Tyrosine Kinase Activity Is Essential for the Association of Phospholipase C- γ with the Epidermal Growth Factor Receptor

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Epidermal growth factor (EGF) treatment of NIH 3T3 cells transfected with wild-type EGF receptor induced tyrosine phosphorylation of phospholipase C- γ (PLC- γ). The EGF receptor and PLC- γ were found to be physically associated such that antibodies directed against PLC- γ or the EGF receptor coimmunoprecipitated both proteins. The association between PLC- γ and wild-type EGF receptor was dependent on the concentration of EGF, but EGF did not enhance the association between PLC- γ and a kinase-negative mutant of the EGF receptor. Oligomerization of the EGF receptor was not sufficient to induce association of the EGF receptor with PLC- γ , since the kinase-negative mutant receptor underwent normal dimerization in response to EGF yet did not associate with PLC- γ . The form of PLC- γ associated with the EGF receptor appeared to be primarily the non-tyrosine-phosphorylated form. It is concluded that the kinase activity of the EGF receptor is essential for association of PLC- γ with the EGF receptor, possibly by stimulating receptor autophosphorylation.

The ability of epidermal growth factor (EGF) to stimulate phosphatidylinositol hydrolysis has been demonstrated in several different cell lines, particularly those that overexpress epidermal growth factor (EGF) receptors (EGFR) (reviewed in reference 30). Although the exact mechanism that results in activation of phospholipase C by EGF are not completely understood, it has been demonstrated that the kinase activity of EGFR is essential for this effect (18). When specific monoclonal and polyclonal antibodies were generated against different phospholipase C (PLC) isozymes (16, 22, 26), it was found that EGF or platelet-derived growth factor (PDGF) stimulation of cells resulted in the phosphorylation of a single isozyme of PLC, now referred to as PLC- γ (16, 17, 28). It has been demonstrated that this phosphorylation can also occur *in vitro* when purified EGFR or PDGF receptor is combined with PLC- γ (17, 20). This finding demonstrated that PLC- γ is a direct substrate of these receptor tyrosine kinases and that an intermediate kinase need not be involved. Phosphorylation of PLC- γ was observed at physiologic concentrations of EGF (16), and it altered PLC- γ mobility on sodium dodecyl sulfate (SDS) gels, demonstrating quantitative phosphorylation (16, 17). Thus, it is hypothesized that tyrosine phosphorylation of PLC- γ is the signal used by certain growth factors to stimulate phosphatidylinositol hydrolysis.

In the course of these studies, it was observed that anti-PLC- γ antibody immunoprecipitates from EGF-treated cells contained two tyrosine-phosphorylated bands, one of 145 kilodaltons (kDa), which was PLC- γ , and one of 170 kDa (16, 28). It was found by immunoblotting that the 170-kDa protein was EGFR (16). The ability of anti-PLC- γ antibodies to coimmunoprecipitate EGFR was specific and could be inhibited by blocking PLC- γ immunoprecipitation with the synthetic peptide used to generate the PLC- γ antiserum (16). Similarly, it was demonstrated that antibodies to EGFR could coimmunoprecipitate PLC- γ . This study examines in further detail the factors that control the association of PLC- γ with EGFR. We demonstrate that treatment of cells with EGF markedly enhanced the association and that EGF

was unable to stimulate the association between PLC- γ and a kinase-negative EGFR mutant. Hence, association requires an activated form of the EGFR kinase.

MATERIALS AND METHODS

Cell culture. NIH 3T3 clone 2.2 cells devoid of endogenous EGFR were transfected with wild-type (HER14) or kinase-negative (K721A) receptor DNA constructs as previously described (7, 10, 13, 14). The kinase-negative receptor mutant was generated by substituting the putative ATP-binding lysine with alanine (7, 10). Cells were grown in 15-cm-diameter dishes in Dulbecco modified Eagle medium (Mediatech) with 10% bovine calf serum (HiClone). When the cells were confluent, they were placed in Dulbecco modified Eagle medium with 1% fetal bovine serum overnight for subsequent study.

Immunoprecipitation and immunoblotting. Cells were stimulated with EGF (ultrapure grade; Toyobo Chemical Co.) or PDGF (porcine platelet; R & D Systems) for 2 min at 37°C and then scraped into lysis buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 10 μ g of leupeptin per ml, 10 μ g of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, 200 μ M sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, and 30 mM *p*-nitrophenyl phosphate. Immunoprecipitation was performed by using either anti-EGFR monoclonal antibody mAb108 or rabbit polyclonal anti-PLC- γ antibody coupled to protein A-Sepharose (Sigma Chemical Co.) as previously described (16). In one experiment, immunoprecipitation was performed with polyclonal antiphosphotyrosine antibodies, in which case *p*-nitrophenyl phosphate was omitted from the lysis buffer. After immunoprecipitation, samples were washed with HNTG (20 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100), sample buffer was added, and the immunoprecipitates were heated to 90°C for 10 min. Immunoprecipitated proteins were next separated on a 6% SDS-gel and transferred to nitrocellulose for immunoblotting. Immunoblotting antibodies were rabbit polyclonal

