Epidermal Growth Factor-induced Activation and Translocation of Phospholipase C-γ1 to the Cytoskeleton in Rat Hepatocytes*

(Received for publication, October 19, 1993)

Li Jun Yang†, Sue Goo Rhee‡, and John R. Williamson

From the Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and the §Laboratory of Biochemistry, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

In this study, we have examined the relationship between epidermal growth factor (EGF)-induced tyrosine phosphorylation of phospholipase C-γ1 (PLC-γ1) and its translocation from the cytosol to the Triton X-100-insoluble cytoskeleton fraction in rat hepatocytes. The translocation of PLC-γ1 was specific for EGF stimulation, because a similar effect was not observed with insulin or vasopressin. EGF caused a transient increase in PLC activity in the cytoskeleton fraction which could be abolished by immunoprecipitating PLC-γ1. Tyrosine phosphorylated PLC-γ1 was seen only in the cytoskeleton fraction, suggesting that tyrosine phosphorylation is required for PLC-γ1 translocation to the cytoskeleton. This process may involve binding of PLC-γ1 to actin filaments, since actin was immunoprecipitated together with PLC-γ1 in the cytoskeleton after EGF treatment. EGF-induced translocation of PLC-γ1 to the cytoskeleton was not inhibited by pertussis toxin, but Gia was translocated in an EGF-dependent manner, suggesting that the interaction of PLC-γ1 with its activated Gi-protein is downstream from both PLC-γ1 tyrosine phosphorylation and its translocation to the cytoskeleton. Taken together, the present studies indicate that EGF-induced tyrosine phosphorylation of PLC-γ1, its association with the cytoskeleton, and its interaction with activated Gia protein are all obligatory for PLC-γ1 activation in hepatocytes.

Numerous studies demonstrate that growth factors promote increased turnover of inositol phospholipids in fibroblasts and other cell types (1, 2). Previous work from our laboratory has shown that occupancy of receptors by growth factors, such as epidermal growth factor (EGF) (3) and hepatocyte growth factor (HGF) (4) triggers a rapid phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2). Hydrolysis of PIP2 produces inositol 1,4,5-trisphosphate (Ins-1,4,5-P3) and diacylglycerol, both of which have second messenger roles in cell function (5). Ins-1,4,5-P3 is responsible for Ca2+ release from specialized intracellular organelles (6–9) and for second messenger-activated Ca2+ entry into cells (10–13). Diacylglycerol is involved in the activation of members of the protein kinase C family of serine/threonine-specific kinases, which have both positive and negative effects on receptor signaling (14, 15). The tyrosine kinase domain of the epidermal growth factor receptor (EGF-R) is essential for expression of growth factor effects, suggesting that a causal relationship exists between tyrosine phosphorylation of specific proteins and activation of inositol lipid metabolism (16, 17). PLC-γ1, a member of the large family of PLC isozymes (18), has been shown to be a physiological substrate for tyrosine phosphorylation by EGF and PDGF receptors and is selectively phosphorylated on specific tyrosine residues (19–21).

The relationship between tyrosine phosphorylation of PLC-γ1 and in its ability to hydrolyze PIP2, however, is not as simple as was first thought (16). Tyrosine phosphorylation of purified PLC-γ1 in vitro does not increase its catalytic activity (22), although PLC-γ1, when tyrosine phosphorylated is less sensitive to detergent inhibition than the nonphosphorylated enzyme (23). Mutational replacement of the essential tyrosine residue (Tyr783) by phenylalanine also does not affect the catalytic activity of PLC-γ1 in vitro (24). Nevertheless, when mutant enzyme is expressed in NIH-3T3 cells, substitution at Tyr783 completely blocks PDGF-promoted hydrolysis of inositol phospholipids by PLC-γ1 (24). These studies suggest that tyrosine phosphorylation of PLC-γ1 does not directly affect the activity of the enzyme, but does regulate its activation by growth hormone receptors in the intact cell by a mechanism that remains to be elucidated. More recent studies show that the EGF receptor in vitro can increase PLC-γ1 activity independently of tyrosine phosphorylation, albeit by an unknown mechanism (25).

