Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 7111–7115, August 1991
Cell Biology

Immunocytochemical localization of phospholipase C-γ in rat embryo fibroblasts

(protein kinase C/signal transduction/cytoskeleton)

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Communicated by Gordon H. Sato, May 17, 1991

ABSTRACT **Rat embryo fibroblasts (REF52) exhibit a distinctive, transformation-sensitive distribution of α-protein kinase C (α-PKC). Receptor-mediated activation of phospholipase C (PLC)-γ generates diacylglycerol, the major cellular activator of PKC. Immunofluorescence techniques were used to investigate the subcellular localization of two PLC isozymes (PLC-γ and PLC-δ) in normal and simian virus 40-transformed REF52 cells to determine (*i*) if PLC colocalizes with α-PKC and (*ii*) if PLC isozyme distribution is sensitive to transformation. PLC-δ was not detected in either cell type. In REF52 cells, PLC-γ was associated with the actin cytoskeleton and was evenly distributed along the length of the actin microfilaments. PLC-γ was coincident with α-PKC at the points where the filaments are anchored to the membrane (i.e., the focal contacts). Cytoskeletal association of PLC-γ was not transformation sensitive, although the actin cytoskeleton was more disordered in simian virus 40-transformed cells. In REF52 cells, platelet-derived growth factor induced tyrosine phosphorylation of both soluble and cytoskeletal PLC-γ. Tyrosine phosphorylation of PLC-γ did not seem to be a determinant of its subcellular localization, but there was a detectable increase in cytoskeleton-associated PLC-γ in response to platelet-derived growth factor treatment.**

Phospholipase C (PLC)-mediated inositol phospholipid metabolism is a critical step in the transduction of a variety of external stimuli across the cell membrane (1, 2). Agonist-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) generates diacylglycerol and inositol trisphosphate (IP_3), both of which have second messenger roles in cells. The diacylglycerol activates protein kinase C (PKC), and IP_3 mobilizes stores of intracellular calcium (3, 4). Individually and synergistically these events regulate many short-term cellular responses and also longer term events such as gene expression and proliferation (5, 6).

Several PLC isoforms have been identified, three of which (PLC-β, -γ, and -δ) have been purified from bovine brain (7, 8). Monoclonal antibodies (mAbs) specific for each of these subtypes were developed (9), and they have been used in a variety of recent studies (10–14). A given tissue or cell type may contain multiple forms of PLC (15), but the specific functions of individual isozymes remain undefined. One approach toward gaining a better understanding of the purpose of PLC heterogeneity is to compare the subcellular localization of the PLC isozymes present in cells. Two studies that examined PLC distribution in rat brain sections demonstrated differential regional distribution of PLC-β, -γ, and -δ, indicating specific roles for these enzymes (16, 17). However, no information is available on the localization of PLC isozymes in single cells.

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Previous work in this laboratory has shown that there are three pools of α-PKC in REF52 rat embryo fibroblasts: soluble, cytoskeletal (CSK), and nuclear (18). The CSK α-PKC is associated with focal contacts in REF52 cells (18) but not in simian virus 40 (SV40) transformants (19). The discrete and transformation-sensitive distribution of α-PKC in these cells makes them an interesting candidate for comparing PLC and α-PKC distribution.

Using type-specific monoclonal PLC antibodies, we have examined the distribution of PLC-γ in normal and SV40-transformed REF52 cells and compared it with that of α-PKC in both cell types.

MATERIALS AND METHODS

Materials. Fluorescein- and rhodamine-conjugated second antibodies were from Jackson ImmunoResearch and Sigma, respectively. Rhodamine-conjugated phalloidin was from Molecular Probes. Rabbit anti-talin was a kind gift from Keith Burridge (Chapel Hill, NC). Platelet-derived growth factor (PDGF) and anti-phosphotyrosine [Tyr(*P*)] mAb were from Upstate Biotechnology (Lake Placid, NY). Alkaline phosphatase-conjugated anti-mouse IgG and alkaline phosphatase substrates were from Promega. Amplify was from Amersham. All other reagents were of the best grade available.

Cell Culture. Normal and SV40-transformed REF52 cells (originally obtained from D. B. McClure, Eli Lilly) were grown in a 3:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 containing 10% fetal bovine serum (20). The normal cells were used only between passages 10 and 20.

mAbs to PKC and PLC. The preparation and characteristics of M4 and M6 mAbs specific for α-PKC have been described (21). The isolation of mAbs to PLC-γ and PLC-δ has also been detailed (9). Each mAb is specific for the isozyme against which it was made. Unless otherwise stated, anti-PLC-γ mAb refers to a mixture of six anti-PLC-γ mAbs.

