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Epidermal growth factor and platelet-derived growth factor promote translocation of phospholipase C-γ from cytosol to membrane

Uh-Hyun Kim, Hee-Sook Kim and Sue Goo Rhee

Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

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Treatment of HER 14 cells with epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) induced a translocation of phospholipase C-γ (PLC-γ) from cytosol to membrane. In such growth factor-treated cells, cytosolic PLC-γ was found to contain more phosphotyrosine than membrane-associated enzyme. Because these growth factors have been shown to promote both the physical association of PLC-γ with their receptors and the subsequent phosphorylation of the enzyme directly by the membrane-bound receptor tyrosine kinases, the membrane assocation of PLC-γ may simply be due to the formation of transient enzyme (receptor)-substrate (PLC-γ) complexes. If this is the case, membrane-associated PLC-γ would be expected to be released from membrane after undergoing tyrosine phosphorylation. However, tyrosine phosphorylation of membrane-associated PLC-γ by the EGF receptor in vitro did not result in the release of PLC-γ from membrane. Thus, the association of PLC-γ with membrane would appear to involve more than enzyme-substrate complex.

Growth factor receptor kinase; Phospholipase C-γ; Translocation

1. INTRODUCTION

The binding of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) to their respective receptors stimulates a phosphoinositide-specific phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) [1]. The two products of PIP_2 hydrolysis are both second messengers: diacylglycerol, which activates the Ca^{2+}/phospholipid-dependent protein kinase C; and inositol 1,4,5-trisphosphate, which mobilizes Ca^{2+} from intracellular stores [1]. The protein-tyrosine kinase activity of the growth factor receptors is essential for growth-factor induced PIP_2 hydrolysis; mutant PDGF [2] and EGF [3] receptors lacking kinase activity fail to induce PIP_2 hydrolysis, even though the ligand binding is not altered.

The substrates of the receptor tyrosine kinases appear to include PLC-γ, one of 4 PLC isoforms identified in mammalian tissues [4]. Treatment of responsive cells with EGF or PDGF results in a rapid increase in the phosphorylation of tyrosine and serine residues in PLC-γ, but not in other isoforms [5-8]. Four sites of tyrosine phosphorylation in PLC-γ have been identified [9,10]. In addition, EGF and PDGF receptors, when stimulated by ligands, bind tightly to and coimmunoprecipitate with PLC-γ [5-8,11,12]. These results suggest that PLC-γ, which exists mainly (>80%) in cytosolic fractions of mammalian tissues and cultured cells, might translocate from cytosol to membrane in response to the growth factors. We investigated this possibility with HER 14 cells, which contain both EGF and PDGF receptors.

Correspondence address: S.G. Rhee, Laboratory of Biochemistry, NHLBI, National Institutes of Health, Building 3, Room 122, Bethesda, MD 20892, USA

2. MATERIALS AND METHODS

HER 14 cells [13], which were derived from NIH 3T3 clone 2.2 by transfecting with human EGF receptor DNA constructs, were generously provided by Dr. J. Schlessinger (Rorer Biotechnology, Inc., King of Prussia, PA). The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) contained in 75 cm^2 stock flasks under a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were subcultured for experiments by plating 2×10^6 cells in 100 mm-diameter dishes and allowing them to become 80-90% confluent, at which point they were placed in DMEM with 1% FBS overnight for subsequent study.

2.1. *Preparation of cell fractions*

The cell dishes were incubated on ice for 30 min before treatment with growth factors for 5 min. The cells were then rinsed twice with ice-cold phosphate-buffered saline, scraped into homogenization buffer (200 μl) containing 10 mM Tris-HCl, pH 7.2, 5 mM EGTA, 0.1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM glycerophosphate, leupeptin (10 μg/ml), and aprotinin (10 μg/ml), and homogenized by 20 strokes with a Kontes Pellet Pestle Motor. The unruptured cells and nuclei were pelleted by centrifugation for 1 min at 1500 rpm in an Eppendorf microcentrifuge. The supernatants were then centrifuged at 100 000 × g for 20 min in a Beckman TL-100 table-top ultracentrifuge. The cytosolic fraction was derived from the supernatant of this centrifugation and the membrane fraction was derived from the pellet by dissolving it with extraction buffer containing 20 mM Hepes, pH 7.2, 1% Triton X-100, 10% glycerol, 50 mM sodium fluoride, 1 mM phenylmethyl-sulfonyl fluoride, 1 mM

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sodium orthovanadate, leupeptin (10 μg/ml), and aprotinin (10 μg/ml).

