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A Site of Tyrosine Phosphorylation in the C Terminus of the Epidermal Growth Factor Receptor Is Required To Activate Phospholipase C

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Cells expressing mutant epidermal growth factor (EGF) receptors have been used to study mechanisms through which EGF increases phospholipase C (PLC) activity. C-terminal truncation mutant EGF receptors are markedly impaired in their ability to increase inositol phosphate formation compared with wild-type EGF receptors. Mutation of the single tyrosine self-phosphorylation site at residue 992 to phenylalanine in an EGF receptor truncated at residue 1000 abolished the ability of EGF to increase inositol phosphate formation. C-terminal deletion mutant receptors that are impaired in their ability to increase inositol phosphate formation effectively phosphorylate PLC-γ at the same tyrosine residues as do wild-type EGF receptors. EGF enhances PLC-γ association with wild-type EGF receptors but not with mutant receptors lacking sites of tyrosine phosphorylation. These results indicate that formation of a complex between self-phosphorylated EGF receptors and PLC-γ is necessary for enzyme activation in vivo. We propose that both binding of PLC-γ to activated EGF receptors and tyrosine phosphorylation of the enzyme are necessary to elicit biological responses. Kinase-active EGF receptors lacking sites of tyrosine phosphorylation are unable to signal increased inositol phosphate formation and increases in cytosolic Ca^{2+} concentration.

The intrinsic protein tyrosine kinase activity of the epidermal growth factor (EGF) receptor is essential for the diverse biochemical responses of cells to the growth factor (8, 15). Stimulation of the phosphatidylinositol pathway to generate the second messengers inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol is a prominent early response to EGF (14, 41) that is dependent on the tyrosine kinase activity of the receptor (32). Phospholipase C-γ (PLC-γ), a member of the family of enzymes catalyzing hydrolysis of phosphatidylinositol bisphosphate to IP_3 and 1,2-diacylglycerol (38), is phosphorylated on tyrosine residues by ligand-activated EGF and platelet-derived growth factor (PDGF) receptors in vivo and in vitro (26, 30, 35, 42). The major identified sites of tyrosine phosphorylation are residues 771, 783, and 1254 with a minor site at 472 in PLC-γ (19, 43). Analysis of site-directed mutation of these residues indicates that Y783 and to a lesser extent Y1254 are essential to PDGF-stimulated inositol phospholipid hydrolysis in vivo (18). In vitro studies indicate that phosphorylated PLC-γ is more active when assayed under carefully defined conditions with Triton X-100 or profilin that may sequester the substrate (13, 34). However, under standard conditions without profilin or Triton X-100, results of in vitro assays of the activity of normal and PLC enzymes mutated to contain phenylalanine replacements of tyrosine at residues 771, 783, and 1254 from control and PDGF-treated cells were similar (18). Conditions that are necessary in vivo are incompletely defined, but these results suggest that factors additional to tyrosine phosphorylation may be necessary to couple ligand-activated growth factor receptors to PLC-γ activation.

Analysis of mutant EGF receptors revealed that deletion of 213 to 228 carboxyl-terminal amino acids abolished EGF-stimulated increases in cytosolic calcium concentrations ($[Ca^{2+}]_i$) (7). These C-truncated EGF receptors exhibited enhanced ligand-dependent protein tyrosine kinase activity in vivo (45) and enhanced mitogenic activity (46). The failure to increase $[Ca^{2+}]_i$ suggested deletion of sequences essential for coupling activated EGF receptors to the phosphatidylinositol pathway. This could result from failure to phosphorylate PLC-γ or altered association of PLC-γ with activated kinase-active EGF receptors. Studies by Mayer and coworkers (27–29) indicate that regions homologous to those present in $p60^{src}$ (*src* homology regions, SH2 domains) interact with tyrosine-phosphorylated and surrounding residues. Such interactions appear to account for the association of PLC-γ, which contains two SH2 domains (38), with ligand-activated but not with control or kinase-inactive EGF and PDGF receptors (1, 24, 25, 33). The identified sites of tyrosine self-phosphorylation in the EGF receptor are located in the C terminus distal to the kinase domain at residues 992, 1068, 1086, 1148, and 1173 (10, 16, 45).

To investigate the mechanisms through which ligand-activated EGF receptors couple to the phosphatidylinositol signaling pathway, we have analyzed the ability of normal and mutant EGF receptors to stimulate inositol phosphate production and to catalyze tyrosine phosphorylation of PLC-γ in vivo. Mutations in the C terminus that remove EGF receptor self-phosphorylation sites severely impair inositol phosphate production and association of PLC-γ with ligand-activated EGF receptors. These truncated receptors efficiently catalyze tyrosine phosphorylation of PLC-γ in vivo, indicating that phosphorylation of PLC-γ alone is not sufficient for its activation. Interaction of PLC-γ with the self-phosphorylated EGF receptor appears required for effi-

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cient in vivo coupling of these two signal transduction pathways.

MATERIALS AND METHODS

Preparation of clonal cell lines expressing mutant EGF receptors. EGF receptor cDNA corresponding to the human wild-type receptor or mutated to contain the indicated carboxyl-terminal truncations or substitutions were placed in the pXER expression vector, which contains a mutant dihydrofolate reductase gene as a selectable marker (8). A truncation mutant (c'1000) was created by removing the coding sequence for the C-terminal 186 amino acids of the EGF receptor. The cDNA for c'1000 F-992 was prepared by site-directed mutagenesis according to the method of Kunkel (22). All mutant sequences were confirmed by the dideoxy chain termination procedure. cDNAs were transfected into recipient mouse B82L cells, which lack endogenous EGF receptor mRNA and protein (7). Permanent clonal transfectants were selected, and the EGF receptor gene was amplified by increasing concentrations of methotrexate from 400 nM to 5 μM. Two or more clonal lines were established for each mutant EGF receptor.

