in cell mitogenesis. In this study, we introduced wild-type PLC-γ1 plasmid and mutants Y771F and Y783F into CCL-39 fibroblasts and investigated their effect on host cell functions. To our surprise, Y771F and Y783F plasmids appeared to have opposite effects on CCL-39 cell actin-cytoskeleton organization and cell proliferation. Y771F transfectants increased cell proliferation by two-fold. Y783F transfectants showed much thicker actin filaments and decreased cell growth rate by 50%. These results suggest that PLC-γ1 mutations have an essential impact on cell mitogenesis.

Key words: Phospholipase C-γ1; Tyr^{771}; Tyr^{783}; Actin; Mitogenesis

1. Introduction

A variety of growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), promote an increased turnover of inositol phospholipids in fibroblasts, transformed cells and some tissues. Stimulation of specific receptors by these growth factors triggers phospholipase C (PLC)-γ1 tyrosine phosphorylation and the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) with production of two important secondary messengers, diacylglycerol and inositol 1,4,5-trisphosphate [1,2]. Recently, some G protein-coupled receptor agonists were also found to induce PLC-γ1 tyrosine phosphorylation by non-receptor tyrosine kinases. These agonists include angiotensin II [3,4], thrombin [5], adenosine triphosphate (ATP) [6], and lysophosphatic acid (Pei, Z.-D. and Williamson, J.R., unpublished observation). The major sites of tyrosine phosphorylation in PLC-γ1 were identified to be at Tyr^{771}, Tyr^{783} and Tyr^{1254} [1]. The role of PLC-γ1 tyrosine phosphorylation in its activation has been studied by substituting Phe for Tyr at the three sites Tyr^{771}, Tyr^{783}, and Tyr^{1254}. It was found that Tyr^{783} phosphorylation was essential for PLC-γ1 activation in vivo, although it was not in vitro [7]. In contrast, Tyr^{771} mutation was found to increase PLC-γ1 enzymatic activity [7].

The role of PLC-γ1 in cell mitogenesis is still not clear [8]. Early studies suggested that PLC-γ1 might have little effect on cell mitogenesis. However, recent studies have provided evidence against this view. Microinjection studies indicated that PLC-γ1 could induce DNA synthesis [9–11]. PLC-γ1 was also found to be involved in nerve growth factor and PDGF-induced cell mitogenesis [12–17]. Furthermore, a more recent report indicated that PLC-γ1 might be essential in embryonal development [18].

In the present study, the effect of Tyr^{771} and Tyr^{783} mutations on cell function was investigated. Introduction of Tyr^{771} mutant into CCL-39 fibroblasts increased the host cell growth rate. In contrast, Tyr^{783} mutation caused morphology changes and an inhibition of cell growth in host cells.

2. Materials and methods

2.1. Materials

The plasmids that encode wild-type (WT) PLC-γ1, mutants Y771F and Y783F and their expression vector pMJ30 [7], and the monoclonal antibody against PLC-γ1 were kindly provided by Dr. S.G. Rhee (National Institute of Health, Bethesda, MD, USA). The monoclonal antibody against the extracellular signal-regulated kinase 2 (ERK2), the mouse IgG, and protein A/G plus agarose beads were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The monoclonal antibody against phosphotyrosine was purchased from Transduction Laboratories (Lexington, KY, USA). The BB dimeric form of PDGF was from Upstate Biotechnology (Lake Placid, NY, USA).

2.2. Cell culture

CCL-39 cells, which are derived from Chinese hamster lung fibroblasts, were purchased from American Type Culture Collection (Rockville, MD, USA) and maintained at 37°C in a humidified 5% CO_2 atmosphere in McCoy's 5A medium (Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum. For cell stimulation experiments, cells were grown to 80% confluence and starved for 20 h in the maintaining medium without serum.

2.3. Transfection

Plasmid DNAs were prepared by Wizard Plus Maxipreps DNA Purification System from Promega (Madison, WI, USA). For stable transfection, plasmid DNAs were introduced into CCL-39 cells by a liposome-mediated electroporation method as described in ref. [19]. In this study, the cells were electroporated at 180 V, 700 μF using BTX Electro Cell Manipulator 600 (Genetronics, Inc., San Diego, CA, USA).

2.4. Actin filament visualization by fluorescence staining

Cells were grown to subconfluency on coverslips and were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 1 h on ice, permeabilized with cold 10% DMSO for 5 min, and labeled for 1 h at room temperature with 0.1 μg/ml FITC-labeled phalloidine (Sigma) in 10% MeOH/PBS. Cells were viewed on a Nikon microscope with an FITC filter and photographed with Kodak T-MAX 400 film.

