Non-catalytic activation of phospholipase C- γ 1 *in vitro* by epidermal growth factor receptor

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To investigate the possible functional role of epidermal growth factor (EGF) receptor–phospholipase C- γ 1 (PLC- γ 1) complexes, we have measured PLC- γ 1 activity *in vitro* in the absence or presence of purified EGF receptor. Immunoprecipitates of PLC- γ 1 from control A-431 cells were incubated without or with purified EGF receptor in the absence or presence of ATP. Under these conditions the EGF receptor increased non-tyrosine-phosphorylated PLC- γ 1 activity 3–4-fold in the absence or presence of ATP, but increased tyrosine-phosphorylated and activated PLC- γ 1 by only 20–50%. Both basal and auto-phosphorylated forms of the purified EGF receptor increased the

activity of the non-tyrosine-phosphorylated PLC- γ 1, and stoichiometric levels of purified receptor were required to increase PLC activity. Other tyrosine kinases such as the platelet-derived growth factor receptor and erbB-2, but not the insulin receptor, also stimulated PLC- γ 1 activity. PLC- γ 1 activity could be activated with the kinase-negative EGF receptor, but a C-terminal truncated receptor was much less effective. Purified EGF receptor could also activate PLC- β 1, but with a much decreased potency compared with PLC- γ 1. Our results suggest that *in vitro* the EGF receptor can increase PLC- γ 1 activity independently of tyrosine phosphorylation.

INTRODUCTION

Phospholipase C (PLC) has a key role in the signal-transduction pathway for several hormones and growth factors. PLC catalyses the hydrolysis of PtdIns(4,5) P_2 , generating two potential intracellular second messengers: diacylglycerol and inositol 1,4,5-trisphosphate (Ins P_3). Approximately eight distinct PLC isoenzymes have been purified, cloned and sequenced [1]. Two of the isoenzymes, PLC- γ 1 and - γ 2, are unique in that they are substrates for the tyrosine kinase activity of several growth-factor receptors (for review see [2]). In intact cells or *in vitro*, tyrosine phosphorylation increases the catalytic activity of PLC- γ 1 [3–10], suggesting that PLC- γ 1 catalytic activity is regulated by tyrosine phosphorylation. Additionally, several reports [5,11–14] suggest a physical association between PLC- γ 1 and activated tyrosine kinases, based on co-precipitation experiments. These association experiments concluded that the epidermal growth factor (EGF) receptor tyrosine kinase activity was essential for association of the two proteins. Primarily, the non-tyrosine-phosphorylated form of PLC- γ 1 was detected in association with the EGF receptor. This bimolecular association has also been detected between PLC- γ 1 and the platelet-derived growth factor (PDGF) receptor in response to PDGF [5,15].

PLC- γ 1 and other signalling proteins such as GTPase-activating protein and PtdIns 3-kinase have in common sequence motifs termed *src* homology (SH) domains 2 and 3 (for review see [16]). It has been shown that SH2 domains bind tyrosine-phosphorylated proteins, such as activated growth-factor receptors [16]. The formation of stable complexes between growth-factor receptors and SH2-containing proteins is dependent on prior growth-factor binding, and receptor autophosphorylation [12,13,17,18]. For PLC- γ 1, association occurs between the SH2 domains of PLC- γ 1 and unidentified sites in the tyrosine-phosphorylated C-terminus of the EGF receptor [12,19–21]. In the PDGF receptor, association of PLC- γ 1 is dependent on the

C-terminal autophosphorylation sites Tyr-1009 and Tyr-1021 [22]. One study compared the wild-type and a C-terminal truncation mutant of the EGF receptor, and concluded that association of PLC- γ 1 and the EGF receptor activated PLC- γ 1 in intact cells [19]. Similar conclusions were reported for PLC- γ 1 activity in PDGF-stimulated cells [5]. However, no studies have examined the consequence of receptor–PLC- γ 1 interactions in a system *in vitro* with purified proteins. The results of such a study are reported in this paper.

MATERIALS AND METHODS

Cell culture

A-431 cells and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium plus 10% calf serum and 50 μ g/ml gentamycin. Cells were grown to confluence in 245 mm² culture dishes.