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FIG. 1. Effects of increasing EGF concentration on PLC- γ tyrosine phosphorylation (A), EGFR autophosphorylation (B), and coimmunoprecipitation of EGFR by anti-PLC- γ antibodies (C). HER14 cells were stimulated with increasing concentrations of EGF for 2 min at 37°C, lysed, and subjected to immunoprecipitation with anti-PLC- γ antibodies or with anti-EGFR mAb108. The immunoprecipitated proteins were separated on a 6% SDS-gel and transferred to nitrocellulose. Immunoblotting was then performed with antiphosphotyrosine antibodies or a rabbit polyclonal antibody directed against the carboxy terminus of the EGFR. Immunoblots were detected with ¹²⁵I-protein A and exposed for 12 h for autoradiography. A total of 15×10^6 cells were used for each lane except those in panel B, for which only 2.5×10^5 cells were used.

antisera directed against EGFR, PLC- γ , or phosphotyrosine (16). Blots were detected by using ¹²⁵I-protein A and developed by autoradiography.

EGFR dimerization in living cells. To detect EGFR dimers, EGFR were chemically cross-linked on living cells after treatment with EGF (2). Cells were grown to confluence in 10-cm-diameter dishes and, before stimulation, were incubated overnight in 1% serum. Cultures were treated with 40 nM EGF for 1 h at 4°C and then treated with 15 mM covalent cross-linking agent 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide for 1 h at 22°C. Cells were washed with phosphate-buffered saline and treated with 150 mM glycine in phosphate-buffered saline for 5 min to react excess cross-linker. After lysis and immunoprecipitation with anti-EGFR mAb108, proteins were separated on a 4 to 6% gradient SDS gel. Immunoblotting was then performed with polyclonal anti-EGFR antibodies, and blots were detected as described above.

RESULTS

NIH 3T3 cells expressing approximately 500,000 human wild-type EGFR per cell (HER14) were used to evaluate the effect of EGF treatment on the coimmunoprecipitation of PLC- γ with EGFR. Cells were treated with increasing concentrations of EGF for 2 min, and then the lysates were subjected to immunoprecipitation with either anti-PLC- γ antibodies or anti-EGFR mAb108. After protein separation by SDS-polyacrylamide gel electrophoresis (PAGE) and transfer to nitrocellulose, immunoblotting was performed with either antiphosphotyrosine or anti-EGFR antibodies. The effect of EGF on PLC- γ phosphorylation can be seen in Fig. 1A. Phosphorylation of PLC- γ was observed at EGF concentrations as low as 1 nM. A similar concentration dependence was seen for both PLC- γ and receptor autophosphorylation (Fig. 1B), although more cells were required to detect PLC- γ phosphorylation. When lysates were

subjected to immunoprecipitation with anti-PLC- γ antibodies and then immunoblotting was performed with anti-EGFR antibody, it was found that increasing amounts of EGFR were coimmunoprecipitated by anti-PLC- γ antibodies as the EGF concentration was increased (Fig. 1C). This result demonstrates that EGF enhances the association of PLC- γ with EGFR in a dose-dependent manner at physiologic EGF concentrations.

Further studies were undertaken to examine the mechanism by which EGF enhanced this association. For these studies, NIH 3T3 cells expressing the kinase-negative EGFR mutant K721A were used. Cells expressing the kinase-negative or wild-type receptor were stimulated with 40 nM EGF, and then immunoprecipitation was carried out with anti-PLC- γ antibodies or anti-EGFR mAb108. In contrast to HER14 cells, no EGFR autophosphorylation or PLC- γ tyrosine phosphorylation was seen in response to EGF in K721A cells (Fig. 2A). Similarly, EGF did not induce coimmunoprecipitation of EGFR by anti-PLC- γ antibodies in the cells expressing the kinase-negative receptor (Fig. 2B). The inability of EGF to enhance the association of PLC- γ with the kinase-negative receptor was not related to the ability of the receptor to undergo EGF-induced dimerization (for recent reviews, see references 23 and 24), since both the kinase-negative and wild-type receptors formed dimers in response to EGF (Fig. 3). It therefore seemed likely that the kinase activity of the receptor was important in mediating the association of PLC- γ with EGFR.