2.5. Cell growth determination

Cells were seeded in quartets in 100-mm petri dishes at a cell density of 5×10^4/dish. Cell numbers were determined daily by mixing with 0.1% trypan blue dye and counted on a hemocytometer.

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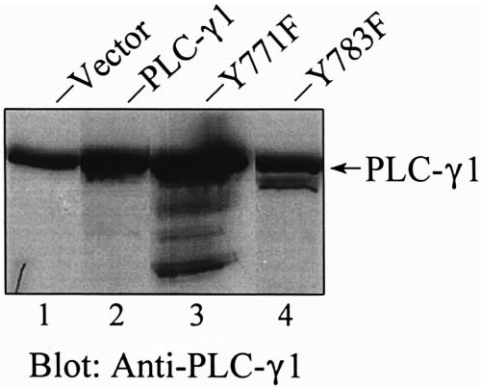

Fig. 1. Transfection and PLC-γ1 expression. 10 μg of WP PLC-γ1, Y771F, Y783F and the vector pMJ30 plasmid DNAs, along with 0.5 μg of pSV2neo plasmid DNA, were introduced into CCL-39 cells by electroporation [19]. Independent cell lines were established from individual G418-resistant colonies. To determine PLC-γ1 expression, cells were collected from 100-mm petri dishes and lysed as described in ref. [4]. Equal amounts of proteins were loaded onto 10% SDS polyacrylamide gel and blotted with PLC-γ1 monoclonal antibody.

2.6. Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting techniques were described elsewhere [4].

2.7. Mitogen-activated protein kinase (MAPK) gel shift assay

Cells in 100-mm tissue culture plates were stimulated with 30 ng/ml of PDGF or vehicle for 5 min. The stimulation was terminated by washing with 10 ml PBS, and the cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% deoxycholic acid, 1% NP-40, 10% glycerol, 50 mM NaF with 10 μg/ml leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride and 1 mM Na_3VO_4 for 30 min on ice. 200 μg of cell lysate from each cell line was used for immunoprecipitation with 1 μg of anti-ERK2 serum. The immunoprecipitates were analyzed by 12% SDS polyacrylamide gel and immunoblotted with anti-ERK2 antibodies.

3. Results

CCL-39 cells have relatively low levels of endogenous PLC-γ1 (data not shown). They were transfected with WT PLC-γ1, mutants Y771F and Y783F, and the vector plasmid pMJ30, along with the selection marker plasmid pSV2-neo. After transfection, cells were selected in cell growth medium containing 0.5 mg/ml G418 (Sigma). G418-resistant colonies were isolated and expanded into independent cell lines. These cell lines were first screened for the expression of PLC-γ1. Fig. 1 shows PLC-γ1 expression in cells transfected respectively by WT-PLC-γ1 plasmid (lane 2, colony #2), Y771F plasmid (lane 3, colony #5) and Y783F plasmid (lane 4, colony #3). They all had a higher expression level of PLC-γ1 than the vector transfectant (lane 1, colony #7). These transfectants were used for later studies.

One of the surprising findings observed with these transfectants was that Y783F transfectants showed a different morphology from the transfectants by WT PLC-γ1 and Y771F plasmids. As shown in Fig. 2, the CCL-39 cells transfected by WT PLC-γ1 and Y771F plasmids appeared to be very similar to those vector-transfected cells in actin-cytoskeleton organization. However, the Y783F transfectants formed much thicker stress fibers, and the cell morphology appeared to be more expanded (Fig. 2D). About 90% of Y783F-transfected colonies showed this type of morphology, as did a repeat of the stable transfection.

The transfectants were further studied for an assessment of cell proliferation. Interestingly, cells containing the Y771F plasmid almost doubled the cell growth rate, while WT PLC-γ1 plasmid did not show much effect (Fig. 3). In contrast, Y783F plasmid inhibited cell proliferation by about 50% (Fig. 3).