Measurement of inositol phosphate production. Cells were grown in 6-cm plates containing Dulbecco modified Eagle medium, 5% dialyzed newborn calf serum, and 5 μM methotrexate. At 24 h before the cells were collected, the medium was replaced with 2 ml of medium containing 5 μCi of [^{3}H]myoinositol per ml. Cells were treated with 20 mM LiCl for 20 min followed by bovine serum albumin (BSA) as control, EGF (100 nM), vasopressin (10 μM), ATP (10 μM), or GTP (10 μM) for the times indicated at 37°C. LiCl was used to increase the inositol phosphate signal by inhibiting inositol phosphate and inositol bisphosphate phosphatases (4). Vasopressin, ATP, or GTP was used to verify the ability of cells to increase inositol phosphate formation. Cells were then washed with phosphate-buffered saline (PBS), and the cells were collected with 10% trichloroacetic acid. The trichloroacetic acid supernatants were extracted with ether, neutralized to pH 7.5, and fractionated on Dowex columns (3). The columns were washed with H_2O and 60 mM sodium formate before the inositol phosphates were eluted with 0.2 M ammonium formate–100 mM formic acid (inositol phosphate), 0.4 M ammonium formate–100 mM formic acid (inositol bisphosphate), and 1 M ammonium formate–100 mM formic acid (IP_3 and inositol tetraphosphate). Inositol phosphate separations were confirmed by using tritium-labeled inositol phosphate standards (New England Nuclear). Samples were counted by diluting them with Aquamix and adding the tubes to a Beckman scintillation counter. Each datum point in Fig. 1 and 2 represents triplicate samples. Inositol phosphates were verified by high-performance liquid chromatography (2).

Measurement of tyrosine phosphorylation of PLC-γ. Cells were collected with radioimmunoprecipitation assay buffer (0.1% sodium dodecyl sulfate [SDS], 1% deoxycholate, 1% Nonidet P-40, 20 mM sodium phosphate [pH 7.2], 150 mM NaCl, 2 mM EDTA) after 5 min of treatment with either BSA (1 μg/ml) or EGF (100 nM). The extracts were cleared with immobilized staphylococcus A before immunoprecipitation with PLC-γ monoclonal antibodies (39). The sample pellets were washed twice with radioimmunoprecipitation assay buffer, once with 0.5 M NaCl, and once with H_2O. The samples were solubilized with Laemmli sample buffer, run on SDS–7.5% polyacrylamide gel electrophoresis (PAGE) gels, and transferred onto Immobilon membranes. PLC-γ protein levels were determined by incubating the blot with a mixture of PLC-γ monoclonal antibodies and developed with goat anti-mouse antibody conjugated with alkaline phosphatase. Protein phosphotyrosine levels were determined on the same blot by incubation with ^{125}I-labeled anti-phosphotyrosine monoclonal antibody PY20 (12) and autoradiography. Densitometry was performed on both the colorimetric immunostained PLC-γ and the phosphotyrosine autoradiogram developed after various times under linear conditions of both color and radioactivity. Levels of PLC-γ tyrosine phosphorylation were determined in relation to the amount of PLC-γ present.

Two-dimensional tryptic phosphopeptide mapping. Solubilized wild-type and c'973 EGF receptors were incubated with 100 nM EGF at 25°C for 20 min. Purified PLC-γ (2.6 μg) was then added with the kinase assay mixture (20 mM HEPES [*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4], 100 μM Na_3VO_4, 2 mM $MnCl_2$, 10 mM $MgCl_2$, 10 μM ATP, 10 μCi [γ-^{32}P]ATP [2×10^5 cpm pmol^{-1}]) in a final assay volume of 25 μl. The samples were incubated for 5 min at 25°C, the reaction was stopped by the addition of Laemmli sample buffer, and the proteins were separated on an SDS–7.5% PAGE gel. PLC-γ, identified by autoradiography, was eluted from the gel with 0.3% ammonium bicarbonate–0.3% SDS–2.3 M β-mercaptoethanol (5). The protein was oxidized with performic acid and treated overnight with 1 mg of trypsin per ml. After trypsinization, the samples were washed and resuspended in 2.5% formic acid–7.5% acetic acid, pH 1.9. The samples were loaded on a cellulose thin-layer chromatography plate (Kodak) and electrophoresed in the first dimension at 1 kV for 30 min at pH 1.9 followed by thin-layer chromatography in the second dimension for 5 h in *n*-butanol–pyridine–acetic acid (38:25:7.5). Phosphoamino acid analysis confirmed that phosphotyrosine was the only phosphoamino acid present.

Coimmunoprecipitation. After EGF treatment, cells were washed with PBS and collected by the addition of solubilization buffer containing 1% Nonidet P-40, 20 mM Tris [pH 8.0], 137 mM NaCl, 10% glycerol, 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 100 μM sodium vanadate, and 10 μM ammonium molybdate. The extracts were cleared by incubation with Sepharose beads, and the supernatant was incubated with a monoclonal antibody directed against the extracellular portion of the EGF receptor (528 immunoglobulin G) (11) coupled to Sepharose beads. The 528 immunoglobulin G-Sepharose beads were washed three times with the solubilization buffer and once with 10 mM Tris, pH 7.4. Proteins were solubilized with Laemmli sample buffer, separated on SDS–7.5% PAGE gels, and transferred onto Immobilon membranes. Blots were stained either with a mixture of PLC-γ monoclonal antibodies or with a rabbit polyclonal antibody directed against the amino-terminal 13 amino acids of the EGF receptor.

RESULTS

In vivo activation of phospholipase C in cells expressing wild-type and mutant EGF receptors. Addition of EGF to B82 cells expressing wild-type EGF receptors rapidly increased $[Ca^{2+}]_i$ ~fourfold from 0.2 to 0.7 to 0.9 μM with a peak response at ~20 s (8). In contrast, EGF failed to increase $[Ca^{2+}]_i$ in B82 cells expressing kinase-inactive (M-721) or kinase-active EGF receptors C-terminally truncated to residue 973 (c'973) or 958 (c'958) (7). Because EGF is reported to stimulate PLC-γ activity, the effect of EGF on inositol phosphate formation was measured to determine whether