In hepatocytes, a pertussis toxin-sensitive G-protein is required for EGF-mediated signal transduction (3, 26). The necessity of a G-protein in the hepatocyte signaling pathway contrasts with studies using cell lines such as transformed A-431 epithelial cells or fibroblasts, which generally do not depend on a G-protein for their responsiveness to EGF or PDGF (27–30). Pretreatment of hepatocytes with pertussis toxin does not prevent EGF-mediated tyrosine phosphorylation of PLC-γ1 in primary cultured rat hepatocytes, although PIP2 hydrolysis is blocked, suggesting that tyrosine phosphorylation of PLC-γ1 by itself is not sufficient to activate the enzyme (31, 32). However, the mechanism of interaction between the EGF receptor, PLC-γ1, and G-protein for EGF-induced signal transduction in hepatocytes is still not clear.

PLC-γ1 is a cytosolic enzyme, but how PLC-γ1 is translocated to the membrane to interact with Gi-protein and its substrate PIP2 remains unresolved. Certain evidence indicates that actin filaments may be involved in EGF-induced signal...
transduction. It has been shown, for instance, that the EGF-R is directly associated with actin filaments in A-431 cells (33) and that the EGF-R containing actin-binding domains, which may provide a structural basis for the association (34). Immunocytochemical studies have shown that PLC-γ1 colocalizes with actin filaments after PDGF stimulation in rat embryo fibroblasts, but it was not shown that this association affected PLC-γ1 activity (35). Since different cell types appear to use different mechanisms for activation of PLC-γ1, it cannot be assumed that an association of PLC-γ1 with actin filaments is a general phenomenon or that it is an essential feature for enzyme activation in vivo. In this paper, we have investigated the role of cytoskeleton in EGF-induced activation of PLC-γ1 in rat hepatocytes. We have found that PLC-γ1 is associated with actin and that EGF-induced translocation of PLC-γ1 to the cytoskeleton fraction is highly correlated with its tyrosine phosphorylation and increased catalytic activity. These results suggest that association of PLC-γ1 with the cytoskeleton may be facilitated by bringing tyrosine phosphorylated PLC-γ1 to the plasma membrane where it interacts with activated Gα subunit for the initiation of its catalytic activity.

EXPERIMENTAL PROCEDURES

Materials—Anti-phosphotyrosine monoclonal antibodies, mouse EGF, and genistein were purchased from Upstate Biotechnology. Anti-phosphotyrosine (PY20), mouse anti-actin (Clone: C4) antibody, [3H]IP, [32P]NAD, and [35S]methionine/cysteine were from ICN Biochemical. Pertussis toxin was purchased from List Biological Research. Protein A-Sepharose CL-4B, Triton X-100, PIP₂, and phosphotyrosine antibody (PY-20), mouse anti-actin (Clone: C4) antibody, EGF, and genistein were purchased from Upstate Biotechnology. Anti-phosphotyrosine monoclonal antibodies were visualized by autoradiography.

Preparation of Soluble and Cytoskeleton Fractions—Primary cultured hepatocytes at a concentration of 5 x 10⁶ cells/dish were treated with or without EGF (200 ng/ml) or with other hormones for the indicated times and the cells were washed twice with cold microtubule stabilization buffer (MSB) containing 0.1 M Pipes (pH 6.9), 2 mM glycerol, 1 mM EGTA, and 1 mM magnesium acetate. Cytoskeleton and soluble fractions were prepared according to McBride et al. (35), with minor modifications. The cells were collected by centrifugation and resuspended in 0.5 ml of MSB by vortexing for 30 s. The suspension was then layered onto a 1.5 M sucrose cushion and centrifuged at 10,000 x g for 30 min. The supernatant was removed and the cytoskeleton fraction was washed twice with MSB and incubated with 100 μCi/ml [35S]methionine in methionine-free DMEM for 18 h prior to EGF treatment. In some experiments, [35S]methionine-labeled hepatocytes were preincubated overnight with 100 ng/ml PT (31) prior to addition of 200 ng/ml EGF.

