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Phospholipase C-γ1 Binds to Actin-Cytoskeleton via Its C-terminal SH2 Domain *in Vitro*

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Association of phospholipase C (PLC)-γ1 with the cytoskeleton has been postulated to be one of the crucial steps for PLC-γ1 activation and translocation to the plasma membrane. In this report, direct binding assays were carried out to study which fragment of PLC-γ1 Src-homology region has been able to bind to the actin-cytoskeleton. Using GST fusion proteins containing various deletions of the PLC-γ1 Src homology region, it was found that PLC-γ1 binds to the actin-cytoskeleton directly via its C-terminal SH2 domain but not the SH3 domain *in vitro*. However, the binding of the C-terminal SH2 domain of PLC-γ1 to actin did not interfere with the SH2 domain's ability to associate with phosphotyrosine, which suggested that actin and phosphotyrosine residues may bind to different sequences in the C-terminal SH2 domain of PLC-γ1. © 1996 Academic Press, Inc.

Stimulation of cells with a great variety of hormone ligands elicits a wide range of biological responses, including mitogenesis, differentiation, metabolic changes, secretion and very frequently, the activation of PLC. PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, both of which have very important roles as second messengers in cell function (1). 1,4,5-trisphosphate induces Ca^{++} release from specialized intracellular organelles while diacylglycerol causes activation of members of the protein kinase C family of serine/threonine-specific kinases.

Previously, we have shown that EGF induces activation of PLC-γ1, a PLC isoform containing two SH2 and one SH3 Src homology domains, and its translocation to the cytoskeleton in rat hepatocytes (2). In the present report, we examined further the interaction between PLC-γ1 and the cytoskeleton. Unexpectedly, we found that PLC-γ1 binds to the actin-cytoskeleton via its C-terminal SH2 (C-SH2) domain but not the SH3 domain *in vitro*. In addition, PLC-γ1 was found to bind directly to pure polymerized F-actin but not to unpolymerized G-actin. Furthermore, the actin binding site in the C-SH2 domain of PLC-γ1 appears not to overlap with the phosphotyrosine binding site.

MATERIALS AND METHODS

Materials. Mouse EGF and purified chick smooth muscle actin were purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-actin antibody was purchased from ICN Biomedicals (Costa Mesa, CA). S-hexylglutathione, reduced glutathione and O-phospho-L-tyrosine on cross-linked 4% beaded agarose were from Sigma Chemical Co.

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Abbreviations: EGF, epidermal growth factor; GST, glutathione *S*-transferase; PLC, phospholipase C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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FIG. 1. Actin-cytoskeleton interaction with C-SH2 domain of PLC-γ1. 80 μg of the Triton X-100 insoluble fraction isolated from EGF 10 min-treated hepatocytes was bound to 5 μg of immobilized GST fusion proteins with various deletions of the PLC-γ1 Src-homology region. After incubation for 1 h at 4°C with shaking, the agarose beads were washed three times with PLC lysis buffer plus 2% Triton X-100 and analyzed by SDS-PAGE and western blotting.

Cytoskeleton preparation. The cytoskeleton extracts from rat hepatocytes were prepared essentially as in Ref. 3. Fresh rat hepatocytes were isolated from collagenase digestion of perfused livers from fed male Sprague-Dawley rats (180-220g) according to our previously published procedures (4). Cell viability, as determined by trypan blue, was routinely >90%. The hepatocytes were incubated in Dulbecco's modified Eagle's medium-nutrient F-12 at 37°C and treated with 200 ng/ml of EGF at 37°C for 10 min, and immediately washed twice with ice-cold Ca^{++} and Mg^{++}-free phosphate-buffered saline. The hepatocytes were separated from the medium and lysed in extraction buffer containing 100 mM Tris-HCl (pH 7.4), 2% Triton X-100, 10 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml aprotinin and 2 mM Na_3VO_4 (3). After incubation on ice for 30 min, the Triton X-100 insoluble extract was collected at 10,000 $\times$ g for 10 min, and then incubated on ice for 30 min in two times-diluted extraction buffer containing 0.5 M NaCl and 0.3% deoxycholic acid followed by centrifuging at 100,000 $\times$ g for 30 min. The supernatants were collected as cytoskeletal fractions.