Cell extracts and PLC- γ 1 Immunoprecipitation

Cells were incubated in Dulbecco's modified Eagle's medium for 20 min, and, after two washes with ice-cold Ca²⁺-/Mg²⁺-free phosphate-buffered saline, cells were scraped into a homogenization buffer (750 μ l/dish, containing 20 mM Hepes, pH 7.4, 100 mM NaCl, 10% glycerol, 50 mM β -glycerophosphate, 0.2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 1 mM EGTA) and lysed with a Teflon homogenizer. The lysates were then centrifuged (400 000 g, 20 min) at 4 °C, and the supernatants were used as the cytosolic fraction. PLC- γ 1 was immunoprecipitated from this fraction. Supernatants were incubated overnight at 4 °C with 1 μ l of polyclonal antibodies to PLC- γ 1 per 100 μ g of cytosolic protein and 10 μ l of 10% heat-inactivated *Staphylococcus aureus* cells. The *S. aureus* cells were then washed twice with cold homogenization buffer containing 0.5% Triton X-100.

Abbreviations used: EGF, epidermal growth factor; PLC- γ 1, phospholipase C- γ 1; Ins P_3 , inositol 1,4,5-trisphosphate; PDGF, platelet-derived growth factor; SH, *src* homology.

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PLC- γ 1 tyrosine phosphorylation

Immunoprecipitated PLC- γ 1 was incubated at 0 °C with wheat-germ-lectin-purified EGF receptor in a phosphorylation reaction mixture containing 20 mM Hepes, pH 7.4, 20 mM MgCl_2 , 4 mM MnCl_2 and 80 μM ATP. After 45 min, the immunoprecipitates were washed and resuspended in homogenization buffer and PLC activity was measured.

PLC assay

PLC activity was measured in PLC- γ 1 immunoprecipitates with $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ as a substrate in a 50 μl reaction volume that contained 200 μM $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ (~ 20000 c.p.m.), 0.05% (v/v) Triton X-100, 0.8 mM EGTA, 0.8 mM CaCl_2 , 35 mM NaH_2PO_4 (pH 6.8) and 70 mM KCl, in the absence or presence of the EGF receptor. The reaction mixtures were incubated for 15 min at 37 °C before the reaction was stopped as described previously [7].

Brain extract

A fresh bovine brain was homogenized in a blender with 100 ml of cold buffer containing 10 mM Tris, pH 7.2, 100 μM orthovanadate, 2 mM phenylmethanesulphonyl fluoride, 2.5 mM EGTA, 2 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin and 0.1 mM dithiothreitol. The homogenate was centrifuged for 30 min at 13000 g , and the pH of the supernatant was adjusted to 5.0 with 1 M acetic acid. After 30 min at 4 °C, precipitates were collected by centrifugation as before. The precipitates were dissolved in 50 mM Tris, pH 8.3, containing 4 mM EGTA, 5 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ leupeptin, 0.1 mM dithiothreitol, 2 mM phenylmethanesulphonyl fluoride and 0.1 mM orthovanadate [23]. PLC- β 1 and PLC- γ 1 were immunoprecipitated with monoclonal antibodies (from S. G. Rhee, National Institutes of Health) [24]. The relative amounts of PLC- γ 1 and - β 1 protein present in the immunoprecipitates were estimated by immunoblot analysis with monoclonal antibodies and ^{125}I -labelled rabbit anti-mouse IgG as reported [24]. For quantification, a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) was used.