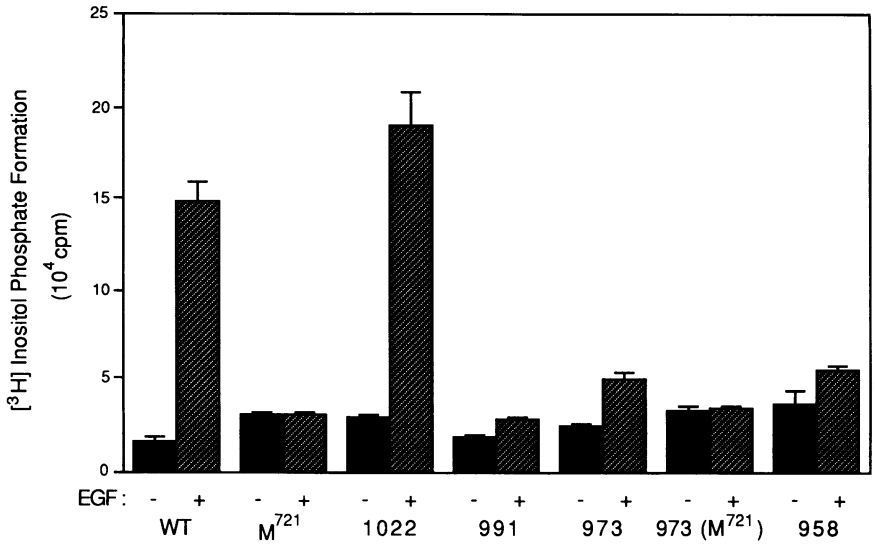

FIG. 1. EGF-stimulated inositol phosphate formation in cells expressing mutant EGF receptors. Inositol phosphates were measured in [^{3}H]myoinositol-labeled cells expressing the indicated mutant EGF receptors (WT, wild type). Cells were treated with 20 mM LiCl for 20 min prior to addition of 100 nM EGF for 30 min. Data represent triplicate dishes of each cell type from two experiments in which various cell types were analyzed in parallel. Similar results were reproducibly observed in multiple experiments.

the differences in $[Ca^{2+}]_i$ responses were correlated with changes in in vivo PLC-γ activity. Figure 1 shows that in cells expressing kinase-active wild-type EGF receptors, EGF increased inositol phosphate formation up to eightfold. EGF had no effect on inositol phosphate formation in cells expressing kinase-inactive M-721 EGF receptors, confirming that tyrosine kinase is essential for this biological effect (32). EGF also strongly increased inositol phosphate formation in cells expressing c'1022 EGF receptors. In contrast, EGF-stimulated inositol phosphate formation was significantly less in cells expressing c'991, c'973, and c'958 EGF receptors, showing a less than twofold increase in total inositol phosphate formation. Cells expressing kinase-inactive M-721 c'973 EGF receptors showed no inositol phosphate response to EGF, indicating that the rise in inositol phosphates in cells expressing c'973 EGF receptors, although markedly diminished, reflected the tyrosine kinase activity of this receptor (Fig. 1). All cells equally increased inositol phosphate formation in response to a mixture of fetal calf serum and 10 μM vasopressin or 10 μM ATP or 10 μM GTP, indicating that the ability to respond was equivalent in all B82 cell clones (data not shown).

A summary of the range of effects of EGF on total inositol phosphate formation in B82 cells expressing wild-type and mutant receptors is shown in Table 1. Data are pooled from 10 separate experiments in which inositol phosphate responses were measured in parallel to compare various cell lines; each experiment contained cells expressing either wild-type or c'1022 EGF receptors as a positive control. These results indicate that wild-type and c'1022 EGF receptors which contain sites of tyrosine self-phosphorylation effectively increase PLC-γ activity measured as in vivo formation of inositol phosphates. EGF receptors which lack self-phosphorylation sites and do not undergo EGF-stimulated tyrosine phosphorylation in vivo (45) have significantly decreased EGF-stimulated inositol phosphate formation to <20% of that measured in parallel in cells expressing wild-type and c'1022 EGF receptors.

TABLE 1. Summary of EGF-stimulated inositol phosphate formation in B82 cells expressing mutant EGF receptors

EGF receptor	Inositol phosphate formation (fold increase[a] ± SEM)
Wild type (c'1186)	5.4 ± 1.6
M-721 wild type (kinase inactive)	1.0 ± 0.0
c'1022	4.7 ± 1.0
c'991	1.3 ± 0.2
c'973	1.9 ± 0.5
c'958	1.8 ± 0.2

[a] EGF-induced increase for 10 separate experiments.

The decreases in inositol phosphate formation in cells expressing EGF receptors that lack self-phosphorylation sites were not due to decreased EGF receptor expression or activity. Cells express $\sim 1.1 \times 10^5$, 2.5×10^5, 2.3×10^6, and 1.6×10^6 EGF receptors cell^{-1} in clonal lines with wild-type c'991, c'973, and c'958 EGF receptors, respectively. c'973 receptors exhibit enhanced in vivo tyrosine kinase activity and stimulation of growth (45, 46). The ability of EGF to increase inositol phosphate formation in cells expressing c'1022 EGF receptors to the same extent as in cells expressing wild-type receptors may be due to the fact that ~10-fold higher numbers of c'1022 EGF receptors are expressed (1.4×10^6 versus 1.1×10^5 EGF receptors cell^{-1}, respectively). The increased expression of a receptor (c'1022) with a single tyrosine self-phosphorylation site resulted in effects equivalent to those seen with lower concentrations of wild-type EGF receptors containing multiple tyrosine self-phosphorylation sites. c'973 and c'958 EGF receptors are expressed at equally high concentrations as c'1022 EGF receptors but exhibit a markedly impaired ability to increase inositol phosphate formation.

To more critically examine the correlation between EGF receptor self-phosphorylation and in vivo activation of PLC-γ, cells expressing c'1000 and c'1000 F-992 EGF receptors were compared. Deletion of sequences between residues 1022 and 991 removed the single site of tyrosine