Immunoprecipitation—Samples from the soluble or cytoskeleton fractions were prepared by collagenase digestion of perfused livers from fed male Sprague-Dawley rats (180-220 g) by slight modifications of our previously published procedure (3). Cell viability, as determined by trypan blue exclusion was normally >95%. For tissue culture, isolated hepatocytes were plated onto collagen-coated dishes at a density of 2.8 x 10⁴/cm² in DMEM-F-12 medium supplemented with 5% fetal calf serum. The medium was changed after 3 h for removal of dead or nonattached cells and was replaced with DMEM-F-12 supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (5 ng/ml) (31, 37). For [35S]methionine labeling, hepatocytes after attachment were incubated with 25 μCi/ml [35S]methionine in methionine-free DMEM medium for 18 h prior to EGF treatment. In some experiments, [35S]methionine-labeled hepatocytes were preincubated overnight with 100 ng/ml PT (31) prior to addition of 200 ng/ml EGF.

RESULTS

EGF-Induced PLC-γ1 Translocation to the Cytoskeleton—[35S]Methionine-labeled hepatocytes were treated with or without EGF (200 ng/ml) for 1 min, and fractions corresponding to the soluble proteins and the cytoskeleton were prepared as described under “Experimental Procedures.” PLC-γ1 immunoprecipitation was assayed with 50 μCi [3H]phosphatidylinositol 4,5-bisphosphate in mixed phospholipid vesicles with 500 μM phosphatidylylamine (39). The final assay solution (60 μl) contained 20 μM Hepes (pH 7.2), 0.08% sodium cholate, 0.83 mM MgCl₂, 1 mM dithiothreitol, 3 mM EGTA, 0.2 mM EDTA, 30 mM KCl, 20 mM NaCl, and varying concentrations of CaCl₂. The assay was started by the addition of 5-10 μg of protein from the soluble or cytoskeleton fraction. The data represent the mean ± S.E. of three separate experiments with assays performed in triplicate.
the cytoskeleton. Cultured hepatocytes were stimulated without or with 200 ng/ml EGF at indicated times, and the cytoskeleton was prepared as described under "Experimental Procedures." PLC-γ1 in the cytoskeleton fraction was immunoprecipitated and the proteins were separated by SDS-PAGE and transferred to nitrocellulose. Separate lanes in the nitrocellulose membrane were blotted with anti-PLC-γ1 antibodies (lane 1) or goat anti-mouse-horseradish peroxidase second antibodies (lane 2) using a Miniblotter. PLC-γ1 was visualized by ECL.

(lanes 1 and 2) and the cytoskeleton fraction (lanes 3 and 4). The protein bands corresponding to PLC-γ1 in the both fractions were detected by immunoblotting with PLC-γ1 antibodies (Fig. 1B) and excised from the nitrocellulose membrane for subsequent counting. EGF is thus shown to induce a rapid translocation of PLC-γ1 from the soluble to the cytoskeleton fraction in hepatocytes. A change in the relative amounts of PLC-γ1 in the soluble and cytoskeleton fractions is apparent from the intensities of the 32P-labeled (Fig. 1A) and alkaline phosphatase-BCIP/NBT color development (Fig. 1B) in the PLC-γ1 bands. From the counts in the radioactive PLC-γ1 bands, the data show that in the absence of EGF, about 75% of the total PLC-γ1 was in the soluble fraction, whereas 1 min after EGF treatment, about 79% of total PLC-γ1 was found in the cytoskeleton fraction. These data indicate that a little more than 50% of the total PLC-γ1 was transported from the cytosol to the cytoskeleton within 1 min of EGF treatment. A prominent 43-kDa 32P-labeled band in the PLC-γ1 immunoprecipitate of the cytoskeleton, but not the soluble, fraction contained actin as shown by immunoblotting with an actin antibody (see also Fig. 8).

To determine the time course of EGF-induced PLC-γ1 translocation, hepatocytes were treated with EGF (200 ng/ml) for different times followed by isolation of the cytoskeleton fraction. PLC-γ1 was immunoprecipitated and separated by SDS-PAGE as in Fig. 1. The proteins were transferred to nitrocellulose, immuno blotted with PLC-γ1 antibodies (Fig. 2, lane 1) and with a goat anti-mouse second antibody as control (Fig. 2, lane 2) and were visualized by ECL. The results show that the amount of PLC-γ1 translocated to the cytoskeleton fraction increased with increasing incubation times of EGF with a peak being reached after 1 min.