Purification of the tyrosine kinases

To prepare purified EGF receptor, plasma-membrane vesicles were obtained from A-431 cells as previously described [25] and solubilized in buffer A (20 mM Hepes buffer, pH 7.4, containing 10% glycerol, 1 mM phenylmethanesulphonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin and 10 $\mu\text{g}/\text{ml}$ aprotinin) containing 5% Triton X-100. The initial membrane protein concentration was 6 mg/ml. The suspension was incubated for 20 min at 0 °C before centrifugation (200000 g , 20 min) at 4 °C. The supernatant (~ 1.5 –2.0 ml) was added to 1 ml of packed wheat-germ lectin–Sephrose 6MB (Sigma) and incubated in a rotator for 2 h at 4 °C. After incubation the gel was washed with 30 column vol. of buffer A containing 0.05% Triton X-100. The EGF receptor was eluted for 1 hr at 4 °C with 0.5–0.75 ml of buffer A containing 0.3 M *N*-acetyl-D-glucosamine. The lectin-purified EGF receptor was stored at –70 °C for no more than 2 weeks. For the purification of receptor tyrosine kinases from other cell lines, the following cell lines were used: NIH 3T3 cells transfected with human wild-type or mutant EGF receptor [20,21], NIH 3T3 cells transfected with human erbB-2 (P. Di Fiore, National Cancer Institute) and NIH 3T3/HIR cells expressing human insulin receptor [26]. The cells were grown to confluence in 245 mm^2 culture dishes and lysed by using the same conditions for preparation of cytosol from A-431 cells. After the lysates were centrifuged (400000 g ,

20 min) at 4 °C, the supernatant was discarded, the pellet, was solubilized, and the receptor tyrosine kinases were purified as described above for the A-431 vesicles.

EGF receptor autophosphorylation

To obtain the purified and autophosphorylated EGF receptor, the Triton extract from A-431 vesicles was incubated on ice in the presence of 20 μM ATP, 25 mM MgCl_2 , 100 μM orthovanadate and 0.3 μM EGF. After 20 min the EGF receptor was purified by wheat-germ-lectin chromatography as described above. Receptor prepared in the presence of this phosphorylation mixture is non-tyrosine-phosphorylated and will be referred to in the text as 'basal receptor'. Receptor prepared in the absence of the phosphorylation mixture is tyrosine-phosphorylated and will be referred to in the text as 'autophosphorylated receptor'. The presence or absence of phosphotyrosine on these receptor preparations was confirmed by Western blotting with antibodies to phosphotyrosine as described below.

Immunoblot analysis

Immunoprecipitates of EGF receptor, PLC- γ 1 or PLC- β 1 were subjected to SDS/PAGE (7.5% gels) and electroblotted on to Nitro Plus transfer membrane (Micron Sep. Inc.) overnight at 30 V or for 6 h at 50 V. The filters were probed with polyclonal phosphotyrosine antibody (1:3000), or polyclonal antibody 986 [27] to the EGF receptor (1:2000) or polyclonal antibody to PLC- γ 1 (1:250) [28], and visualized with ^{125}I -Protein A. For immunodetection of PLC- γ 1 and - β 1 with monoclonal antibodies, a 1:500 dilution of a mixture of monoclonal antibodies was used and bound antibody was detected with ^{125}I -labelled rabbit anti-mouse IgG.

Materials

EGF was isolated from mouse submaxillary glands as described previously [29]. $\text{PtdIns}(4,5)\text{P}_2$ (ammonium salt) was obtained from Boehringer Mannheim. $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ was obtained from DuPont–New England Nuclear. *S. aureus* cells were obtained from Calbiochem. ^{125}I -labelled rabbit anti-mouse IgG and ^{125}I -Protein A were obtained from ICN Radiochemicals. Calf serum was from GIBCO. Rabbit polyclonal antibodies to PLC- γ 1 were developed against a C-terminal peptide [28]. Polyclonal antibody to phosphotyrosine was purchased from Zymed Inc.

RESULTS

In previous experiments, it was shown that tyrosine phosphorylation of PLC- γ 1 *in vitro* by the EGF receptor increases PLC activity 3–4-fold [6]. Because Mn^{2+} and Mg^{2+} inhibit PLC activity, the EGF receptor was removed after PLC- γ 1 phosphorylation, but before the assay of PLC activity in those experiments [6]. In the present study, we examined the potential role of the EGF receptor in the activation of PLC- γ 1 when the receptor was present during the PLC activity assay. In these experiments, non-tyrosine-phosphorylated PLC- γ 1 was obtained by immunoprecipitating the enzyme from unstimulated A-431 cells. Tyrosine-phosphorylated PLC- γ 1 was obtained by phosphorylating the immunoprecipitated enzyme *in vitro* with the EGF receptor and then removing the receptor by washing. Using $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ as a substrate, PLC- γ 1 activity was measured in the immunoprecipitate in the absence or presence of wheat-germ-lectin-purified EGF receptor, but not ATP, Mn^{2+} or Mg^{2+} , during the phospholipase assay.