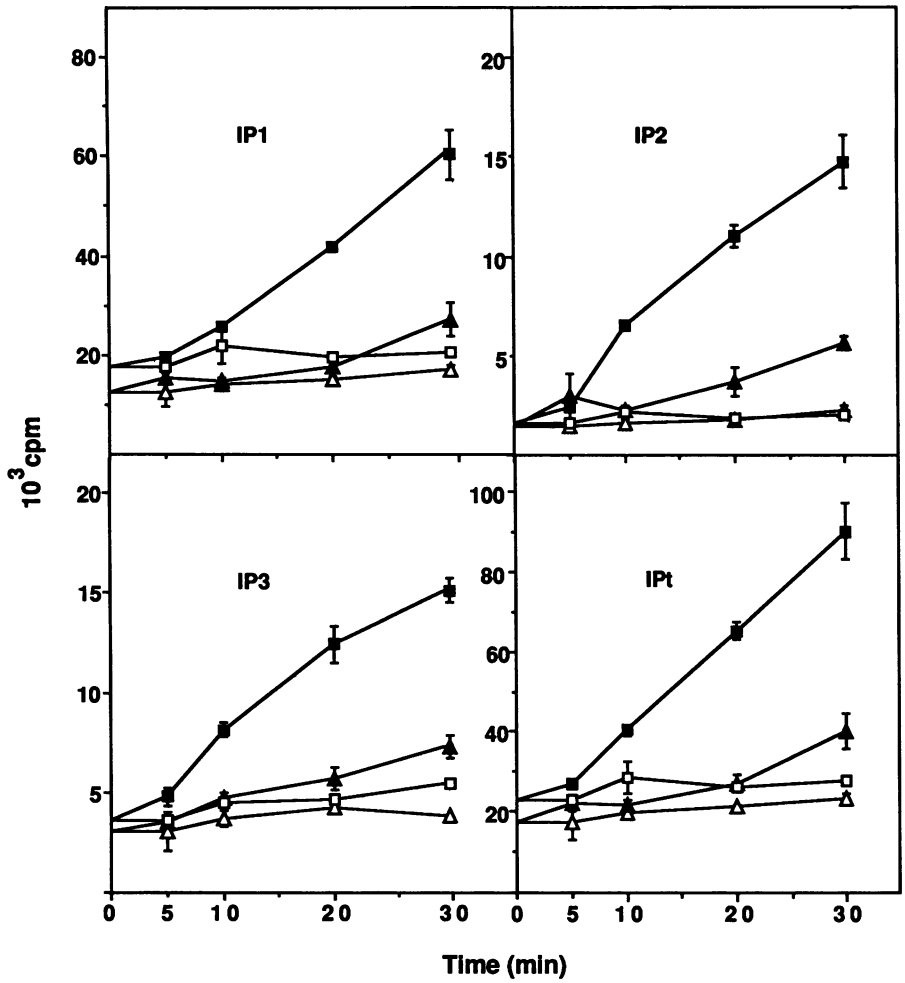

FIG. 2. Kinetics of formation of inositol phosphates in cells expressing c'1000 and c'1000 F-992 EGF receptors. Cells were labeled with ^{3}H-myoinositol for 24 h and treated with 20 mM LiCl for 20 min and 100 nM EGF for the indicated times at 37°C. Inositol phosphates were measured in triplicate dishes under each experimental condition. Data are for cells expressing c'1000 EGF receptors treated without (□) or with (■) EGF and cells expressing c'1000 F-992 EGF receptors treated without (△) or with (▲) EGF. IPt, total inositol phosphates.

self-phosphorylation at residue 992 (45) and severely impaired EGF-stimulated inositol phosphate formation. Replacement of Tyr-992 with Phe also removed this site of self-phosphorylation, yielding a kinase-active EGF receptor that does not undergo in vivo self-phosphorylation (data not shown). Figure 2 shows that EGF effectively stimulated formation of each of the measured inositol phosphates in cells expressing c'1000 EGF receptors. IP_3 was significantly increased by 5 to 10 min following addition of EGF and was increased ~fivefold at 30 min. In contrast, EGF stimulated IP_3 accumulation minimally in cells expressing c'1000 F-992 receptors at all times assayed. Proportional changes were seen in inositol phosphate, inositol bisphosphate, and total inositol phosphates. Scatchard analysis indicated similar expression of EGF receptors in the two cell types (2.5×10^6 and 1.6×10^6 c'1000 and c'1000F-992 EGF receptors cell^{-1}, respectively). Because these two receptors differ only at residue 992, it appears that a site of tyrosine self-phosphorylation is essential for activation of PLC-γ by EGF receptors in vivo.

In vivo tyrosine phosphorylation of PLC-γ. Because tyrosine phosphorylation of PLC-γ is associated with enzyme activation in response to EGF and PDGF (43, 44), one explanation for the diminished inositol phosphate formation in cells expressing c'973 EGF receptors is an inability of this receptor to phosphorylate PLC-γ on tyrosine residues. To investigate this possibility, cells expressing wild-type or c'973 EGF receptors were treated with EGF and PLC-γ was isolated by immunoprecipitation with a mixture of monoclonal antibodies (39). Immunoisolated PLC-γ was resolved on SDS-PAGE gels, transferred to Immobilon membranes, and analyzed by immunoblotting with monoclonal PLC-γ and phosphotyrosine antibodies. This procedure allowed comparison of the relative in vivo tyrosine phosphorylation per unit of mass of PLC-γ. Figure 3 shows that EGF effectively stimulated tyrosine phosphorylation of PLC-γ in cells expressing either wild-type or c'973 EGF receptors. Comparison of the results of densitometric scanning of the autoradiogram of ^{125}I-labeled antiphosphotyrosine antibody staining with the mass of PLC-γ quantitated by colorimetric immunostaining showed that EGF caused a 5.2-fold increase in tyrosine phosphorylation of PLC-γ in cells expressing c'973 EGF receptors compared with a 3.6-fold increase in cells expressing wild-type EGF receptors. The reproducibly greater increase in EGF-stimulated tyrosine phosphorylation of PLC-γ in cells expressing c'973 EGF receptors is in agreement with the

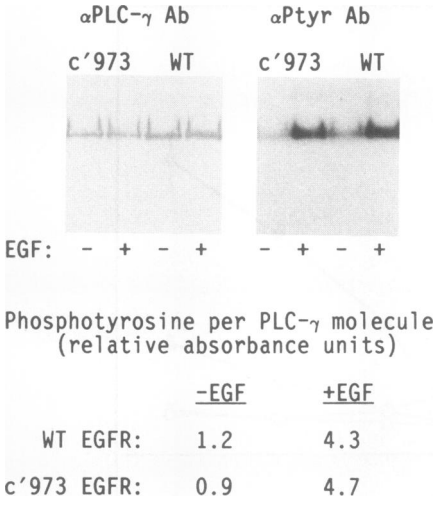

FIG. 3. In vivo tyrosine phosphorylation of PLC-γ by wild-type (WT) and c'973 EGF receptors. Cells were incubated without or with 100 nM EGF for 5 min. PLC-γ was immunoprecipitated with a mixture of six monoclonal antibodies, and immunoprecipitates were analyzed following SDS-PAGE and transfer to Immobilon membranes. PLC-γ was immunostained with monoclonal anti-PLC-γ antibody and detected by using a second antibody conjugated with alkaline phosphatase (αPLC-γ Ab); phosphotyrosine was detected by using ^{125}I-labeled monoclonal antibody PY-20 (αPTyr Ab). The lower panel shows the results of densitometry of blots carried out under linear conditions of detection.

enhanced in vivo tyrosine kinase activity of this receptor (45). EGF-dependent increases in tyrosine phosphorylation of PLC-γ were also similar (three- to fivefold) in cells expressing c'958 and c'1000 F-992 EGF receptors in which PLC-γ activity is not increased and in cells expressing c'1022 and c'1000 EGF receptors in which PLC-γ activity is increased (data not shown). These results indicate that the defect in PLC-γ activation is not due to diminished tyrosine phosphorylation by activated EGF receptors. Rather, tyrosine-phosphorylated PLC-γ is less active in cells expressing EGF receptors which lack a site of tyrosine self-phosphorylation than in cells expressing EGF receptors which contain a site of tyrosine self-phosphorylation.