Preparation of CSKs. Cells grown on coverslips (12 mm) were washed twice with a modified microtubule stabilization buffer [MSB; 0.1 M Pipes (pH 6.9), 2 M glycerol, 1 mM EGTA, and 1 mM magnesium acetate] as described (18). The soluble proteins were extracted (4-min incubation in MSB containing 0.2% Triton X-100, aprotinin at 10 μg/ml, and leupeptin at 10 μg/ml), and the samples were washed twice more in MSB. Fixation and staining of these preparations was as for whole cells (described below).

Immunofluorescence. Cells grown on coverslips were washed twice in either phosphate-buffered saline (PBS) (con-

Abbreviations: CSK, cytoskeleton; mAb, monoclonal antibody; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PDGF, platelet-derived growth factor; PDGF-R, PDGF receptor(s); PLC, phospholipase C; Tyr(*P*), phosphotyrosine; SV40, simian virus 40; IP_3, inositol trisphosphate.

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taining Ca^{2+} and Mg^{2+}) or MSB, for cells or CSK preparations, respectively. Cells or CSKs were fixed for 10 min in 3.7% formaldehyde in PBS or MSB and washed twice in PBS or MSB, respectively. After permeabilization in methanol at −20°C for 6 min, the samples were washed twice in PBS and then washed for 30 min in PBS containing 1% bovine serum albumin (PBA). The coverslips were incubated with first antibody (diluted in PBA) for 1 hr, washed three times (5 min each) with PBA, and incubated with second antibody for a further 30 min. Fluorescein- and rhodamine-conjugated second antibodies were diluted 1:300 and 1:1000, respectively. Rhodamine-conjugated phalloidin was diluted 1:30,000 in PBA. The M6 α-PKC mAb (ascites fluid or ammonium sulfate fraction from hybridoma conditioned medium) was diluted 1:20, and the purified M4 mAb was used at 30–100 μg/ml. The M6 and M4 mAb preparations produced identical results. Anti-PLC-γ and -δ mAbs were used at 50 and 53 μg/ml, respectively. Coverslips were examined with a Nikon Optiphot microscope (×60 objective), and photomicrographs were taken on Kodak T Max 400 film.

Immunoprecipitation. REF52 cells were grown in 75-cm^2 tissue culture flasks. After a 1-hr incubation in methionine-free DMEM (containing 1% fetal bovine serum, 10 mM Hepes, and 2 mM glutamine), cells were labeled overnight (18 hr) with 3 ml of methionine-free DMEM containing 100 μCi (1 Ci = 37 GBq) of [^{35}S]methionine. Soluble proteins were extracted in 0.75 ml of MSB containing 0.2% Triton X-100, aprotinin at 10 μg/ml, leupeptin at 10 μg/ml, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1 mM Na_3VO_4. The CSKs were washed once with 0.75 ml of cold MSB, which was combined with the soluble protein extract, and the sample was made 1× with respect to RIPA (1% deoxycholate/1% Triton X-100/0.1% SDS/0.15 M NaCl/1 mM EDTA in 50 mM Tris Cl, pH 7.4). CSKs were scraped directly into RIPA (1 ml). Samples were precleared by incubation with formalin-fixed *Staphylococcus aureus* for 1 hr. After preclearing, the samples (300 μl) were shaken for 1 hr with a mixture of either three or six anti-PLC-γ mAbs (2.9 or 0.5 μg, respectively) or mouse IgG (0.4 μg). This was followed by a 1-hr incubation with protein A-Sepharose that had been previously equilibrated with rabbit anti-mouse IgG. All incubations were performed at 4°C. Immunocomplexes were collected and washed several times. Pellets were boiled in sample preparation buffer and electrophoresed on SDS/7.5% polyacrylamide gels according to Laemmli (22). Gels were stained, destained, treated with Amplify, dried, and exposed to film (Kodak X-OMAT AR) at −70°C for 3 days.