For the phosphorylation studies, cell proteins were metabolically labeled by incubating the cells with 0.5 mCi/ml [^{32}P]orthophosphate (ICN) in phosphate-free Minimal Essential Medium plus 10% dialyzed FBS for 4 h at 37°C. Cells were then incubated on ice, stimulated with EGF, washed, and lysed with extraction buffer as described above.

2.2. *Immunoprecipitation and immunoblotting*

Immunoprecipitation was performed with a mixture of monoclonal antibodies to PLC-γ [14], as previously described [15]. The immunoprecipitates were washed with a buffer containing 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, and 50 mM Tris, pH 8.5. Immunoprecipitated proteins were released by the addition of 200 μl of Laemmli buffer and heating for 5 min at 95°C. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 7.5% polyacrylamide gel by the method of Laemmli [16], and the separated proteins were then transferred to nitrocellulose for 2 h at 50 V. After blocking with 2% bovine serum albumin, the filters were probed with a mixture of monoclonal antibodies to PLC-γ or monoclonal antibodies to phosphotyrosine (ICN Immunochemicals). Immune complexes were detected with ^{125}I-labeled protein A (Amersham) and autoradiography.

2.3. *Phosphoamino acid analysis*

[^{32}P]Phosphoproteins were separated by SDS-PAGE with a 7.5% polyacrylamide gel. The [^{32}P]PLC-γ band was cut from the dried gel and the protein was eluted as described [6] and treated with 5.7 N HCl at 100°C for 1 h. Phosphoamino acid analysis was performed as described [6].

3. RESULTS

To investigate whether EGF and PDGF cause a translocation of PLC-γ, cytosolic and membrane fractions were prepared from HER 14 cells that had or had not been treated with either EGF (100 ng/ml) or PDGF (50 ng/ml) for 5 min at 4°C. After proteins in each fraction were separated by SDS-PAGE and transferred to nitrocellulose sheets, the sheets were probed with monoclonal antibodies to PLC-γ. The antibody-PLC-γ complex was visualized with ^{12}I-labeled protein A and quantitated by measuring the ^{125}I-radioactivity associated with PLC-γ bands cut from the immunoblot. As shown in Fig. 1, EGF and PDGF treatment both caused a significant increase of PLC-γ in membrane fractions (lane 3 vs. lane 4). Five independent experiments with EGF were performed. The results revealed that 5 ± 2% of the total PLC-γ protein was associated in untreated cells. But upon treatment with EGF, PLC-γ in the membrane fraction increased 3-fold to 16 ± 3%.

We next investigated whether the state of tyrosine phosphorylation is different in PLC-γ immunoprecipitated from cytosolic and membrane fractions of HER 14 cells. As shown in lanes 5 and 6 of Fig. 1, cytosolic PLC-γ exhibited stronger reactivity to anti-tyrosine phosphate antibodies than did membrane PLC-γ in both EGF- and PDGF-treated cells. To confirm this observation, PLC-γ was immunoprecipitated from HER 14 cells metabolically labeled with [^{32}P]orthophosphate and separated by SDS-PAGE. We then analyzed the phosphoamino acid content of PLC-γ