Relationship between EGF-induced Tyrosine Phosphorylation and Translocation of PLC-γ1—In order to define a possible relationship between EGF-induced translocation of PLC-γ1 to the cytoskeleton and its tyrosine phosphorylation, PLC-γ1 was immunoprecipitated from the cytoskeleton fraction, and the nitrocellulose membrane was immunoblotted with anti-P-tyrosine antibodies, followed by visualization of the proteins by ECL (Fig. 3). The protein band corresponding to PLC-γ1 was identified by immunoblotting with anti-PLC-γ1 antibodies. The results in Fig. 3 show that tyrosine-phosphorylated PLC-γ1 could not be detected in the control sample and that EGF increased tyrosine phosphorylation of PLC-γ1 in the cytoskeleton fraction, with a peak being seen after 1 min of EGF stimulation. In contrast, no tyrosine phosphorylation of PLC-γ1 was observed in the soluble fraction after EGF treatment, even though unphosphorylated PLC-γ1 was present (Fig. 4). These results indicate that PLC-γ1 is only associated with the cytoskeleton after it is tyrosine phosphorylated. A second EGF-dependent tyrosine phosphorylated protein with a relative molecular mass of 110 kDa (labeled X in Fig. 3) was also observed in the PLC-γ1 immunoprecipitate (see also Fig. 8).

The Specificity of EGF-induced PLC-γ1 Translocation to the Cytoskeleton—In order to determine whether translocation of PLC-γ1 to the cytoskeleton was specific for EGF stimulation, the effects of EGF were compared with those of vasopressin (a Ca2+-mobilizing hormone) and insulin (a receptor tyrosine kinase with a different substrate specificity). The results in Fig. 5 show immunoblots of PLC-γ1 from the PLC-γ1 immunoprecipitate of the cytoskeleton fraction in control samples and after stimulation with different hormones (lanes 1–5). Lanes 6 and 7 show the effect of pretreatment of hepatocytes with 10 μM genistein for 30 min followed by incubation with or without 200 ng/ml of EGF for 1 min. The results demonstrate that translocation of PLC-γ1 from the soluble to the cytoskeleton fraction is specific to EGF, since a similar translocation was not observed with vasopressin or insulin. Treatment of hepatocytes with genistein, a nonspecific tyrosine kinase inhibitor, blocked the EGF-induced PLC-γ1 translocation, suggesting that translocation of the enzyme to the cytoskeleton is dependent on EGF receptor tyrosine kinase activity.

Relationship of Activation of PLC-γ1 with Its Translocation—To investigate a possible relationship between EGF-stimulated PLC-γ1 translocation and its activation, PLC activity was assayed in both cytoskeleton and soluble fractions. Phospholipase C activity in the cytoskeleton fraction increased after 1 min and subsequently decreased to a level slightly higher than the control (Fig. 6A, open circles). Since the PLC assay measures total PIP2-dependent phospholipase C activity, it was important to determine whether PLC-γ1 was responsible for the increased PLC activity. Consequently, PLC-γ1 was removed from the solubilized cytoskeleton fractions by immunoprecipitation with anti-PLC-γ1 antibodies prior to measuring PLC activity. Results given in Fig. 6A show that the EGF-induced increase of total PLC activity in the cytoskeleton fraction was essentially abolished by this treatment (Fig. 6A, filled circles). As a further control to ascertain the specificity of...
PLC-γ1 activation by EGF, hepatocytes were treated with vasopressin for 1 min, followed by separation and solubilization of the cytoskeleton fraction. PLC-γ1 was removed by immunoprecipitation as before, and the supernatant was used for assay of PLC activity. For this experiment, lane 1 was immunoblotted with antibodies to PLC-β1, and lane 2 was immunoblotted with anti-phosphotyrosine antibodies. The results show that PLC-β1 was present in the cytoskeleton fraction, but that EGF had no effect on either the translocation or tyrosine phosphorylation of this PLC isoform.