PDGF Treatment of REF52 Cells. REF52 cells were grown in 100-mm dishes to 80% confluence. After overnight incubation in medium [3:1 (vol/vol) mixture of DMEM and Ham's F-12] containing 0.1% fetal bovine serum, the cells were treated with PDGF (30 ng/ml) for 10 min at 37°C. Medium was aspirated, and the dishes were washed twice with 5 ml of ice-cold MSB, followed by a 4-min incubation (with shaking) in 1 ml of MSB containing 0.2% Triton X-100, aprotinin at 10 μg/ml, leupeptin at 10 μg/ml, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1 mM Na_3VO_4. The extracts were removed; CSKs were washed twice with 1 ml of cold MSB and then scraped into 1 ml of RIPA. PLC-γ and Tyr(*P*)-containing proteins were immunoprecipitated from 1 ml of sample as described above (except that the soluble extracts were not made 1× in RIPA) by using 0.5 μg and 5 μg of anti-PLC-γ and anti-Tyr(*P*) mAbs, respectively. Immunocomplexes were collected, and bound proteins were eluted by boiling in sample preparation buffer for electrophoresis. Equal volumes of each sample were applied to separate lanes for detection of PLC-γ and Tyr(*P*) on separate blots. The nitrocellulose blots were blocked in 5% instant milk in 50 mM Tris Cl (pH 7.4) containing 0.5 M NaCl (TBS), washed three times in TBS, and incubated for 1 hr with either anti-PLC-γ mAb (4 μg/ml) or anti-Tyr(*P*) mAb (3.3 μg/ml) diluted in TBS containing 1% bovine serum albumin (TBA). PLC-γ and Tyr(*P*) were detected after sequential incubations in alkaline phosphatase-conjugated anti-mouse IgG (133 ng/ml in TBS containing 1% bovine serum albumin, 30 min), TBS (three washes), and alkaline phosphatase substrate.

RESULTS

Immunoprecipitation of PLC-γ in REF52 Cells. The specificity of PLC-γ mAbs in REF52 cells was verified by immunoprecipitation and immunoblotting of PLC-γ from REF52 cell extracts (Fig. 1). PLC-γ was specifically detected in the soluble and CSK fractions of REF52 cells.

Immunofluorescence of α-PKC, PLC-γ, and PLC-δ in REF52 Cells. REF52 cells and detergent-extracted preparations (CSKs) stained with PKC and PLC antibodies are shown in Fig. 2. The most striking aspects of α-PKC distribution are its localization in the nucleus and at the focal contacts (Fig. 2 *A* and *B*; see ref. 18).

In whole cells stained with PLC-γ antibodies, perinuclear cytoplasmic and fiber-associated staining were found (Fig. 2*C*). PLC-γ was only weakly detected in the nucleus. A significant portion of PLC-γ was stable to detergent extraction (Fig. 2*D*) and was associated with large fibers. Whole cells and CSKs stained with PLC-δ antibody were very similar to controls (which were not incubated with first antibody), suggesting that a very small amount (if any) of PLC-δ is present in REF52 cells.

Immunocytochemical Colocalization of PLC-γ and CSK Proteins in REF52 Cells. The staining pattern observed for PLC-γ suggested that this isozyme was associated with the actin CSK in these cells. Double labeling of CSK preparations with anti-PLC-γ antibody and rhodamine-conjugated phalloidin, which stains filamentous actin, revealed that PLC-γ colocalized with the actin microfilaments, also known as stress fibers (Fig. 3 *A* and *B*). PLC-γ was evenly distributed along the length of the fibers. The ends of the stress fibers are known to be anchored to the membrane at sites known as focal contacts (23–25). To determine if PLC-γ is a component of focal contacts, CSKs were double-stained with antibodies to PLC-γ and talin, a focal contact protein (Fig. 2 *C* and *D*).

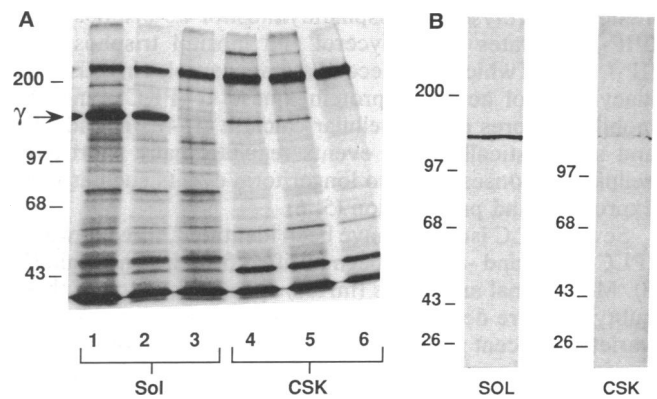

FIG. 1. Immunoprecipitation and immunoblotting of PLC-γ in REF52 cell extracts. (*A*) PLC-γ was immunoprecipitated from soluble (Sol) and CSK fractions. Samples were treated either with a mixture of three PLC-γ mAbs (2.9 μg, lanes 1 and 4), with a mixture of six mAbs (0.5 μg, lanes 2 and 5), or with mouse IgG (0.4 μg, lanes 3 and 6) to detect nonspecific binding. The positions of molecular mass markers (in kDa) are shown on the left. PLC-γ is indicated by a γ. The intensity of the high molecular mass band relative to PLC-γ is diminished in lane 5 compared to lane 4, although the immunofluorescence pattern observed with these antibody mixtures is similar. This high molecular mass protein may be a coimmunoprecipitating protein because it is not apparent in immunoblots of soluble (SOL) and CSK fractions stained with the mixture of six mAbs (*B*).