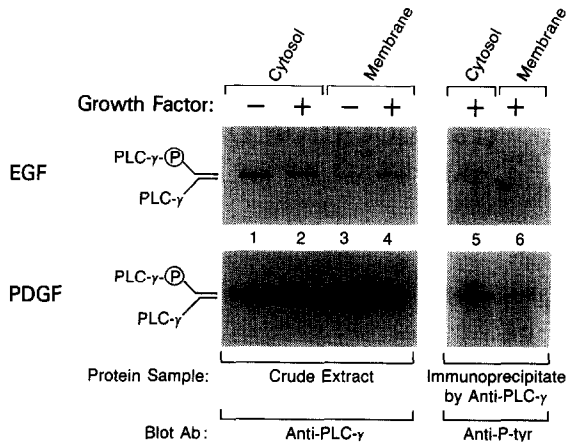

Fig. 1. Effects of growth factors on the distribution and the tyrosine phosphorylation of PLC-γ in cytosol and membrane. Lanes 1–4. Cytosolic and membrane fractions were obtained from HER 14 cells treated with 100 ng/ml EGF or 50 ng/ml PDGF as described in 'Materials and Methods'. The final volumes of cytosol and membrane fractions were adjusted to 200 μl and different volumes of the fractions were loaded onto 7.5% SDS-PAGE. For the EGF-treated cells (upper panel), the sample volume loaded was 10 μl for cytosol (lanes 1 and 2) and 50 μl for membrane (lanes 3 and 4); for the PDGF-treated cells (lower panel), the sample volume was 5 μl for cytosol (lanes 1 and 2) and 100 μl for membrane (lanes 3 and 4). Proteins were transferred from the gel to a nitrocellulose sheet and blotted with antibodies to PLC-γ. Finally, the antibody-PLC-γ complex was detected with ^{125}I-labeled protein A and autoradiography. PLC-γ bands were cut from the filter and the amount of ^{125}I-radioactivity determined. The specific radioactivity of ^{125}I-labeled protein A and the exposure time to X-ray film varied from one experiment to another. Radioactivity for lanes 1–4 was 252, 234, 72, and 212 cpm, respectively, for the experiment shown in the upper panel, and 498, 484, 604, and 1710 cpm, respectively, for that shown in the lower panel. Lanes 5 and 6. To compare the tyrosine phosphate content of cytosolic and membrane PLC-γ from the growth factor-treated cells, we prepared immunoprecipitates from the cytosolic and membrane fractions and adjusted the volumes of the dissolved immunoprecipitates loaded onto the gel on the basis of the amount of ^{125}I-radioactivity present in the samples loaded in lanes 2 and 4, such that the lanes for cytosol (lane 5) and membrane (lane 6) received the same amount of PLC-γ. After separation by SDS-PAGE, proteins were transferred to nitrocellulose and the filter was probed with antibodies to phosphotyrosine (anti-*p*-tyr). Again the antibody-PLC-γ complex was visualized by using ^{12}I-protein A. The positions of the PLC-γ bands are indicated. PLC-γ phosphorylated at multiple serine and tyrosine residues shows retarded mobility.

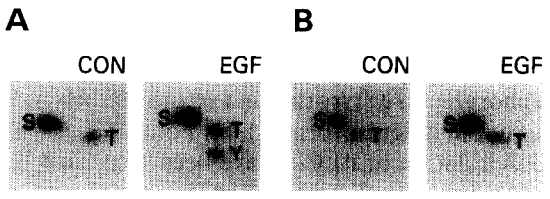

Fig. 2. Phosphoamino acid analysis of PLC-γ from cytosol and membrane of HER 14 cells. HER 14 cells were metabolically labeled with [^{32}P]orthophosphate and treated with or without (control) EGF (100 ng/ml). PLC-γ was immunoprecipitated from the cytosol (A) and membrane (B), separated by SDS-PAGE, and visualized by autoradiography. Protein was extracted from the PLC-γ bands and subjected to acid hydrolysis. Phosphoamino acids were separated on thin-layer chromatography plates as described [6] and the plates were exposed to Kodak XAR film for 12 days. The positions of spots associated with serine (S), threonine (T), and tyrosine (Y) are shown.

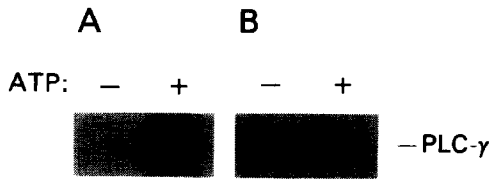

Fig. 3. Effect of in vitro tyrosine phosphorylation on the association of PLC-γ with membrane. The membrane in the 100 000 × g pellet (see Materials and Methods) prepared from HER 14 cells that had been treated with 100 ng/ml EGF for 10 min at 4°C were suspended in 100 μl of 20 mM Hepes, pH 7.4, 5 mM $MnCl_2$, and 0.1 mM Na_3VO_4, and divided into two equal portions. To one portion we added [γ-^{32}P]ATP (30 μM, 10 μCi/nmol) and to the other (control) we added water. After 5 min, membranes were precipitated by centrifugation and membrane proteins were solubilized by adding 100 μl of extraction buffer. PLC-γ was immunoprecipitated with antibodies to PLC-γ, separated on 7.5% SDS-PAGE, transferred to nitrocellulose, and visualized first by autoradiography (A) and then by immunoblotting with antibodies to PLC-γ (B).