Purification of GST fusion proteins. Various GST-truncated PLC-γ1 Src-homology region plasmids were kindly provided by Drs. Gerald Gish and Tony Pawson (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada). cDNA fragments corresponding to the whole Src-homology regions of bovine PLC-γ1 containing the two SH2 domains, one SH3 domain and the two tyrosine-phosphorylation sites Tyr^{771} and Tyr^{783} (residues 545-850, designated as 22Y3), N- and C-terminal SH2 domains (residues 545-759, designated as N+C), N-terminal SH2 domain (residues 545-659), C-terminal SH2 domain (residues 663-759), and the SH3 domain (residues 798-850) were subcloned into a bacterial vector pGEX-kt (5). The transformed bacteria were grown in Luria-Bertani medium (10 g/L of bacto-trypton, 5 g/L of bacto-yeast extract and 10 g/L of NaCl) containing 100 μg/ml ampicillin, and fusion protein production was induced by addition of isopropyl-D-thio-galactopyranoside to 0.1 mM for 6 hours. The bacteria were then harvested and sonicated in PLC-lysis buffer (50 mM Hepes, pH 7.4, 10% glycerol, 150 mM NaCl, 100 mM NaF, 1 mM EGTA, 50 mM pyrophosphate, 1.5 mM $MgCl_2$, 10 mM dithiothreitol with 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 1 mM Na_3VO_4). Fusion proteins were purified from clarified cell lysates by affinity chromatography on glutathione-agarose beads.

In vitro cytoskeleton binding assay. Purified GST fusion proteins were shaken overnight at 4°C with a 50% slurry of glutathione-agarose beads (5 mg of fusion protein/25 ml of beads). 80 μg of cytoskeleton extracts were then added to the fusion protein bound glutathione-agarose beads in a total volume of 60 μl. After incubation for 1 h at 4°C with shaking, the beads were washed three times with PLC lysis buffer plus 2% Triton X-100, and analyzed by SDS-PAGE. The actin was visualized by western blotting using anti-actin monoclonal antibody.

Actin Co-sedimentation assay. The sedimentation assay followed the method of Ref. 3 with slight modifications. Briefly, 5 μg of various GST fusion proteins were mixed with 10 μg of purified chick smooth muscle actin in 200 ul F-actin buffer containing 2 mM Tris-HCl (pH 7.4), 0.2 mM $CaCl_2$, 0.2 mM dithiothreitol, 0.5 mM ATP, 75 mM KCl and 2 mM $MgCl_2$. After incubation at room temperature for 1 h, 10 μl of 20% Triton X-100 was added and incubated for an additional 10 min to disrupt unspecific binding. The mixtures were then centrifuged at 100,000 $\times$ g for 60 min at 25°C. The supernatants were carefully removed and concentrated by ultrafiltration. Both pellets and supernatants were analyzed by SDS-PAGE and stained with Coomassie blue.

F-actin and C-SH2 binding phosphotyrosine beads assay. 5 μg of various fusion proteins were first bound to 120 μl of 50% slurry of phosphotyrosine on cross-linked agarose beads. After overnight shaking at 4°C, the agarose beads were washed twice with F-actin buffer, and 10 μg of purified chick smooth muscle actin was added and incubated at room temperature for 2 h with gentle shaking. After incubation, the agarose beads were washed three times with PLC lysis buffer plus 2% Triton X-100. The samples were analyzed by SDS-PAGE and western blotting.

RESULTS

Freshly isolated hepatocytes from rat livers were treated with EGF for 10 min and the insoluble cytoskeletal fraction was then isolated and used for binding to various GST-truncated PLC-γ1 fusion proteins. The samples were shaken at 4°C for 1 h and then stringently washed. As shown in Fig. 1, the 22Y3 which includes the whole Src-homology regions (two SH2 domains and one SH3 domain), the N + C (N-terminal and C-terminal SH2 domains) and the