**EGF-induced Association of PLC-γ1 with Actin and a 110-kDa Protein**—The role of the cytoskeleton in PLC-γ1 activation was explored further by determining whether actin was able to form a stable complex with PLC-γ1 and whether this was affected by EGF. Fig. 8 shows the results of an experiment in which hepatocytes were treated with 200 ng/ml of EGF for different times up to 10 min, followed by Triton X-100 extraction to obtain the cytoskeleton fraction. The proteins associated with PLC-γ1 were immunoprecipitated with PLC-γ1 antibodies, separated by SDS-PAGE, and transferred to nitrocellulose. The nitrocellulose membrane was blotted with a mixture of anti-actin and a 110-kDa protein antibody (Fig. 8A) or with 200 ng/ml of insulin for 1 min (lane 4), and 200 ng/ml of insulin for 1 min (lane 5), respectively. Lanes 6 and 7 show the effect of pretreatment of hepatocytes with 10 μM genistein for 30 min in the absence (lane 6) and presence (lane 7) of 200 ng/ml of EGF for 1 min. PLC-γ1 in the cytoskeleton fraction was immunoprecipitated with anti-PLC-γ1 antibodies, separated by SDS-PAGE, and transferred to nitrocellulose paper. The nitrocellulose membrane was immunoblotted with anti-phosphotyrosine antibodies. The results of this experiment confirmed that a 110-kDa protein was required to enable PLC-γ1 to be translocated to the cytoskeleton.

**Role of Gi-protein in EGF-induced PLC-γ1 Translocation to the Cytoskeleton**—Our previous results (3, 31) have shown that PT-sensitive Giα is physically associated with PLC-γ1 after EGF stimulation. If Giα is obligatorily involved in PLC-γ1 activation in hepatocytes, it would be expected to be present in the cytoskeleton fraction along with PLC-γ1. Fig. 9 shows the results of an experiment in which hepatocytes were stimulated with EGF, followed by separation of the cytoskeleton fraction. After solubilization of this fraction, Giα antibodies were used for immunoprecipitation, and Giα subunits were detected by ADP-ribosylation using [35S]NAD for visualization on x-ray film. Some Giα was present in the cytoskeleton of control cells (Fig. 9, lane 2), but its amount was greatly increased after 1 min (Fig. 8, lane 4) before diminishing to undetectable amounts after 10 min.

In further experiments, the effects of PT on EGF-induced translocation of PLC-γ1 to the cytoskeleton were investigated to determine whether activation and dissociation of the Gi-protein were required to enable PLC-γ1 to be translocated to the cytoskeleton. [35S]Methionine-labeled hepatocytes were preincubated for 12 h with 100 ng/ml of pertussis toxin prior to addition of 200 ng/ml of EGF (Fig. 10, lanes 3 and 4). The cytoskeleton fraction was collected and PLC-γ1 was immunoprecipitated with PLC-γ1 antibodies (Fig. 10A), followed by subsequent immunoblotting of the nitrocellulose membrane with PLC-γ1 antibodies (Fig. 10B). Lanes 1 and 2 of Fig. 10 are controls run in the absence and presence of EGF, respectively. Since PT did not prevent EGF-induced translocation of PLC-γ1 to the cytoskeleton, the data suggest that the interaction of PLC-γ1 with the Gi-protein is downstream from PLC-γ1 translocation or tyrosine phosphorylation.

**DISCUSSION**

Our previous work with rat hepatocytes has shown that EGF promotes the association of a PT-sensitive Gi-protein with both PLC-γ1 and the EGF receptor (3, 31). These studies also showed that PT blocked the EGF-induced stimulation of phosphoinositide breakdown but did not affect tyrosine phosphorylation of PLC-γ1. The major conclusions from these studies were that a PT-sensitive Gi-protein coupled the EGF receptor to stimulation of PLC activity in rat hepatocytes and that tyrosine phosphorylation of PLC-γ1 could be dissociated from PLC activation. These conclusions are different from current dogma, which is based on studies using transformed cells with overexpressed EGF receptors. Thus, it is generally considered that activation of PLC-γ1 by receptor tyrosine kinases is mediated directly by tyrosine phosphorylation of the enzyme without the involvement of a G-protein (40, 41). There are, however, a number of reports in the older literature that were interpreted as showing participation of a G-protein in coupling certain growth factor receptors to signal transduction pathways (for review, see Refs. 3 and 26). Nevertheless these findings have been largely ignored as the story linking tyrosine phosphorylation of PLC-γ1 and other signaling molecules to activation of growth factor receptors unfolded. An interesting possibility is that the different conclusions drawn from studies with a variety of cell types may reflect fundamental differences between normal and transformed cells for the regulation of cell growth. In either case, a common unsolved problem is how...
EGF-Induced Translocation of PLC-γ₁ to the Cytoskeleton

**Fig. 6. Changes of PLC activity in the cytoskeleton.** Hepatocytes were treated with 200 ng/ml EGF for different times (A) or 100 nM vasopressin for 1 min (B), and cytoskeleton fractions were collected as described under "Experimental Procedures." Samples (10 μg) of cytoskeleton protein were removed at each time point and assayed for PLC activity, as shown by the open circles in A and the open bars in B. PLC activity of the cytoskeleton fraction after removing PLC-γ₁ with anti-PLC-γ₁ antibodies is shown by the filled circles (A) and shaded bars (B). Values plotted in the y axis are the ratio of experimental to control Ins-1,4,5-P₃ production. The results are the means of three separate experiments.