These transfectants were further characterized by measuring PLC-γ1 tyrosine phosphorylation and MAPK/ERK2 activation. The cells were stimulated with PDGF for 1 min and cell

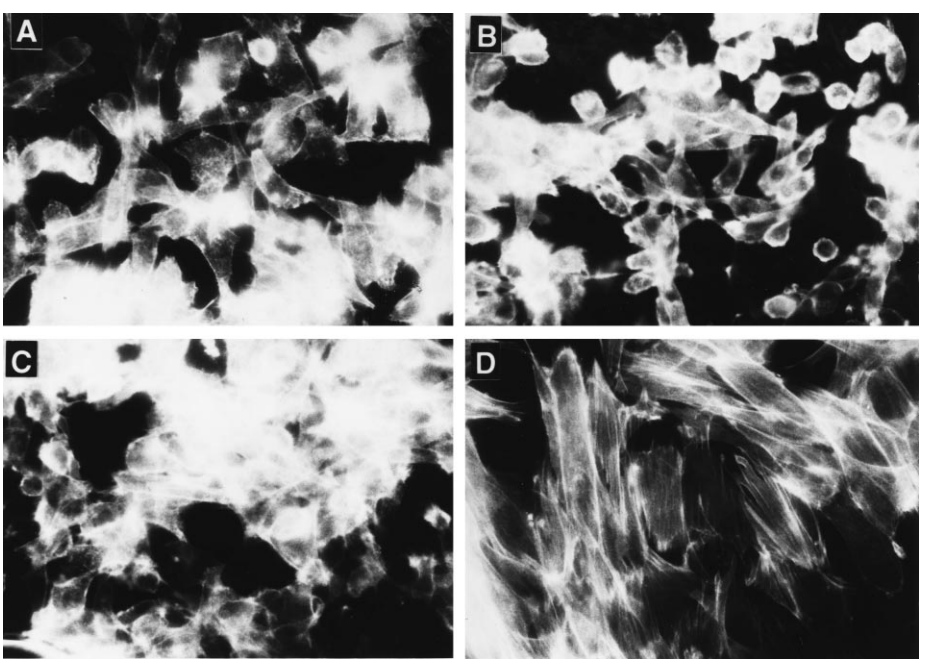

Fig. 2. Y783F plasmid induced actin reorganization in CCL-39 cells. Cells were seeded on coverslips, fixed and stained as described in Section 2. A: Vector #7; B: WT-PLC-γ1 #2; C: Y771F #5; D: Y783F #3.

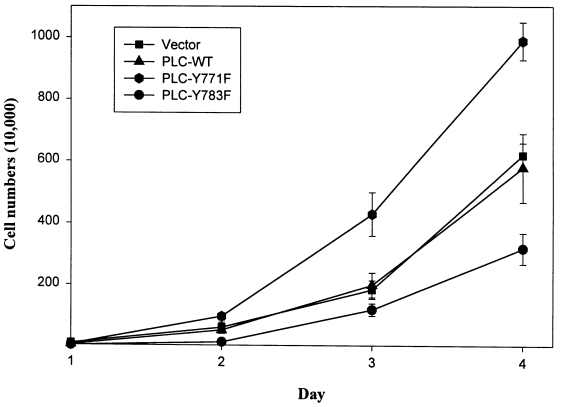

Fig. 3. Y771F and Y783F plasmids had opposite effects on cell proliferation in CCL-39 cells. Cells were equally seeded at day 1 in cell growth medium and cell numbers were then determined by hemocytometer for 4 consecutive days.

lysates were immunoprecipitated (IP) with anti-PLC-γ1 antibody and blotted with anti-phosphotyrosine antibody. As shown in Fig. 4, the intensity of PLC-γ1 tyrosine phosphorylation in each cell line was directly correlated with the expression level of PLC-γ1. These results suggest that mutations at Tyr^{771} and Tyr^{783} do not have much effect on total PLC-γ1 tyrosine phosphorylation.

Since Y771F and Y783F transfectants showed altered cell proliferation rates (Fig. 3), we wondered whether these transfectants showed any difference in MAPK/ERK2 activity from the vector transfectant. The cells were stimulated with PDGF for 5 min, and MAPK phosphorylation (which is correlated with MAPK activation) was studied by the gel shift assay. The Y771F transfectant was found to be much more activated than vector-transfected cells (Fig. 5), which is consistent with the results in Fig. 3 that the Y771F transfectant had a higher growth rate. However, the Y783F transfectant did not show decreased MAPK activation (Fig. 5), although their growth rate was about 50% less than vector-transfected cells (Fig. 3). EGF stimulation gave similar results (data not shown).