Table 1 Influence of the presence of EGF receptors on PLC- γ 1 activity *in vitro*

Immunoprecipitates of PLC- γ 1 from non-stimulated A431 cells were phosphorylated *in vitro* as described in the Materials and methods section. After two washes, PLC activity was measured in the immunoprecipitates in the absence or presence of either basal or autophosphorylated EGF receptor (0.2 μ g). Results are means $\pm$ S.E.M. of duplicate determinations in four experiments. ^a $P < 0.001$ versus PLC- γ 1 non-tyrosine-phosphorylated in the absence of EGF receptor; ^bno significant difference compared with tyrosine-phosphorylated PLC- γ 1 in the absence of EGF receptor.

EGF receptor added	InsP ₃ formation (pmol/min)	
	PLC- γ 1 non-tyrosine-phosphorylated	PLC- γ 1 tyrosine-phosphorylated
None	8.3 $\pm$ 0.6	36.3 $\pm$ 4.8 ^a
Basal	31.0 $\pm$ 1.6 ^a	44.8 $\pm$ 8.0 ^b
Autophosphorylated	30.5 $\pm$ 3.3 ^a	36.0 $\pm$ 6.23 ^b

Activation of PLC- γ 1 activity by EGF receptors

To determine whether the presence of the EGF receptor would alter the activity of PLC- γ 1, the following experiment was performed (Table 1). Either non-tyrosine-phosphorylated or tyrosine-phosphorylated PLC- γ 1 was assayed in the presence of EGF receptor. Basal EGF receptor increased the activity of non-tyrosine-phosphorylated PLC approx. 3.7-fold. To determine whether autophosphorylated EGF receptor would have the same effect, EGF receptor was autophosphorylated by incubation in the presence of EGF, ATP and Mg²⁺, and the autophosphorylated EGF receptor was purified from this mixture by wheat-germ-lectin chromatography. As shown in Table 1, the autophosphorylated receptor did not have a significantly larger effect on PLC activity (3.6-fold) than the basal form of the receptor. To determine whether EGF receptors were able to activate the tyrosine-phosphorylated form of PLC- γ 1, this form of the enzyme was assayed with basal and autophosphorylated EGF receptor. Tyrosine-phosphorylation of the enzyme *in vitro* increased activity 4.3-fold relative to the non-tyrosine-phosphorylated PLC- γ 1, but addition of either basal or autophosphorylated EGF receptors did not further increase PLC activity (5.4- and 4.3-fold respectively) to a significant degree. In these experiments, the amounts of basal and autophosphorylated EGF receptor were equivalent as judged by anti-receptor Western blots. Anti-phosphotyrosine blots of the EGF receptor preparations confirmed that the basal receptor did not contain significant phosphotyrosine, but the activated EGF receptor was highly tyrosine-phosphorylated (results not shown). EGF receptor preparations were shown by specific immunoblots not to contain endogenous PLC- γ 1 protein or PLC activity. These results indicate that 200 ng of EGF receptor is able to increase PLC- γ 1 activity significantly in the absence of phosphotyrosine.

The possibility that the activation might be due to an unknown glycoprotein that co-purified on the wheat-germ-lectin column with the EGF receptor was considered. When the receptor was purified by using a ligand affinity column (EGF-Affigel), however, the receptor was as potent as the wheat-germ-purified receptor. Also, the stimulatory effect was abolished when the EGF receptor preparation was heated for 15 min at 45 °C, a condition previously shown to inactivate the cytoplasmic kinase domain of the EGF receptor [30].

Figure 1 Relationship of increasing EGF receptor concentration to PLC- γ 1 activity

Immunoprecipitates of PLC- γ 1 (~1 pmol), tyrosine-phosphorylated (+P~Y) (●) or non-tyrosine-phosphorylated (−P~Y) (○) were used to measure PLC activity in the presence of increasing amounts of basal (—) and autophosphorylated (---) EGF receptor. Error bars were omitted for clarity, but they were < 10% of the mean.