**Fig. 7. Lack of effects of EGF on PLC-β₁ translocation and tyrosine phosphorylation in the cytoskeleton.** Samples were prepared as in Fig. 2. PLC-β₁ in the cytoskeleton fraction was immunoprecipitated with PLC-β₁ antibodies. PLC-β₁ in the nitrocellulose paper was blotted either with anti-PLC-β₁ (lane 1) or anti-Tyr(P) (lane 2) antibodies and visualized by ECL.

**Fig. 8. Association of actin and 110 kDa protein with PLC-γ₁ in the cytoskeleton.** Hepatocytes were treated with EGF for different times, and the cytoskeleton was collected as described previously. Proteins associated with PLC-γ₁ were detected by immunoprecipitation with PLC-γ₁ antibodies and immunoblotting first with anti-actin antibody (A) and then with PLC-γ₁ antibodies without stripping off the actin activity (B).

EGF-induced change in the amount of Gα in the cytoskeleton. Hepatocytes were treated with or without 200 ng/ml of EGF, and the cytoskeleton was collected. Gα subunits in the cytoskeleton were immunoprecipitated with Gα antibody, and the immunocomplexes were used for in vitro ADP-ribosylation with [¹²⁵I]NAD.

**Fig. 9. Effects of PT on PLC-γ₁ translocation to the cytoskeleton.** [³⁵S]Methionine-labeled hepatocytes were preincubated overnight with 100 ng/ml pertussis toxin (PT) (lanes 3 and 4) prior to addition of 200 ng/ml EGF. The cytoskeleton fraction was collected, and PLC-γ₁ was immunoprecipitated with PLC-γ₁ antibodies. A shows a radioautograph, whereas B is an immunoblot of the nitrocellulose membrane using PLC-γ₁ antibodies.
EGF-Induced Translocation of PLC-γ1 to the Cytoskeleton

7161

actin filaments in A-431 cells (33, 34, 43). EGF also increases the number of cytoskeleton-associated EGF receptors (44, 45). An active role for the cytoskeleton in EGF-mediated signal transduction is also suggested from the work of Payraudeau et al. (46) who demonstrated that PLC and several lipid kinases become associated with the cytoskeleton of A-431 cells upon EGF stimulation. Actin filaments make frequent contact with plasma membrane actin binding proteins at focal contact sites and an association of PLC-γ1 with the actin cytoskeleton, as observed by immunofluorescence techniques (35), may provide a physical mechanism to promote interaction of an activated PLC-γ1 with its lipid substrate and kinetic modifiers. However, in the study of McBride et al. (35) the authors were unable to demonstrate that the association of PLC-γ1 with actin filaments was EGF-dependent.

Data presented in this paper show that addition of EGF to hepatocytes induces a rapid translocation of about half the soluble PLC-γ1 to the cytoskeleton and that this process correlates kinetically with an increase in PLC activity attributable to PLC-γ1, tyrosine phosphorylation of PLC-γ1, and to an increase in the proportion of Gia associated with the cytoskeleton. Since no tyrosine-phosphorylated PLC-γ1 could be detected in the soluble fraction before or after EGF treatment, it is reasonable to assume that once PLC-γ1 becomes tyrosine-phosphorylated it associates rapidly with the cytoskeleton.