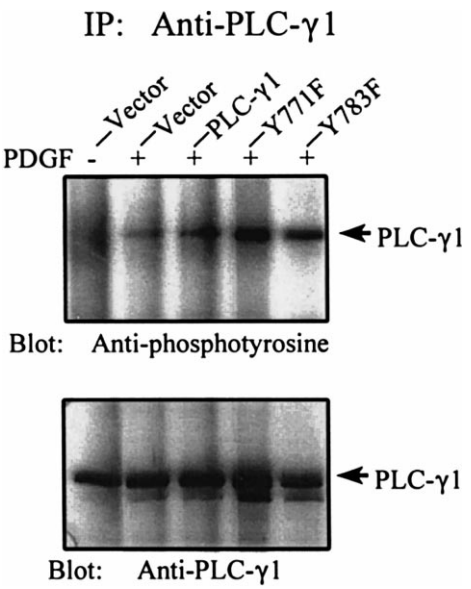

Fig. 4. PLC-γ1 tyrosine phosphorylation induced by PDGF. Cells were starved overnight and stimulated with 30 ng/ml PDGF for 1 min, and cell lysates were immunoprecipitated (IP) with the PLC-γ1 monoclonal antibody. The immunoprecipitates were separated by 10% SDS polyacrylamide gel and blotted with anti-phosphotyrosine and anti-PLC-γ1 antibodies, respectively.

4. Discussion

The substrate for PLC-γ1, PIP2, is a membrane lipid, and has been suggested to be a regulator of actin filament reorganization. PIP2 binds to actin-regulatory proteins such as profilin, cofilin, gelsolin, gCap, and α-actinin, and therefore regulates actin polymerization and depolymerization [20]. It is proposed that PIP2 promotes actin polymerization, while decreased levels of PIP2 together with increased cytosolic Ca^{2+} favors actin depolymerization [21]. In the current study, we demonstrate that PLC-γ1 mutation at Tyr^{783}, which was shown to be essential for PLC-γ1 activation in vivo [7], could actually induce CCL-39 cells to form much thicker actin filament bundles (Fig. 2). This result is consistent with the hypothesis that PIP2 accumulation promotes actin polymerization.

Another finding of interest in this study was that Y771F and Y783F plasmids showed opposite effects on CCL-39 cell proliferation. In the colonies we studied, Y771F increased cell proliferation by about 50% but Y783F inhibited cell growth by 50% (Fig. 3). The WT PLC-γ1 plasmid showed no significant effect on host cell proliferation, which is consistent with a previous report [22]. Y771F plasmid was previously found to increase PLC-γ1 enzymatic activity in vivo [7]. Therefore, the Y771F plasmid-induced higher growth rate in CCL-39 cells is possibly due to an increased enzymatic activity. Interestingly, the Y771F transfectant also showed a more activated MAPK/ERK2 in response to PDGF stimulation (Fig. 5), which indicates that Y771F-induced cell growth might be through the MAPK pathway.

The Y783F transfectant showed inhibited cell growth, but the ERK2 activation was not less than the vector and WT PLC-γ1 transfectants (Fig. 5). The reason is possibly due to the altered actin structure in Y783F transfectants. Cell actin organization has been correlated with the cell proliferation, but how this is achieved is not known. From our current results, actin may affect cell proliferation by promoting a MAPK independent pathway, or perhaps by interrupting cell signaling downstream of ERK2.

Our current studies provide a new insight on how PLC-γ1 may affect cell mitogenesis. Tyr^{771} is a potential site that may

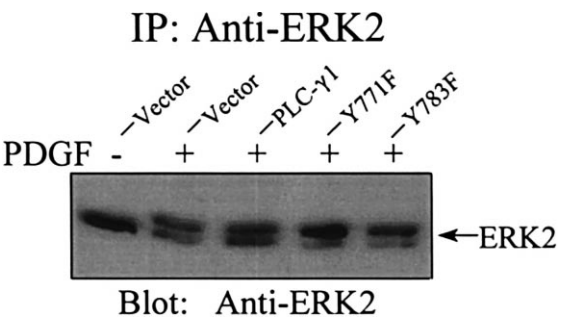

Fig. 5. MAPK activation induced by PDGF. Serum-starved transfected cells were stimulated with 30 ng/ml PDGF for 5 min, and cell lysates were immunoprecipitated with anti-ERK2 serum. The immunoprecipitates were separated by 12% SDS polyacrylamide gel and blotted with ERK2 monoclonal antibody.

cause cell-transforming phenotype. Mutation at Tyr^{783} seems to do just the opposite, which may be used to reverse cell-transforming phenotype or to induce cell apoptosis. The finding that Tyr^{783} mutation promotes actin polymerization suggests that PLC-γ1 may also be linked to signaling pathways like the Rho GTPase pathway or the integrin-mediated pathway.

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