The data in Figure 1 show that receptor activation of PLC- γ 1 is dependent on the concentration of EGF receptor added, as the activity of non-tyrosine-phosphorylated PLC- γ 1 in the presence of increasing amounts of basal or autophosphorylated EGF receptor was measured. Increasing levels of receptor stimulated non-tyrosine-phosphorylated PLC- γ 1 activity, producing a maximum enhancement of 3.7-fold. At this point, both proteins (PLC- γ 1 and EGF receptor) are present in approximately equimolar amounts (1 pmol), suggesting that the activation process requires a stoichiometric ratio of EGF receptor and PLC- γ 1. In a separate experiment the ratio of receptor to PLC- γ 1 was increased to 2:1. This did not produce activation of PLC- γ 1 to a greater extent than a 1:1 ratio of receptor to PLC- γ 1.

The data in Figure 1 also show that the tyrosine-phosphorylated form of PLC- γ 1 is not substantially activated by increasing amounts of EGF receptor. In addition, the results also demonstrate that the autophosphorylated EGF receptor was similar in potency to the basal receptor with regard to PLC- γ 1 activation *in vitro*. Since there was no evidence that autophosphorylated EGF receptors were more potent than basal receptors, we decided to use only basal EGF receptor for the following experiments.

Receptor requirement for activation

To explore the structural requirements of the EGF receptor for PLC- γ 1 activation in this system, receptor was purified from NIH 3T3 cells transfected with wild-type and mutant EGF receptors [20,21]. Mutant receptors included a kinase-negative receptor (kin[−]) containing a Lys-721 → Ala mutation, a truncation mutant with the C-terminal 123 amino acids deleted and four known autophosphorylation sites (DC-123), and DC-123 with a Tyr-992 → Phe (DC-123F) substitution to eliminate a fifth reported autophosphorylation site. To compare equivalent

Table 2 Influence of different tyrosine kinases on PLC- γ 1 activation

Activity in the immunoprecipitates of non-tyrosine-phosphorylated PLC- γ 1 was measured in the presence of different tyrosine kinases ($\sim 0.2 \mu\text{g}$ of protein), as described in the Materials and methods section. Results are means $\pm$ S.E.M. of duplicate determinations from 2–5 experiments. The results are expressed as a percentage of the basal activity, which was $9.1 \pm 0.6 \text{ pmol/min}$. Abbreviations: wt, wild-type human EGF receptor; kin⁻, kinase-negative receptor (Ala substitution of Lys-721); DC-123, deletion of the C-terminal 123 amino acids of the EGF receptor; DC-123F, deletion of the C-terminal 123 amino acids of the EGF receptor and phenylalanine substitution of Tyr-992. ^a $P < 0.001$ versus basal; ^b $P < 0.01$ versus wt EGF receptor; ^c $P < 0.001$ versus wt EGF receptor; ^dno significant difference versus basal.

Receptor added	InsP ₃ formation (% of basal)
wt EGF receptor	619 ± 30^a
kin ⁻	512 ± 6.98^{ba}
DC-123	455 ± 23^{ba}
DC-123F	210 ± 21^{ca}
PDGF receptor	424 ± 21^a
erbB-2	609 ± 54^a
Insulin receptor	118 ± 5.7^d

Table 3 Influence of EGF receptor on the PLC- β 1 activity

PLC- γ 1 and PLC- β 1 were immunoprecipitated from bovine brain crude extracts as described in the Materials and methods section, and PLC activity was measured in the absence or presence of basal EGF receptor (1 pmol). Results are means $\pm$ S.E.M. of duplicate determinations in 3–4 experiments. ^a $P < 0.001$ versus basal PLC- γ 1; ^bno significant difference versus basal PLC- β 1 ($\sim 1 \text{ pmol}$); ^c $P < 0.001$ versus basal PLC- β 1 ($\sim 0.1 \text{ pmol}$).