Comparison of tryptic phosphopeptide maps of PLC-γ phosphorylated by wild-type and c'973 EGF receptors. Because specific sites of tyrosine phosphorylation of PLC-γ appear essential to growth factor activation of the enzyme (18), the sites of tyrosine phosphorylation of purified PLC-γ catalyzed by wild-type and c'973 EGF receptors were compared. Two-dimensional tryptic phosphopeptide maps of PLC-γ indicate that both wild-type and c'973 EGF receptors catalyze tyrosine phosphorylation at the same sites (Fig. 4). Peptide a contains tyrosine 783, peptide b represents an incompletely digested peptide containing both tyrosine 771 and tyrosine 783, and peptide c contains tyrosine 1254 (18). Mixing the two tryptic digestions prior to separation confirmed the identity of the sites of tyrosine phosphorylation (Fig. 4C). Further confirmation was provided by comigration with electrophoresis at pH 7.2 rather than 1.9 in the first dimension (data not shown). These in vitro studies indicate that the wild-type and c'973 EGF receptors catalyze tyrosine phosphorylation of PLC-γ on the same sites. Although insufficient material was available for comparison of in vivo phosphorylated sites, previous studies indicated strict concordance between in vivo and in vitro sites of tyrosine phosphorylation of PLC-γ (18, 19, 42).

Comparison of the association of PLC-γ with activated wild-type and c'973 EGF receptors in vivo. Because tyrosine phosphorylation of PLC-γ was equivalent in cells expressing wild-type and c'973 EGF receptors, we examined the interaction between PLC-γ and ligand-activated EGF receptors. Cells were treated with EGF, and EGF receptors were isolated by using a monoclonal antibody. Immunoisolated receptors were analyzed by SDS-PAGE, transfer to Immobilon, and immunostaining with both a monoclonal anti-PLC-γ antibody and an anti-EGF receptor peptide antibody. As shown in Fig. 5, PLC-γ is associated with ligand-activated wild-type EGF receptors. The slower migration of wild-type EGF receptors from EGF-treated cells shown in panel B is due to receptor self-phosphorylation. In contrast, EGF did not enhance association of PLC-γ with c'973 EGF receptors (panel A). The small amount of PLC-γ detected from untreated wild-type and from c'973 EGF receptor-expressing cells is nonspecific because these low levels of association are also seen in immunoprecipitates from cells that express c'688 EGF receptors that contain only 40 amino acids of the cytoplasmic domain. Equivalent amounts of EGF receptor were loaded (Fig. 5B), indicating that PLC-γ specifically associates with the self-phosphorylated C terminus of the EGF receptor. There is thus a direct correlation between in vivo activation of PLC-γ and its association with the EGF receptor.

DISCUSSION

PLC-γ is a well-defined in vivo substrate for ligand-activated EGF and PDGF receptors (26, 30, 35, 43). The correlation between in vivo PLC-γ activation and tyrosine phosphorylation of PLC-γ suggested that this covalent modification resulted in enzyme activation. Mutation of tyrosine-phosphorylated residues 783 and 1254 in PLC-γ abolished the ability of ligand-activated PDGF receptors to stimulate activity of transfected mutant PLC-γ in vivo, supporting an essential role for tyrosine phosphorylation in PLC-γ activation (18).

The present results indicate that a site of tyrosine phosphorylation in the EGF receptor is necessary for EGF to activate PLC-γ activity in vivo. Removal of the five identified sites of tyrosine self-phosphorylation in the C terminus of the EGF receptor (c'991, c'973, and c'958 EGF receptors) markedly impaired the ability of EGF to stimulate inositol phosphate formation. The necessity for at least one site of tyrosine self-phosphorylation was demonstrated by mutating Tyr-992 to Phe in a C-terminal truncation mutant EGF receptor containing only this proximal self-phosphorylation site (c'1000). This mutation (c'1000 F-992) severely impaired the ability of EGF to increase inositol phosphate formation in vivo. Although the four additional sites of tyrosine self-phosphorylation in the C terminus of wild-type EGF receptors may similarly function to couple activated EGF receptors to PLC-γ in vivo, the single site of tyrosine self-phosphorylation at Tyr-992 is sufficient. A mutant EGF receptor in which residues 959 to 1023 are deleted retains tyrosine self-phosphorylation of C-terminal tyrosines and increases $[Ca^{2+}]_i$ to the same extent (70%) as c'1022 EGF receptors which contain a single tyrosine self-phosphorylation site at residue 992 (5a). These results suggest that other sites of tyrosine self-phosphorylation may serve the same function as Tyr-992. Kinase-active C-terminal deletion mutant EGF receptors which fail to self-phosphorylate in vivo