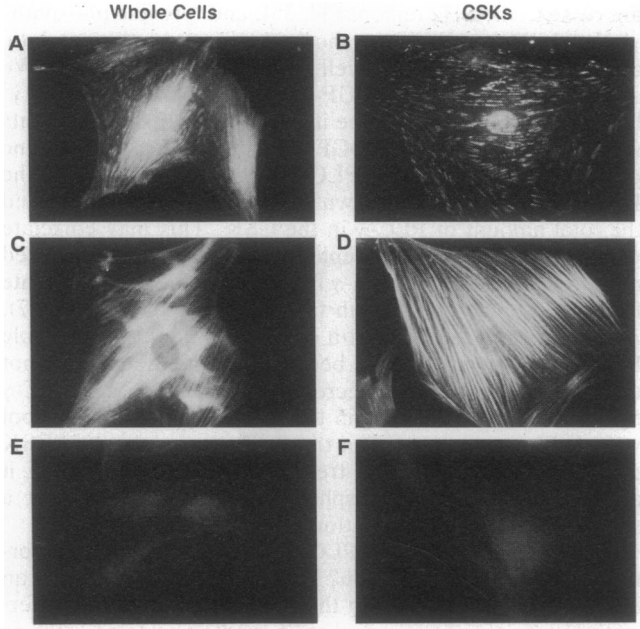

FIG. 2. Immunofluorescence of α-PKC, PLC-γ, and PLC-δ in REF52 cells. Whole cells and CSKs were prepared and stained. Samples were treated with first antibodies specific for α-PKC (*A* and *B*), PLC-γ (*C* and *D*), and PLC-δ (*E* and *F*).

CSK-associated PLC-γ was coincident with talin at discrete points along the stress fibers, implying that PLC-γ is indeed present although not concentrated in focal contacts.

Immunofluorescence of α-PKC and PLC-γ in SV40-Transformed REF52 Cells. The immunofluorescence of α-PKC was markedly different in transformed cells (compare Fig. 4 *A* and *B* with Fig. 2 *A* and *B*; see ref. 19). The nuclear staining pattern in these cells was similar to normal cells (Fig. 2 *A* and *B*), but the focal contact staining was lost. Most of the diffuse extranuclear α-PKC in whole cells (Fig. 4*A*) was detergent soluble, and what little remained in CSK preparations was randomly distributed (Fig. 4*B*). Thus, α-PKC is associated with focal contacts in normal but not SV40-transformed REF52 cells.

The distribution of PLC-γ in SV40-transformed REF52 cells (Fig. 4 *C* and *D*) was similar to normal cells in that it was apparently actin fiber-associated and was only weakly detected in the nucleus. The fibrous staining pattern was more apparent in CSKs than in whole-cell preparations (Fig. 4*D*). The brightly staining perinuclear PLC-γ found in whole-cell preparations of REF52 cells (Fig. 2*C*) was less apparent in the transformed cells. Again, PLC-δ was not detectable (data not shown).

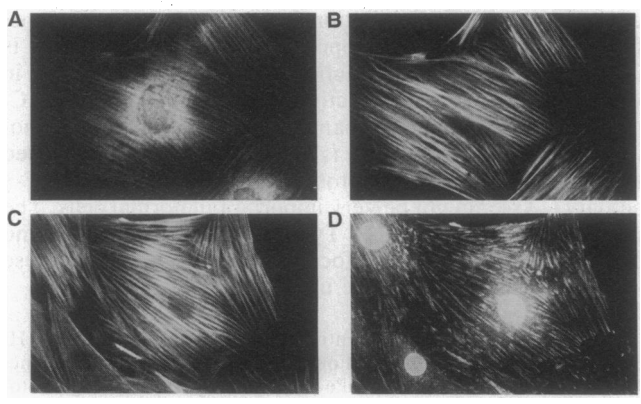

FIG. 3. Immunocytochemical colocalization of PLC-γ and CSK proteins in REF52 cells. REF52 CSKs were prepared as described in *Materials and Methods*. They were double-stained by incubating with anti-PLC-γ mAb (*A* and *C*) followed by either rhodamine-conjugated phalloidin (*B*) or rabbit anti-talin (*D*). In *C* and *D*, second antibodies were a mixture of fluorescein isothiocyanate-conjugated anti-mouse and tetramethylrhodamine isothiocyanate-conjugated anti-rabbit antibodies.