Structural motifs within the amino acid sequence of PLC-γ1 as well as tyrosine-phosphorylated sites are likely to account for its interaction with other proteins. PLC-γ1, together with a number of other proteins involved in signal transduction, contain conserved noncatalytic domains termed SH2 homology, SH2 and SH3 regions (47, 48). The SH2 domains function as adaptor molecules that bind with high affinity to specific tyrosine residues of a variety of autophosphorylated growth factor receptors, including PDGF, EGF, and fibroblast growth factor (49). Located near specific phosphorylated tyrosine residues within the COOH-terminal region of the receptor, there is a short peptide sequence that determines the specificity of binding to proteins containing particular SH2 motifs (50). PLC-γ1, like a number of other proteins containing SH2 domains, also possesses a distinct sequence termed the SH3 domain, which contains a region of about 60 amino acids having a high homology between different proteins (51). SH3-like sequences have been identified in a variety of proteins that comprise or associate with the cytoskeleton (52), and the SH3 domain of PLC-γ1 has recently been shown to account for the interaction of PLC-γ1 with the cytoskeleton (53). By microinjection of glutathione S-transferase fusion proteins encoding the SH3 and SH2 domains of PLC-γ1 into rat embryo fibroblasts (REF-52), and using immunofluorescence microscopy for determining their cellular distribution, it was demonstrated that the SH3 domain of PLC-γ1 was responsible for targeting it to the actin microfilament network (53). The SH3 binding sites for PLC-γ1 in the cytoskeleton have not been identified, but two proteins (3BPL and 3BP2) that bind specifically to the SH3 domain of the tyrosine kinase product of the abl proto-oncogene have been isolated and their SH3 binding sites were localized to 9-10-amino acid stretches especially rich in proline (54).

Binding of the SH3 domain of PLC-γ1 to components of the actin cytoskeleton in hepatocytes may account for the association of a fraction of the total PLC-γ1 (approximately 25% from the data in Fig. 1) with the cytoskeleton under control conditions and may explain the localization of PLC-γ1 along the actin filaments rather than in the plasma membrane in the study by McBride et al. (35). However, other mechanisms must account for the EGF-dependent translocation of PLC-γ1 to the cytoskeleton and its accompanying enzymatic activation. Tyrosine phosphorylation of PLC-γ1 appears to be a necessary requirement, since genistein pretreatment of the hepatocytes abolished EGF-induced translocation of PLC-γ1 to the cytoskeleton (Fig. 5) as well as its activation (4). No tyrosine-phosphorylated PLC-γ1 could be detected in the Triton X-100-soluble fraction of the hepatocyte after EGF treatment (Fig. 4), suggesting that once it is phosphorylated by the activated EGF receptor tyrosine kinase, it rapidly becomes associated with the actin cytoskeleton. Possible mechanisms in addition to the binding of SH3 domains of PLC-γ1 to the cytoskeleton that could account for the translocation of tyrosine phosphorylated PLC-γ1 to the cytoskeleton include the specific interaction of a tyrosine phosphorylated residue of PLC-γ1 with the SH2 domain of an actin binding protein. Alternatively, the SH2 domains of PLC-γ1 may bind to a tyrosine-phosphorylated cytoskeleton protein. The latter possibility may be the more likely one in hepatocytes, since a 110-kDa protein is present in the PLC-γ1 immunoprecipitate of the cytoskeleton fraction that is recognized by actin antibodies (Fig. 8) and becomes transiently tyrosine-phosphorylated after EGF treatment (Fig. 3).

Activation of PLC-γ1 by EGF, at least in hepatocytes, requires its interaction with a G-protein subunit. According to the data in Fig. 6, activation of PLC-γ1 occurs after it is translocated to the cytoskeleton. Significantly, the amount of Gia in the cytoskeleton fraction of hepatocytes increased after EGF treatment with similar kinetics to the increase in amount of tyrosine-phosphorylated PLC-γ1 as well as its activity in the cytoskeleton (cf. Figs. 2, 3, 6, and 9). The involvement of other mechanisms for PLC-γ1 activation, including its interaction with profilin, remain speculative (55, 56), but a number of reports are in agreement that tyrosine phosphorylation of PLC-γ1 can be dissociated from activation of the enzyme (57-59).

In summary, we can conclude from the present studies that EGF-induced activation of PLC-γ1 in hepatocytes requires not only tyrosine phosphorylation of PLC-γ1, but also its translocation from the cytosol to the cytoskeleton where it interacts with activated Gia subunits. Tyrosine phosphorylation of PLC-γ1 appears to promote its association with actin components of the cytoskeleton, but is not sufficient to produce enzyme activation.

Acknowledgments—We thank Xi-Chen Yang and Vadim Gurvitz for technical assistance and Dr. David Manning for the Gia antibody.

REFERENCES