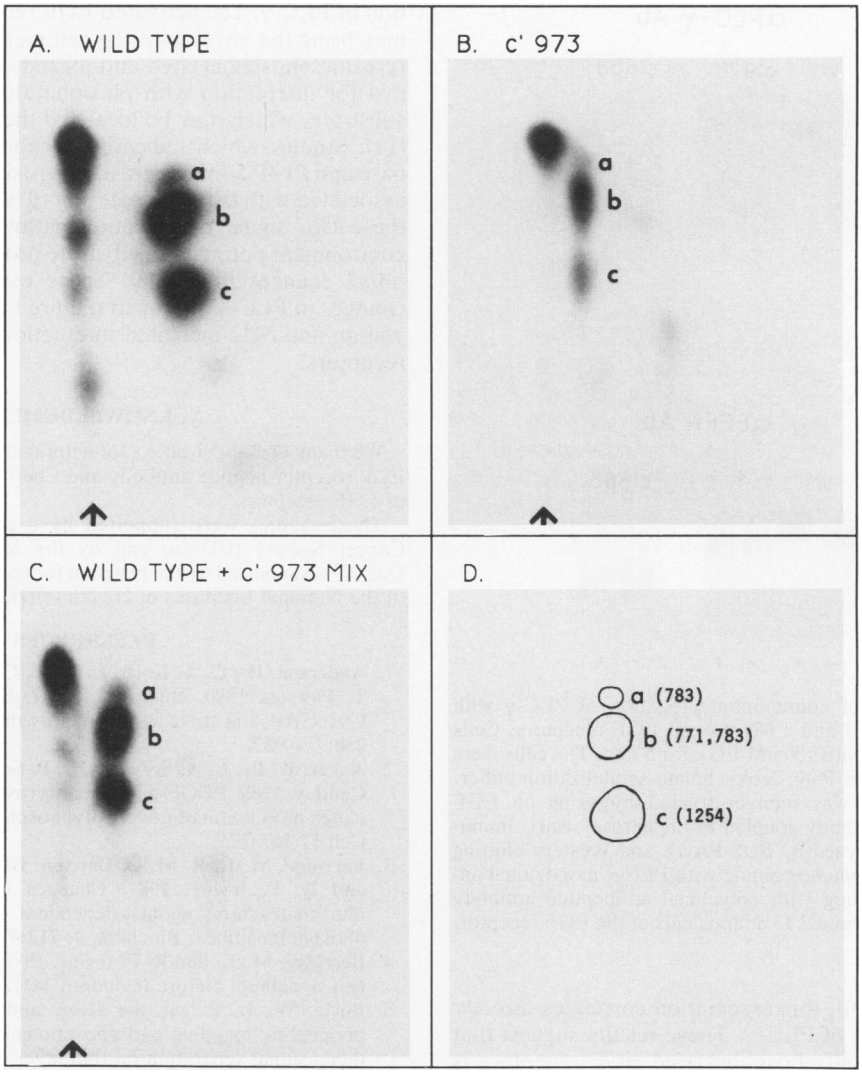

FIG. 4. Two-dimensional tryptic phosphopeptide maps of PLC-γ. Purified PLC-γ was phosphorylated by the indicated EGF receptors in the presence of [γ-^{32}P]ATP, isolated, and digested with trypsin, and the fragments were separated in the first dimension (horizontal) by high-voltage electrophoresis (pH 1.9) and in the second dimension (vertical) by thin-layer chromatography. (A) PLC-γ phosphorylation by wild-type EGF receptor; (B) PLC-γ phosphorylation by c'973 EGF receptor; (C) the phosphorylated samples run mixed; (D) schematic showing the sites of tyrosine phosphorylation. Arrows indicate the position of sample application in panel A to C.

(45) effectively signal other responses to EGF such as morphological changes, gene induction, and cell proliferation (7, 46, 47). Sites of tyrosine self-phosphorylation thus appear necessary for PLC-γ activation but not for other cell responses to ligand-activated EGF receptors.

EGF stimulated increases in $[Ca^{2+}]_i$ also require a site of tyrosine self-phosphorylation in the C terminus of the EGF receptor. Chang et al. (6) analyzed a series of mutant EGF receptors and demonstrated that EGF-stimulated increases in $[Ca^{2+}]_i$, quantitated in single cells by the fluorescent indicator Fura 2, showed strict dependence on a site of tyrosine self-phosphorylation. Because maximum increases in $[Ca^{2+}]_i$ occur within 30 s (37), it is not possible to causally link the in vivo changes in inositol phosphate directly to changes in $[Ca^{2+}]_i$. Although additional mechanisms may be involved in EGF-stimulated increases in $[Ca^{2+}]_i$, the identical requirements for a site of tyrosine self-phosphorylation in the EGF receptor for activation of PLC-γ and formation of IP_3, a known mediator of increased $[Ca^{2+}]_i$ (4, 31, 36), suggest that these may be causally linked. Development of methods for measuring single-cell IP_3 will be necessary to critically test whether a causal link exists.

Mutation of the kinase insert region of the PDGF receptor to remove a site of tyrosine self-phosphorylation abolished PDGF-stimulated increases in phosphatidylinositol 3-kinase activity and association of this enzyme with the PDGF receptor (17). The present study indicates that activation of PLC-γ by EGF similarly requires a site of tyrosine self-phosphorylation in the EGF receptor. The SH2 domains of PLC-γ bind tightly to sites of tyrosine phosphorylation in EGF and PDGF receptors (1, 25). Similar to other proteins, tyrosine self-phosphorylation sites are proposed to create a high-affinity binding site for PLC-γ via the ~100-amino-acid SH2 domain (20, 25). Deletion of tyrosine phosphorylation sites in the EGF receptor prevents PLC-γ binding as assayed by immunoprecipitation of complexes formed in vivo. Although only a fraction of activated EGF and PDGF receptors complex with PLC-γ in vivo as revealed by coimmunopre-

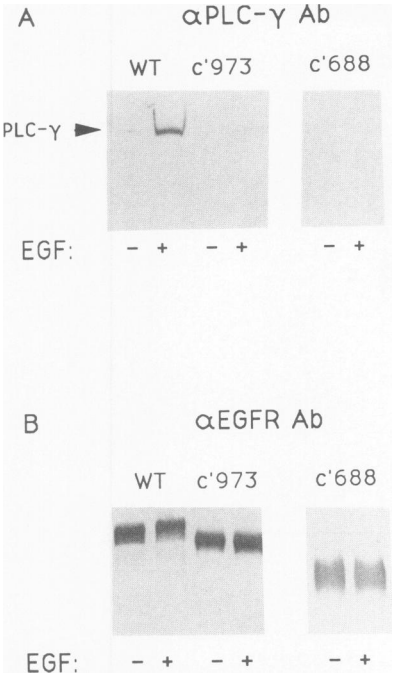

FIG. 5. Comparison of coimmunoprecipitation of PLC-γ with wild-type (WT) and c'973 and c'688 mutant EGF receptors. Cells were treated without or with 100 nM EGF for 5 min. The cells were then lysed with a Nonidet P-40–deoxycholate solubilization buffer, and the EGF receptor was immunoisolated by using an EGF receptor monoclonal antibody coupled to Sepharose beads. Immunoprecipitates were analyzed by SDS-PAGE and Western blotting (immunoblotting). (A) Immunostaining with PLC-γ monoclonal antibody; (B) immunostaining with polyclonal antipeptide antibody directed against the N-terminal 13 amino acids of the EGF receptor.

cipitation (1, 21, 26, 30), this association correlates directly with in vivo activation of PLC-γ. These results suggest that the association with self-phosphorylated EGF receptors is required for in vivo activation of PLC-γ.

Although tyrosine phosphorylation of PLC-γ appears essential for activation (18, 34), the observation that c'973 EGF receptors catalyze tyrosine phosphorylation of PLC-γ in vivo as effectively as wild-type EGF receptors but fail to activate the enzyme indicates that tyrosine phosphorylation of PLC-γ is not sufficient for effective enzyme activation in vivo. Recent studies of a chimeric receptor composed of the EGF receptor-binding domain and the c-*kit* cytoplasmic domain also indicate a dissociation between tyrosine phosphorylation of PLC-γ and in vivo enzyme activation (23).