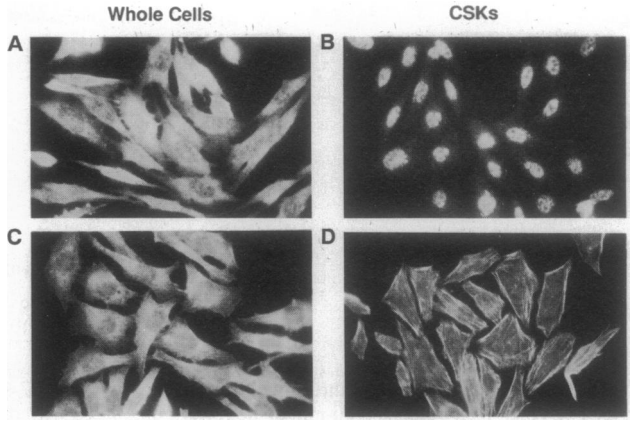

FIG. 4. Immunofluorescence of α-PKC and PLC-γ in SV40-transformed REF52 cells. The preparation and staining of whole cells and CSKs is detailed in *Materials and Methods*. Particular subtypes of PKC and PLC were detected by using antibodies specific for α-PKC (*A* and *B*) and PLC-γ (*C* and *D*).

Colocalization of PLC-γ with CSK Proteins in SV40-Transformed REF52 Cells. As in REF52 cells, PLC-γ appeared to be associated with fibers in SV40-transformed REF52 cells (Fig. 4*D*). To determine if these were microfilaments, PLC-γ and phalloidin staining were compared in a double-label experiment (Fig. 5 *A* and *B*). Although the actin fibers were smaller and less organized than in REF52 cells, PLC-γ still colocalized with actin fibers in the SV40 transformants. The PLC-γ and talin distribution were also compared in the SV40-transformed REF52 cells (Fig. 5 *C* and *D*), and it appeared that PLC-γ was also a focal contact protein in these cells, although the focal contacts were smaller and more peripheral than in normal REF52 cells.

Effect of PDGF on the Subcellular Localization of PLC-γ in REF52 Cells. We compared the subcellular distribution of PLC-γ in control and PDGF-treated cells to ascertain whether tyrosine phosphorylation of PLC-γ may be a determinant of subcellular location. PDGF caused increased tyrosine phosphorylation of PDGF receptors (PDGF-R) and

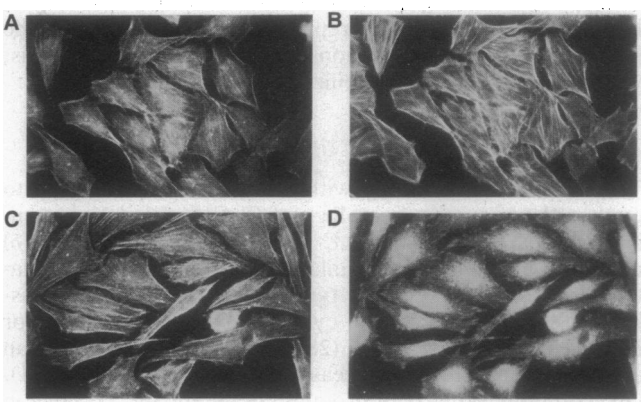

FIG. 5. Colocalization of PLC-γ with CSK proteins in SV40-transformed REF52 cells. CSKs were prepared and double-stained to examine PLC-γ (*A* and *C*) distribution with respect to that of actin (*B*) and talin (*D*) in the same preparations. Samples were treated as described in *Materials and Methods* except for those double-stained for PLC-γ and actin (*A* and *B*), which were permeabilized using ethanol instead of methanol.