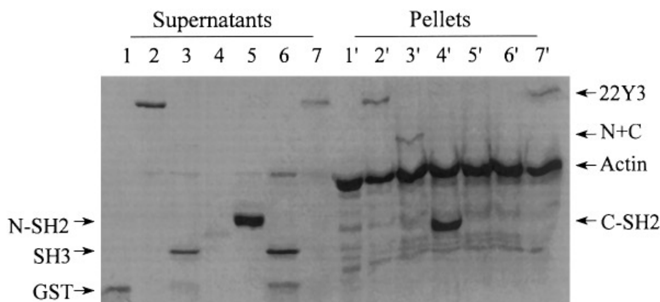

FIG. 2. F-actin co-sedimentation assay. Aliquots of GST-truncated PLC-γ1 Src-homology domains were incubated with chicken smooth muscle actin in F-actin buffer at room temperature for 1 h as described under Materials and Methods. The pellets and supernatants were both analyzed in 10% SDS acrylamide gel and stained with Coomassie blue. Lane 1 and 1′, GST; lane 2 and 2′, GST-22Y3; lane 3 and 3′, GST-N + C; lane 4 and 4′, GST-C-SH2; lane 5 and 5′, GST-N-SH2; lane 6 and 6′, SH3; lane 7 and 7′, GST-22Y3-R694A (C-SH2 mutant). The background bands in the pellet fraction may be from actin degradation.

C-SH2 (C-terminal SH2 domain) were able to bind to the actin-cytoskeleton *in vitro.* The N-SH2 domain (N-terminal SH2 domain) and the SH3 domain demonstrated much less actin binding, essentially the same as the GST protein. These results indicate that the C-SH2 domain is responsible for the binding of PLC-γ1 to the actin-cytoskeleton.

To determine whether the C-SH2 domain can bind to actin directly, an actin cosendimentation assay was employed. Aliquots of GST-truncated PLC-γ1 fusion proteins and chicken smooth muscle actin were incubated at room temperature in the F-actin buffer for 60 min. After incubation, the polymerized actin filaments (F-actin) were sedimented, and the supernatants were concentrated by ultrafiltration. The pellets and concentrated supernatants were both analyzed by SDS-PAGE. As shown in Fig. 2, the 22Y3, N+C and C-SH2 fragments were able to bind to F-actin directly, but no F-actin binding was observed with the GST, N-SH2 and SH3 proteins. As a negative control, the C-SH2 domain was found not to bind to bovine serum albumin protein (data not shown).

We further examined whether unpolymerized actin (G-actin) could bind to the C-SH2 domain. The G-actin was mixed with GST-fusion proteins bound to glutathione-agarose beads in a total volume of 60 μl as in the 22Y3-cytoskeleton binding assay. After washing 3 times with PLC lysis buffer plus 2% Triton X-100, the samples were separated on SDS-PAGE and analyzed by Coomassie blue staining. As shown in Fig. 3, no significant actin protein was detected that associated with GST alone or with 22Y3, N+C and C-SH2 proteins.

Since SH2 domain generally binds to phosphotyrosine residues, we therefore studied whether F-actin and phosphotyrosine bind to the same sequence in the C-SH2 domain. A C-SH2 mutant in which Arg^{694} (corresponding to Arg^{175} of Src-SH2 domain that is required for SH2 binding to phosphotyrosine residues) was changed to Ala showed similar F-actin-binding affinity to the wild-type 22Y3 protein (Fig. 2, lane 7 and 7′). Furthermore, to determine whether the C-SH2 domain can still associate with actin after binding to phosphotyrosine, phosphotyrosine cross-linked agarose beads were employed in the actin binding assay. The GST fused C-SH2 domain was first bound to an excess of phosphotyrosine beads overnight, and F-actin was then added. After 2 h of incubation at room temperature, the beads were extensively washed with PLC lysis buffer plus 2% Triton X-100 and analyzed by SDS-PAGE. As shown in Fig. 4, there was no binding of F-actin or GST to the phosphotyrosine beads. However, C-SH2 alone or with F-actin, and N+C with F-actin bound to the phosphotyrosine beads. Since F-actin alone did not show a significant binding, F-actin must bind to the C-SH2 domain. The data suggest that phosphotyrosine and F-actin may not bind to the same site in the C-SH2 domain.

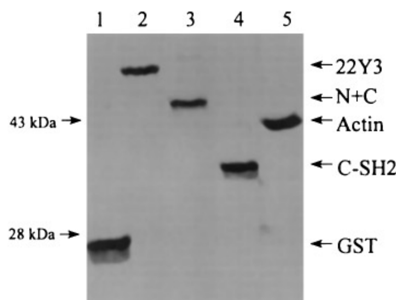

FIG. 3. G-actin binding assay. 10 μg of G-actin was bound to 5 μg of immobilized GST-truncated PLC-γ1 Src-homology domains by following the *in vitro* cytoskeleton binding assay as described under Materials and Methods. The samples were analyzed by 10% SDS acrylamide gel and stained with Coomassie blue. Lane 1, GST only; lane 2, GST-22Y3; lane 3, GST-N + C; lane 4, GST-C-SH2; lane 5, actin control.