bands cut from the gel. As shown in Fig. 2, EGF treatment enhanced the extent of serine phosphorylation of PLC-γ in both cytosol and membrane fractions, whereas the extent of threonine phosphoryation remained virtually unchanged. Phosphotyrosine was detected only in PLC-γ from cytosolic fractions of EGF-treated cells. Thus, the extent of tyrosine phosphorylation of cytosolic PLC-γ appears to be greater than that of membrane PLC-γ.

We then prepared membrane fractions from EGF-treated cells and incubated the fractions under a condition where PLC-γ becomes phosphorylated by the EGF-receptor tyrosine kinase, and evaluated if phosphorylation induced the release of PLC-γ from membrane. Fig. 3A shows that incubation of membrane fractions with Mn^{2+} and [γ-^{32}P]ATP resulted in the phosphorylation of PLC-γ. After SDS-PAGE, subsequent phosphoamino acid analysis of the PLC-γ band cut out from the gel indicated that phosphorylation occurred mainly at tyrosine residues (data not shown). Nevertheless, as shown in Fig. 3B, the content of PLC-γ in membrane was not altered as a result of the phosphorylation. Thus, tyrosine phosphorylation alone may not be the signal for the release of PLC-γ from membrane.

4. DISCUSSION

To avoid the complexity arising from the internalization of growth factor receptors at 37°C, PLC-γ distribution was measured in cells equilibrated at 4°C. At this temperature, endocytosis ceases, and PLC-γ remains associated with the receptors and is phosphorylated by the receptor kinases [5,11]. In the absence of EGF, PLC-γ was distributed in a ratio of 95 : 5 between cytosol and membrane, respectively. After treatment with EGF for 5 min at 4°C, the distribution ratio was 84 : 16. PDGF treatment also caused a similar translocation of PLC-γ.

Translocation of PLC-γ has been evaluated in A431 cells, which express abnormally high concentrations of EGF receptors [17]. The amount of membrane-associated PLC-γ increased from 12% to 68% of total PLC-γ after treatment with EGF at 4°C. This is a more extensive translocation than we observed with HER 14 cells. Although the concentration of PLC-γ is nearly equal in the two cell types (T. Kim and S.G. Rhee, unpublished results), the number of EGF receptors is much larger in A431 cells (2×10^6 receptors/cell) than in HER 14 cells (3×10^5 receptors/cell) [13,18]. Because the receptors for EGF and PDGF are the enzymes which phosphorylate PLC-γ at multiple tyrosine residues [9,10], we considered the possibility that the transient enzyme-substrate interaction might be responsible for the growth factor-induced change in the subcellular distribution of PLC-γ. This possibility is consistent with the observations that in EGF-treated cells cytosolic PLC-γ contained more phosphorylated tyrosine residues that membrane-associated PLC-γ, and that the extent of translocation appears to be dependent on the number of receptors. We investigated, therefore, whether PLC-γ becomes dissociated from membrane after it becomes phosphorylated by the EGF receptor. Membrane fractions containing EGF receptors and PLC-γ were incubated in the presence of [γ-^{32}P]ATP and Mn^{2+}. During this incubation, PLC-γ became phosphorylated at tyrosine residue(s), but the phosphorylation did not reduce the concentration of PLC-γ associated with membrane. This result suggests that PLC-γ translocation is not solely due to the formation of transient EGF receptor-PLC-γ complexes.

Possible modulators of PLC-γ translocation are the proteins with which it has been shown to be associated. Immunoprecipitation of PLC-γ with antibodies to PLC-γ coprecipitates three proteins with molecular masses of 100 000, 84 000, and 47 000 Da in addition to the EGF or PDGF receptor [5,6]. These proteins are also phosphoproteins and their state of phosphorylation varies when cells are treated with EGF and PDGF and activators of protein kinase C and cAMP-dependent protein kinase [6,15]. It is possible that one of these proteins associated with PLC-γ modulates the translocation of PLC-γ, and its capacity to modulate the translocation may vary depending on its state of phosphorylation.

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