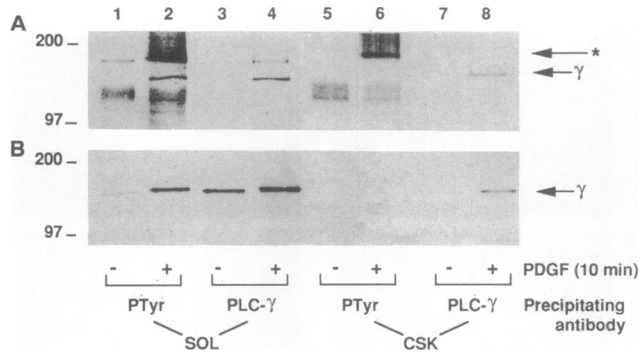

FIG. 6. Effect of PDGF on the subcellular localization of PLC-γ in REF52 cells. REF52 cells (100-mm dishes) were incubated with or without PDGF (30 ng/ml) for 10 min at 37°C. Soluble (SOL) and CSK fractions were collected, and duplicate samples were immunoprecipitated with mAbs to PLC-γ or Tyr(*P*). Equal volumes of precipitates were loaded in separate lanes of an SDS/7.5% polyacrylamide gel. After electroblotting, the samples were probed for either Tyr(*P*) (*A*) or PLC-γ (*B*). PLC-γ is indicated by a γ; the putative PDGF-R is indicated by a star. The positions of molecular mass markers (in kDa) are shown on the left.

PLC-γ in the soluble fractions. PDGF increased the Tyr(*P*) content of soluble PLC-γ because (*i*) PLC-γ was immunoprecipitated with anti-Tyr(*P*) mAb from PDGF-treated cells but not control cells (Fig. 6, lanes 1 and 2), and (*ii*) PLC-γ was effectively precipitated from control and PDGF-treated cells with anti-PLC-γ mAb (Fig. 6*B*, lanes 3 and 4); however, only PLC-γ from the treated cells was recognized by the anti-Tyr(*P*) mAb (Fig. 6*A*, lanes 3 and 4). In fact, the staining intensity of PLC-γ was very similar in immunoblots stained with anti-Tyr(*P*) and anti-PLC-γ mAbs (compare lanes 2 and 4 in Fig. 6*A* with their corresponding lanes in Fig. 6*B*), indicating that a large fraction of the total soluble PLC-γ contained Tyr(*P*). PDGF-R coimmunoprecipitated with PLC-γ after PDGF treatment, whether anti-Tyr(*P*) (Fig. 6*A*, lane 2) or anti-PLC-γ mAb (Fig. 6*A*, lane 4) was used. Much less PDGF-R was recovered with the anti-PLC-γ mAb, implying that a relatively small fraction of the total soluble PDGF-R pool is coupled to PLC-γ upon stimulation.

PDGF increased the total amount of CSK-associated PLC-γ (Fig. 6*B*, lanes 7 and 8). Immunoblots of the PLC-γ immunoprecipitates were stained with anti-Tyr(*P*) mAb, demonstrating that the CSK pool of PLC-γ was tyrosine phosphorylated (Fig. 6*A*, lane 8). However, CSK-associated PLC-γ was not precipitated with the anti-Tyr(*P*) mAb (Fig. 6, lane 6). This apparent discrepancy may be attributed to the high detergent concentrations in the CSK preparations, which are required to solubilize the CSK proteins.

DISCUSSION

The association of PLC-γ with the actin CSK may help to explain the sequence homology of PLC-γ with other seemingly unrelated proteins. PLC-γ (but not PLC-α, -β, and -δ) contains an SH3 domain similar to that found in the regulatory domain of nonreceptor tyrosine kinases (26, 27), translocatable 47- and 67-kDa cytosolic factors required for NADPH oxidase activation (28, 29), myosin 1A and 1B, an actin-binding protein from yeast, and other proteins (30, 31). One common property of proteins containing the SH3 region is their association with the actin CSK. Our observation substantiates the hypothesis that the SH3 domain acts as an actin binding site (31).

PLC-γ also contains two SH2 domains. These domains regulate protein–protein interactions, including association with growth factor receptors (32, 33). Some sites of epidermal growth factor-induced tyrosine phosphorylation of PLC-γ are close to the SH2 regions (34, 35), and tyrosine phosphorylation has recently been shown to increase the catalytic activity of PLC-γ in A431 cell immunoprecipitates (36). We examined the effect of PDGF-induced tyrosine phosphorylation of PLC-γ to determine if this modification affected its subcellular localization. PDGF treatment increased tyrosine phosphorylation of soluble PLC-γ and its association with the PDGF-R, as previously shown (10, 11). PDGF also increased the total amount of PLC-γ in the CSK. This may correlate with a recent report that epidermal growth factor caused rapid redistribution of PLC-γ from a soluble to a particulate fraction in epidermal growth factor-treated A431 cells (37). Although we did not detect a corresponding loss of soluble PLC-γ, this is most likely because our methods were not designed to detect a small decrease in the large soluble PLC-γ pool, whereas an increase in the relatively small CSK pool was readily apparent. Given that both the soluble and CSK pools of PLC-γ from PDGF-treated cells contained Tyr(*P*), it appears that tyrosine phosphorylation of PLC-γ is not a sufficient signal for association with the CSK.