DISCUSSION

The SH3 domain has been a leading candidate for cytoskeletal interactions because it has been identified in a number of actin-binding proteins, including certain isoforms of myosin II (6), the cytoskeletal protein α-spectrin (7), and the yeast actin-binding protein ABP-1 (8), and also because many proteins found to interact with the cytoskeleton posses at least one SH3 domain such as PLC-γ1, Grb2, PI-3 kinase, c-Src and c-Abl (9-14). In this study, our data demonstrate that the SH3 domain of PLC-γ1 is not sufficient for cytoskeleton association, since the SH3 domain cannot bind to the actin-cytoskeletal fraction *in vitro* (Fig. 1 and 2). However, the results do not necessarily rule out a role for the SH3 domain in subcellular localization since many SH3-containing proteins also interact directly or indirectly with proline-rich proteins in the plasma membrane (12). It has been suggested that SH3 domains of Grb2 function in the recruitment of hSos1, the human homologue of the *Drosophila* guanine-nucleotide releasing factor for Ras, to the membrane where cytoskeletal and other protein-protein interactions can take place (15). In addition, it has been suggested that PLC-γ1 may bind to Shc directly via its SH3 domain (16), and Shc was found to be able to bind to actin directly (3). Therefore, it is possible that PLC-γ1 may also bind to cytoskeleton via Shc *in vivo.*

It also appears that many signaling molecules bind to actin-cytoskeleton through non-SH3 domains. The SH2 domain of v-Src was demonstrated to be required for its binding to cytoskeleton (12,17), and c-Abl was reported to possess a specific F-actin binding sequence at its

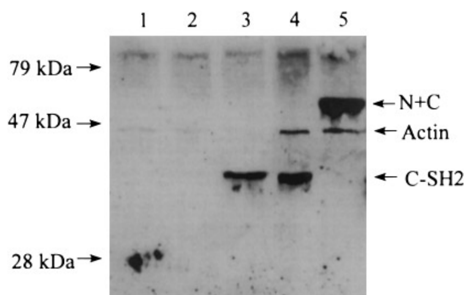

FIG. 4. Phosphotyrosine beads binding assay. 5 μg of GST fusion proteins were incubated with excess amount of phosphotyrosine beads overnight, and F-actin was then added as described under Materials and Methods. The samples were analyzed by SDS-PAGE and blotted with anti-actin monoclonal antibody. Lane 1, F-actin only; lane 2, GST only; lane 3, GST-C-SH2 only; lane 4, GST-C-SH2 with F-actin; lane 5, GST-N + C with F-actin.

extreme C-terminus (13). Also the EGF receptor can bind directly to actin via its cytoplasmic domain (18). More recently, the adaptor protein Shc was shown to bind to F-actin directly by its amino-terminal domain (3). In addition, an SH2 domain has been found in the actin-binding protein, tensin (19).

It is well established that SH2 domains directly recognize phosphotyrosine, and Arg^{175} may be the most critical residue in Src SH2 domain binding to phosphotyrosine (20). Our data have demonstrated that the C-SH2 domain of PLC-γ1 can bind to phosphotyrosine and F-actin simultaneously (Fig. 4), and the mutation of Arg^{694} (corresponding to Arg^{175} in Src SH2 domain) in the C-SH2 domain of PLC-γ1 has no significant effect on 22Y3 binding to F-actin (Fig. 2). The significance of these observations is that, although the C-SH2 domain binds to actin-cytoskeleton, this binding does not interfere with the binding of the C-SH2 domain to phosphotyrosine residues of other signaling molecules. Interestingly, a previous study has shown that, in the Src SH2 domain, a sequence (amino acids 149-169) outside the phosphotyrosine binding box (sequence around Arg^{175}) was shown to be required for cytoskeleton association, and a mutant deleted for amino acids 169-204, which includes Arg^{175}, retained significant levels of cytoskeleton association (17). A similarity may exist between Src and PLC-γ1 in their properties of association with cytoskeleton.

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