Taken together, these data indicate that both tyrosine phosphorylation of PLC-γ and binding to the EGF receptor via SH2 domains of PLC-γ to the EGF receptor sites of tyrosine phosphorylation are necessary for PLC-γ activation in vivo. Enzyme substrate interactions are not expected to require high-affinity interactions such as those dictated by SH2 regions, and c'973 EGF receptors efficiently catalyze tyrosine phosphorylation of PLC-γ in vivo. Because there is evidence that tyrosine-phosphorylated PLC-γ binds less tightly than does nonphosphorylated enzyme to self-phosphorylated EGF and PDGF receptors (21, 25) and because mutant nonphosphorylated PLC-γ associates with self-phosphorylated PDGF receptors (18), the present experiments suggest that although binding correlates directly with PLC-γ activation, it may not be sufficient to explain in vivo activation of PLC-γ. The activated EGF receptor–PLC-γ complex may bring the enzyme to the cell membrane (40) where it is tyrosine phosphorylated and placed in a conformation optimal for interaction with phosphatidylinositol bisphosphate substrate, which may be localized there or bound to profilin (13). Studies which indicate that phosphatidylinositol 4-kinase and PI 4P 5-kinase are also tyrosine phosphorylated and associated with the EGF receptor (9) suggest that coupling of these two signal transduction pathways occurs in a local environment optimal for substrate production and conformational changes in PLC-γ. These essential conformational changes in PLC-γ appear to require both tyrosine phosphorylation and SH2-mediated interactions with activated EGF receptors.

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REFERENCES

1. **Anderson, D., C. A. Koch, L. Grey, C. Ellis, M. F. Moran, and T. Pawson.** 1990. Binding of SH2 domains of phospholipase Cγ1, GAP, and src to activated growth factor receptors. Science **250**:979–982.
2. **Auger, K. R., L. A. Serunian, S. P. Soltoff, P. Libby, and L. C. Cantley.** 1989. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell **57**:167–175.
3. **Berridge, M. J., R. M. C. Dawson, C. P. Downes, J. P. Heslop, and R. F. Irvine.** 1983. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem. J. **212**:473–482.
4. **Berridge, M. J., and R. F. Irvine.** 1989. Inositol phosphates and cell signalling. Nature (London) **341**:197–205.
5. **Boyle, W. J., P. van der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin layer cellulose plates. Methods Enzymol. **201**:110–148.

5a. **Chang, C.-P.** Unpublished data.

6. **Chang, C.-P., J. P. Kao, C. S. Lazar, J. B. Welsh, A. Wells, H. S. Wiley, G. N. Gill, and M. G. Rosenfeld.** 1991. Ligand-induced internalization and increased cell calcium are mediated via distinct structural elements in the carboxy terminus of the epidermal growth factor receptor. J. Biol. Chem., in press.
7. **Chen, W. S., C. S. Lazar, K. A. Lund, J. B. Welsh, C. Chang, G. M. Walton, C. J. Der, H. S. Wiley, G. N. Gill, and M. G. Rosenfeld.** 1989. Functional independence of the epidermal growth factor receptor from a domain required for ligand-induced internalization and calcium regulation. Cell **59**:33–43.
8. **Chen, W. S., C. S. Lazar, M. Poenie, R. Y. Tsien, G. N. Gill, and M. G. Rosenfeld.** 1987. Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. Nature (London) **328**:820–823.
9. **Cochet, C., O. Filhol, B. Payrastre, T. Hunter, and G. N. Gill.** 1991. Interaction between the epidermal growth factor receptor and phosphoinositide kinases. J. Biol. Chem. **266**:637–644.
10. **Downward, J., P. Parker, and M. D. Waterfield.** 1984. Autophosphorylation sites on the epidermal growth factor receptor. Nature (London) **311**:483–485.
11. **Gill, G. N., T. Kawamoto, C. Cochet, A. Le, J. D. Sato, H. Masui, C. McCloud, and J. Mendelsohn.** 1984. Monoclonal anti-epidermal growth factor receptor antibodies which are inhibitors of epidermal growth factor binding and antagonists of epidermal growth factor-stimulated tyrosine protein kinase activity. J. Biol. Chem. **259**:7755–7760.
12. **Glenney, J. R., L. Zokas, and M. P. Kamps.** 1988. Monoclonal