Could CSK-associated PLC-γ play a role in receptor-mediated signal transduction? Inositol phospholipids play an important role in regulating the polymerization/depolymerization state of actin. Several actin binding proteins, such as profilin, gelsolin, cofilin, and destrin, have been shown to interact with PIP_2 (38–40). Association of PIP_2 with the actin capping protein, profilin, disrupts the profilin–actin complex, thus increasing the concentration of monomeric actin available for filament elongation (38). It has recently been shown that PIP_2-associated with profilin is a good substrate for phosphorylated, but not unphosphorylated, PLC-γ (41). Thus, activation of receptor tyrosine kinase activity and phosphorylation of PLC-γ would trigger hydrolysis of PIP_2-associated profilin. This would promote formation of the profilactin complex, thus preventing nucleation and filament elongation. Furthermore, the accompanying IP_3-mediated increase in intracellular calcium activates actin-severing and barbed end nucleating proteins, such as gelsolin. All of these events favor actin depolymerization (42). Changes in cell shape observed after addition of growth factors may thus be a function of increased PIP_2 hydrolysis by CSK-associated PLC-γ.

Both α-PKC and PLC-γ were found in focal contacts of normal cells. Possibly, colocalization correlates with their strongly adherent and stationary phenotype because focal contacts of the less adherent and more migratory SV40-transformed cells contain PLC-γ but not α-PKC. Relative changes in α-PKC distribution between normal and transformed cells suggest that appropriate α-PKC localization is important in the normal functioning of the cell. The way in which focal contact PLC-γ activity, in the absence of α-PKC, might contribute to maintenance of the transformed phenotype remains unknown. It is possible that the suggested ability of PKC to limit receptor-mediated increases in PLC-γ activity (43–45) might be very important in normal cells. The presence of PLC-γ, α-PKC, Tyr(*P*)-containing proteins, and other regulatory proteins in focal contacts suggests that these structures have a major role in transmembrane signaling.

K.M. is deeply grateful for continued support from Dr. Gordon H. Sato and the W. Alton Jones Foundation. We thank Carol Baine, Polly Butler, and Marina LaDuke for their help in preparing photographs and figures. This work was supported in part by Council for Tobacco Research Grant 2375 and grants from the National Cancer Institute (CA46530 and CA37589).

1. Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360.
2. Hokin, L. E. (1985) *Annu. Rev. Biochem.* **54**, 205–235.
3. Nishizuka, Y. (1986) *Science* **233**, 305–312.
4. Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **341**, 197–205.

5. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56,** 159–193.
6. Berridge, M. J. (1987) *Biochim. Biophys. Acta* **907,** 33–45.
7. Ryu, S. H., Cho, K. S., Lee, K.-Y., Suh, P.-G. & Rhee, S. G. (1987) *J. Biol. Chem.* **262,** 12511–12518.
8. Ryu, S. H., Suh, P.-G., Cho, K. S., Lee, K.-Y. & Rhee, S. G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6649–6653.
9. Suh, P.-G., Ryu, S. H., Choi, W. C., Lee, K.-Y. & Rhee, S. G. (1988) *J. Biol. Chem.* **263,** 14497–14504.
10. Morrison, D. K., Kaplan, D. R., Rhee, S. G. & Williams, L. T. (1990) *Mol. Cell. Biol.* **10,** 2359–2366.
11. Meisenhelder, J., Suh, P.-G., Rhee, S. G. & Hunter, T. (1989) *Cell* **57,** 1109–1122.
12. Margolis, B., Rhee, S. G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ullrich, A., Zilberstein, A. & Schlessinger, J. (1989) *Cell* **57,** 1101–1107.
13. Smith, M. R., Liu, Y.-L., Kim, H., Rhee, S. G. & Kung, H.-F. (1990) *Science* **247,** 1074–1077.
14. Rath, H. M., Fee, J. A., Rhee, S. G. & Silbert, D. F. (1990) *J. Biol. Chem.* **265,** 3080–3087.
15. Rhee, S. G., Suh, P.-G., Ryu, S.-H. & Lee, S. Y. (1989) *Science* **244,** 546–550.
16. Gerfen, C. R., Choi, W. C., Suh, P. G. & Rhee, S. G. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3208–3212.
17. Choi, W. C., Gerfen, C. R., Suh, P. G. & Rhee, S. G. (1989) *Brain Res.* **499,** 193–197.
18. Jaken, S., Leach, K. & Klauck, T. (1989) *J. Cell Biol.* **109,** 697–704.
19. Hyatt, S. L., Klauck, T. & Jaken, S. (1990) *Mol. Carcinog.* **3,** 45–53.
20. McClure, D. B., Hightower, M. J. & Topp, W. C. (1982) in *Growth of Cells in Hormonally Defined Media*, eds. Sato, G. H., Pardee, A. & Sirbasku, D. A. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 345–364.
21. Leach, K. L., Powers, E. A., McGuire, J. C., Dong, L., Kiley, S. C. & Jaken, S. (1988) *J. Biol. Chem.* **263,** 13223–13230.
22. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
23. Woods, A. & Couchman, J. R. (1988) *Collagen Relat. Res.* **8,** 155–182.
24. Burridge, K. (1986) *Cancer Rev.* **4,** 18–78.
25. Burridge, K., Fath, K., Kelly, T., Nuckolls, G. & Turner, C. (1988) *Annu. Rev. Cell Biol.* **4,** 487–525.
26. Suh, P.-G., Ryu, S. H., Moon, K. H., Suh, H. W. & Rhee, S. G. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5419–5423.
27. Stahl, M. L., Ferenz, C. R., Kelleher, K. L., Kriz, R. W. & Knopf, J. L. (1988) *Nature (London)* **332,** 269–272.
28. Lomax, K. J., Leto, T. L., Nunoi, H., Gallin, J. I. & Malech, H. L. (1989) *Science* **245,** 409–412.
29. Leto, T. L., Lomax, K. J., Volpp, B. D., Nunoi, H., Sechler, J. M. G., Nauseef, W. M., Clark, R. A., Gallin, J. I. & Malech, H. L. (1990) *Science* **248,** 727–730.
30. Rodaway, A. R. F., Sternberg, M. J. E. & Bentley, D. L. (1989) *Nature (London)* **342,** 624.
31. Drubin, D. G., Mulholland, J., Zhu, Z. & Botstein, D. (1990) *Nature (London)* **343,** 288–290.
32. Moran, M. F., Koch, C. A., Anderson, D., Ellis, C., England, L., Martin, G. S. & Pawson, T. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8622–8626.
33. Anderson, D., Koch, C. A., Grey, L., Ellis, C., Moran, M. F. & Pawson, T. (1990) *Science* **250,** 979–982.
34. Kim, J. W., Sim, S. S., Kim, U.-H., Nishibe, S., Wahl, M. I., Carpenter, G. & Rhee, S. G. (1990) *J. Biol. Chem.* **265,** 3940–3943.
35. Wahl, M. I., Nishibe, S., Kim, J. W., Kim, H., Rhee, S. G. & Carpenter, G. (1990) *J. Biol. Chem.* **265,** 3944–3948.
36. Todderud, G., Wahl, M. I., Rhee, S. G. & Carpenter, G. (1990) *Science* **249,** 296–298.
37. Nishibe, S., Wahl, M. I., Hernandez-Sotomayor, S. M. T., Tonks, N. K., Rhee, S. G. & Carpenter, G. (1990) *Science* **250,** 1253–1256.
38. Lassing, I. & Lindberg, U. (1985) *Nature (London)* **314,** 472–474.
39. Janmey, P. A. & Stossel, T. P. (1987) *Nature (London)* **325,** 362–364.
40. Yonezawa, N., Nishida, E., Iida, K., Yahara, I. & Sakai, H. (1990) *J. Biol. Chem.* **265,** 8382–8386.
41. Goldschmidt-Clermont, P. J., Kim, J. W., Machesky, L. M., Rhee, S. G. & Pollard, T. D. (1991) *Science* **251,** 1231–1233.
42. Stossel, T. P. (1989) *J. Biol. Chem.* **264,** 18261–18264.
43. Rhee, S. G., Suh, P. G., Ryu, S. H. & Lee, S. Y. (1989) *Science* **244,** 546–550.
44. Boyer, J. L., Hepler, J. R. & Harden, T. K. (1989) *Trends Pharmacol. Sci.* **10,** 360–364.
45. Huckle, W. R., Hepler, J. R., Rhee, S. G., Harden, T. K. & Earp, H. S. (1990) *Endocrinology* **127,** 1697–1705.