	InsP ₃ formation (pmol/min)			
	PLC- γ 1 ($\sim 1 \text{ pmol}$)	PLC- β 1 ($\sim 1 \text{ pmol}$)	PLC- γ 1 ($\sim 1 \text{ pmol}$)	PLC- β 1 ($\sim 0.1 \text{ pmol}$)
Basal	11.1 ± 1.6	60.7 ± 7.1	19.4 ± 1.2	20 ± 1.5
+ EGF receptor	40.0 ± 4.0^a	68.5 ± 9.7^b	69.5 ± 6.3^a	79 ± 6.3^c

amounts of purified receptors, each receptor preparation was normalized for amount of protein. As shown in Table 2, both the wild-type and the kinase-negative receptors were able to activate non-tyrosine-phosphorylated PLC- γ 1 to approximately the same extent (6.2- and 5.0-fold respectively). When the truncated form of the EGF receptor (DC-123) was tested, its ability to activate PLC- γ 1 was significantly diminished only when the truncated receptor also contained a phenylalanine substitution of Tyr-992 (DC-123F) to eliminate all known autophosphorylation sites.

We also tested the capacity of other receptor tyrosine kinases to activate non-tyrosine-phosphorylated PLC- γ 1 in this assay. As shown in Table 2, the insulin receptor was not able to increase PLC- γ 1 activity, but basal PDGF receptor as well as basal erbB-2 tyrosine kinase were able to stimulate phospholipase activity. The insulin receptor tested positive for autophosphorylation when ligand was added, indicating that it was biologically active.

Specificity of PLC activation

PLC- γ 1 and - γ 2 are the only isoenzymes from the PLC family which have SH domains and are substrates for tyrosine kinases [16]. Therefore, PLC- β 1 was also tested to determine whether it

could be activated by the EGF receptor. For this comparison, PLC- γ 1 and PLC- β 1 were separately immunoprecipitated from a bovine brain homogenate, such that each immunoprecipitate contained an equivalent amount of PLC protein (approx. 1 pmol). Phospholipase activity for both enzymes was then assayed in the absence or presence of EGF receptor (approx. 1 pmol). The results are presented in Table 3. Parallel immunoprecipitates were assayed for PLC activity and quantified for amount of isoenzyme by Western blotting with the respective antibodies. Although the immunoblot showed equivalent amounts of both isoenzymes, the basal catalytic activity of PLC- β 1 was nearly 6-fold higher than that of PLC- γ 1. The source of the differences in basal activity is possibly due to the influence of each antibody on the enzyme activity of its antigen. Alternatively, the differences in basal activity could also reflect the different catalytic activities inherent in PLC- γ 1 and PLC- β 1. These data show that under these conditions addition of the EGF receptor only activated PLC- γ 1.

To determine whether PLC- β 1 could be activated if the ratio of receptor to isoenzyme was increased (Table 3), the concentration of PLC- β 1 was decreased so that equivalent levels of enzyme activity were present in both PLC- γ 1 and - β 1 immunoprecipitates. Under these conditions, addition of EGF receptor resulted in activations of 3.5- and 3.9-fold for PLC- γ 1 and - β 1 respectively. In those experiments the molar ratio of EGF receptor to PLC- β 1 was about 10:1, whereas in the previous experiments the ratio was 1:1. In both cases (Table 3) there was a 1:1 ratio of receptor and PLC- γ 1.

DISCUSSION

In this paper the results suggest that, at least *in vitro*, the EGF receptor can also activate PLC- γ 1 independently of tyrosine phosphorylation. The data shown that this process does not require EGF binding or autophosphorylated receptor, and is ATP-independent, and that the non-tyrosine-phosphorylated form of PLC- γ 1 responds to the EGF receptor.

Previous reports have indicated that only the autophosphorylated form of the receptor is able to associate with PLC- γ 1 and promote tyrosine phosphorylation of the substrate [5,11–14]. However, in none of these experiments was the receptor concentration equivalent to the concentration of PLC- γ 1, and in most of those experiments PLC- γ 1 activity was not measured. Two reports have concluded from studies *in vivo* that receptor association with PLC- γ 1 is part of a mechanism to activate this enzyme [5,19]. It is also clear, however, that once PLC- γ 1 is tyrosine-phosphorylated, its catalytic activity is increased, regardless of the presence or absence of the receptor [6–9].