antibodies to phosphotyrosine. J. Immunol. Methods **109**:277–285.
13. **Goldschmidt-Clermont, P. J., J. W. Kim, L. M. Machesky, S. G. Rhee, and T. D. Pollard.** 1991. Profilin allows phosphorylation of PLC-γ_1 by the EGF receptor to regulate the production of IP_3 from PIP_2. Science **251**:1231–1233.
14. **Hepler, J. R., N. Nakahata, T. W. Lovenberg, J. DiGuiseppi, B. Herman, H. S. Earp, and T. K. Harden.** 1987. Epidermal growth factor stimulates the rapid accumulation of inositol (1,4,5)-trisphosphate and a rise in cytosolic calcium mobilized from intracellular stores in A431 cells. J. Biol. Chem. **262**:2951–2956.
15. **Honegger, A. M., D. Szapary, A. Schmidt, R. Lyall, E. Van Obberghen, T. J. Dull, A. Ullrich, and J. Schlessinger.** 1987. A mutant epidermal growth factor receptor with defective protein tyrosine kinase is unable to stimulate proto-oncogene expression and DNA synthesis. Mol. Cell. Biol. **7**:4568–4571.
16. **Hsuan, J. J., N. Totty, and M. D. Waterfield.** 1989. Identification of a novel autophosphorylation site (P4) on the epidermal growth factor receptor. Biochem. J. **262**:659–663.
17. **Kaziauskas, A., and J. A. Cooper.** 1989. Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins. Cell **58**:1121–1133.
18. **Kim, H. K., J. W. Kim, A. Zilberstein, B. Margolis, J. G. Kim, J. Schlessinger, and S. G. Rhee.** 1991. PDGF stimulation of phospholipid hydrolysis requires PLC-γ phosphorylation on tyrosine 783. Cell **65**:435–441.
19. **Kim, J. W., S. S. Sim, U. Kim, S. Nishibe, M. I. Wahl, G. Carpenter, and S. G. Rhee.** 1990. Tyrosine residues in bovine phospholipase C-γ phosphorylated by the epidermal growth factor receptor in vitro. J. Biol. Chem. **265**:3940–3943.
20. **Koch, C. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson.** 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science **252**:668–674.
21. **Kumjian, D. A., A. Barnstein, S. G. Rhee, and T. O. Daniel.** 1991. Phospholipase Cγ complexes with ligand-activated platelet-derived growth factor receptors. J. Biol. Chem. **266**:3973–3980.
22. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
23. **Lev, S., D. Givol, and Y. Yarden.** 1991. A specific combination of substrates is involved in signal transduction by the *kit*-encoded receptor. EMBO J. **10**:647–654.
24. **Margolis, B., F. Bellot, A. M. Honegger, A. Ullrich, J. Schlessinger, and A. Zilberstein.** 1990. Tyrosine kinase activity is essential for the association of phospholipase C-γ with the epidermal growth factor receptor. Mol. Cell. Biol. **10**:435–441.
25. **Margolis, B., N. Li, A. Koch, M. Mohammadi, D. R. Hurwitz, A. Zilberstein, A. Ullrich, T. Pawson, and J. Schlessinger.** 1990. The tyrosine phosphorylated carboxy terminus of the EGF receptor is a binding site for GAP and PLC-γ. EMBO J. **9**:4375–4380.
26. **Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger.** 1989. EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signalling. Cell **57**:1101–1107.
27. **Matsuda, M., B. J. Mayer, Y. Fukui, and H. Hanafusa.** 1990. Binding of transforming protein, p47gag-crk to a broad range of phosphotyrosine-containing proteins. Science **248**:1537–1589.
28. **Mayer, B. J., M. Hamaguchi, and H. Hanafusa.** 1988. A novel oncogene with structural similarity to phospholipase C. Nature (London) **332**:272–275.
29. **Mayer, B. J., and H. Hanafusa.** 1990. Association of the v-crk oncogene product with phosphotyrosine-containing proteins and protein kinase activity. Proc. Natl. Acad. Sci. USA **87**: 2638–2642.
30. **Meisenhelder, J., P. Suh, S. G. Rhee, and T. Hunter.** 1989. Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. Cell **57**:1109–1122.
31. **Meyer, T., D. Holowka, and L. Stryer.** 1988. Highly cooperative opening of calcium channels by inositol 1,4,5-trisphosphate. Science **240**:653–656.
32. **Moolenaar, W. H., A. J. Bierman, B. C. Tilly, I. Verlaan, L. H. Defize, A. M. Honegger, A. Ullrich, and J. Schlessinger.** 1988. A point mutation at the ATP-binding site of the EGF-receptor abolishes signal transduction. EMBO J. **7**:707–710.
33. **Moran, M. F., C. A. Koch, D. Anderson, C. Ellis, L. England, G. S. Martin, and T. Pawson.** 1990. Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc. Natl. Acad. Sci. USA **87**:8622–8626.
34. **Nishibe, S., M. I. Wahl, S. M. Hernandez-Sotomayer, N. K. Tonks, S. G. Rhee, and G. Carpenter.** 1990. Increase of the catalytic activity of phospholipase C-γ1 by tyrosine phosphorylation. Science **250**:1253–1256.
35. **Nishibe, S., M. I. Wahl, S. G. Rhee, and G. Carpenter.** 1989. Tyrosine phosphorylation of phospholipase C-II *in vitro* by the epidermal growth factor receptor. J. Biol. Chem. **264**:10335–10338.
36. **Parker, I., and I. Ivorra.** 1990. Localized all-or-none calcium liberation by inositol trisphosphate. Science **250**:977–979.
37. **Poenie, M., J. Alderton, R. Y. Tsien, and R. A. Steinhardt.** 1986. Calcium rises abruptly and briefly throughout the cell at the onset of anaphase. Science **233**:886–889.
38. **Rhee, S. G., P. Suh, S. Ryu, and S. Y. Lee.** 1989. Studies of inositol phospholipid-specific phospholipase C. Science **244**: 546–550.
39. **Suh, P., S. H. Ryu, W. C. Choi, K. Lee, and S. G. Rhee.** 1988. Monoclonal antibodies to three phospholipase C isozymes from bovine brain. J. Biol. Chem. **263**:14497–14504.
40. **Todderud, G., M. I. Wahl, S. G. Rhee, and G. Carpenter.** 1990. Stimulation of phospholipase C-γ1 membrane association by epidermal growth factor. Science **249**:296–298.
41. **Wahl, M., and G. Carpenter.** 1988. Regulation of epidermal growth factor-stimulated formation of inositol phosphates in A-431 cells by calcium and protein kinase C. J. Biol. Chem. **263**:7581–7590.
42. **Wahl, M. I., S. Nishibe, J. W. Kim, H. Kim, S. G. Rhee, and G. Carpenter.** 1990. Identification of two epidermal growth factor-sensitive tyrosine phosphorylation sites of phospholipase C-γ in intact HSC-1 cells. J. Biol. Chem. **265**:3944–3948.
43. **Wahl, M. I., S. Nishibe, P.-G. Suh, S. G. Rhee, and G. Carpenter.** 1989. Epidermal growth factor stimulates tyrosine phosphorylation of phospholipase C-II independently of receptor internalization and extracellular calcium. Proc. Natl. Acad. Sci. USA **86**:1568–1572.
44. **Wahl, M. I., N. E. Olashaw, S. Nishibe, S. G. Rhee, W. J. Pledger, and G. Carpenter.** 1989. Platelet-derived growth factor induces rapid and sustained tyrosine phosphorylation of phospholipase C-γ in quiescent BALB/c 3T3 cells. Mol. Cell. Biol. **9**:2934–2943.
45. **Walton, G. M., W. S. Chen, M. G. Rosenfeld, and G. N. Gill.** 1990. Analysis of deletions of the carboxyl terminus of the epidermal growth factor receptor reveals self-phosphorylation at tyrosine 992 and enhanced in vivo tyrosine phosphorylation of cell substrates. J. Biol. Chem. **265**:1750–1754.
46. **Wells, A., J. B. Welsh, C. S. Lazar, H. S. Wiley, G. N. Gill, and M. G. Rosenfeld.** 1990. Ligand induced transformation by a non-internalizing epidermal growth factor receptor. Science **247**:962–964.
47. **Welsh, J. B., G. N. Gill, M. G. Rosenfeld, and A. Wells.** 1991. A negative feedback loop attenuates EGF-induced morphological changes. J. Cell Biol. **114**:533–543.