Recent papers have shown that only certain autophosphorylation sites on the PDGF, fibroblast growth factor, and to a lesser extent EGF, receptors are able to recognize PLC- γ 1 [12,13,19–21]. Thus it appears that the interaction site between receptors and PLC- γ 1 is not simply a phosphotyrosine residue, but rather a linear or conformational determinant around particular phosphotyrosine residues. It seems possible that, in the experiments reported in this paper, receptor concentration is sufficiently high that a low-efficiency interaction is possible between receptor and PLC- γ 1. That tyrosine phosphorylation on the receptor is not essential in this system is demonstrated by two means: the use of non-tyrosine-phosphorylated receptors and the effectiveness of a kinase-negative mutant receptor.

A previous study [19] showed that PLC- γ 1 associates with a C-terminal autophosphorylated fragment of the EGF receptor. Our data (Table 2) also indicate that the C-terminus of the EGF receptor is necessary for this activation of PLC- γ 1 *in vitro*.

Studies *in vivo* [19] have suggested that Tyr-992 is important for receptor recognition of PLC- γ 1, which is confirmed by our results with the mutant receptor DC-123F (Table 2). Also, a previous study concluded that tyrosine phosphorylation of PLC- γ 1 decreased its capacity to associate with the receptor [11]. Likewise, we do not find that tyrosine-phosphorylated PLC- γ 1 is as sensitive as the non-tyrosine-phosphorylated enzyme to activation by the EGF receptor.

In terms of specificity, we have observed activation by the EGF, PDGF and erbB-2 receptor tyrosine kinases (Table 2), all of which are able to stimulate the hydrolysis of PtdIns(4,5) P_2 in intact cells. The insulin receptor was ineffective in our assay, and insulin is not a hormone that stimulates PtdIns(4,5) P_2 hydrolysis *in vivo*. Interactions between tyrosine kinases and their substrates are presumed to occur most efficiently through the SH2 domains present in many of these substrates, such as PLC- γ 1 [16]. Our results show that, although PLC- γ 1 activation by receptor occurs at an approximate equimolar stoichiometry, activation of PLC- β 1, which contains no SH2 domains, can occur only in the presence of excess receptor. This would suggest a basal and inefficient recognition between the receptor and PLC isoenzymes that does not rely on SH2 domains. The nature of sites that contribute to this interaction is unknown. However, it would seem likely that these low-efficiency sites are of little consequence physiologically and that the higher-efficiency interactions between PLC- γ 1 and the receptor would predominate.

Low-efficiency interactions between basal receptors and effector proteins have been observed in other systems also. Studies *in vitro* of interactions between β -adrenergic receptors and G_s α -subunit showed that, in the absence of ligand, the non-activated receptor was able to stimulate G_s activity 3–4-fold, indicating that low-efficiency interaction sites are present in that system [31]. Also, studies to delineate receptor–G-protein coupling have failed to show receptor-mediated specificity within G_i or G_o isoforms, and reconstitution studies with partially or completely purified receptors have shown no detectable differences in receptor–G-protein activation. For example, β -adrenergic receptors, which generally stimulate adenylate cyclase via G_s , can also stimulate the activation of the inhibitory G_i *in vitro* [32]. Recently, another example of a phospholipase activated by a protein kinase independent of phosphorylation was reported. Conricode et al. [33] found that a partially purified preparation of protein kinase C can stimulate phospholipase D activity in isolated membranes approx. 3-fold in the absence of added ATP and under conditions that preclude protein kinase C auto-phosphorylation.

Under physiological conditions of low to moderate numbers of EGF receptors, it is likely that catalytic activation of PLC- γ 1 by tyrosine phosphorylation is the mechanism that predominates. Recently [28], it has been shown that a high percentage of mammary carcinomas display increased levels of receptor tyrosine kinases (such as EGF receptor or erbB-2) and increased levels of PLC- γ 1. Similar over-expression of EGF receptors and PLC- γ 1 has been reported for hyperproliferative but benign skin lesions, such as psoriasis [34]. It is not yet clear, however, in these instances of exaggerated levels of EGF receptor and PLC- γ 1 whether a non-catalytic activation of PLC- γ 1 could occur.

The technical assistance of Deirdre Sánchez, Usha Barnela and Tanya Sorkina is gratefully acknowledged. We also thank Laura Hein and Sue Carpenter for typing the

manuscript. This research was supported by NIH grant CA43720 and a Fogarty International Research Fellowship to S.M.T.H.-S. (TWO4600).

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