Confirmation of this conclusion was obtained when HER14 cells were pretreated with the phorbol ester 12-*O*-tetradecanoyl-13-acetate (TPA). Phorbol esters are known to reduce both the kinase activity of the receptor and EGF binding affinity toward the receptor (1, 11, 12, 25). TPA caused a marked reduction in PLC- γ tyrosine phosphorylation but appeared to have less effect on receptor autophosphorylation (Fig. 4). The ability of TPA to decrease EGF-

FIG. 2. Tyrosine phosphorylation of PLC- γ (A) and coimmunoprecipitation of EGFR by anti-PLC- γ (B) in HER14 and K721A cells. NIH 3T3 clone 2.2 cells expressing wild-type (HER14) or kinase-negative (K721A) receptor cells were treated with 40 nM EGF for 2 min at 37°C. Immunoprecipitation and immunoblotting were then carried out as described for Fig. 1. To control for nonspecific immunoprecipitation of EGFR, immunoprecipitations were also performed with normal rabbit serum (NRS). A total of 15×10^6 cells were used in all anti-PLC and normal rabbit serum immunoprecipitations; 5×10^5 and 2.5×10^5 cells were used in the mAb108 (108) immunoprecipitations of panels A and B, respectively. Immunoblots were exposed for 16 h.

induced PLC- γ phosphorylation correlated with the well-known effect of phorbol esters to inhibit phosphatidylinositol turnover in response to EGF (21, 27). TPA also reduced the association of PLC- γ with EGFR, and this effect was quantitatively similar to its ability to reduce EGFR autophosphorylation. These data also suggested that inhibition of tyrosine kinase activity blunts the association of PLC- γ with EGFR but did not clearly demonstrate whether EGFR or PLC- γ phosphorylation was essential for the association. Further insight into this question was obtained when HER14 cells were stimulated with PDGF. PDGF treatment of HER14 cells resulted in tyrosine phosphorylation of PLC- γ similar to that seen with EGF. A 190-kDa tyrosine-phosphorylated molecule was coimmunoprecipitated with PLC- γ in PDGF-treated cells, but this protein was shown by immunoblotting not to be EGFR (Fig. 5). We and others have demonstrated previously that PDGF receptor is associated with PLC- γ in PDGF-stimulated NIH 3T3 cells (3a, 17). In fact, no enhancement of the PLC- γ -EGFR association was seen after PDGF treatment, suggesting that PLC- γ phosphorylation alone cannot be responsible for the association.

Additional insights were gained by studying the coimmunoprecipitation of PLC- γ by anti-EGFR mAb108. Cells were treated with 40 nM EGF for 2 min, and then immunoprecipitation was performed with either mAb108 or anti-PLC- γ antibodies. After separation by SDS-PAGE, immunoblotting was performed with anti-PLC- γ antibodies (Fig. 6). In EGF-treated HER14 cells, PLC- γ migrated in the gel as a doublet as a result of the decreased mobility of a heavily tyrosine phosphorylated PLC- γ (16, 17). A single band was seen in EGF-treated K721A cells, in which PLC- γ was not phosphorylated. mAb108 coimmunoprecipitated PLC- γ from lysates of HER14 cells treated with EGF but not from lysates of either unstimulated or EGF-treated K721A cells. This result is consistent with the data shown in Fig. 2, which shows that anti-PLC- γ antibodies immunoprecipitated EGFR only from EGF-treated HER14 cells, not from K721A

FIG. 3. Demonstration of dimerization of wild-type and kinase-negative EGFR in living cells. Cells expressing wild-type (HER14) or kinase-negative (K721A) receptors were treated with 40 nM EGF for 1 h at 4°C. Cells were then warmed to room temperature in the presence of the chemical cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). After 1 h, cells were lysed and immunoprecipitated with mAb108. After SDS-PAGE on a 4 to 6% gradient gel, proteins were transferred to nitrocellulose, probed with polyclonal anti-EGFR antibodies, and detected with 125 I-protein A and autoradiography. M and D, migration positions of monomers and dimers, respectively.

FIG. 4. Effects of phorbol esters on PLC- γ tyrosine phosphorylation, EGFR autophosphorylation, and coimmunoprecipitation of EGFR by anti-PLC antibodies. HER14 cells were treated with 50 nM TPA for 15 min at 37°C and then stimulated with 40 nM EGF for 2 min. Immunoprecipitation and immunoblotting were then carried out as described for Fig. 1. To quantitate the effects of TPA, the bands were cut from the nitrocellulose and counted in a gamma counter. TPA reduced the EGF-induced PLC- γ tyrosine phosphorylation to $8.0 \pm 0.1\%$ of the control value, reduced EGFR autophosphorylation to $50 \pm 6.2\%$ of the control value, and reduced coimmunoprecipitation of EGFR by anti-PLC- γ antibodies to $68 \pm 8.6\%$ of control (mean $\pm$ standard error; $n = 3$). A total of 15×10^6 cells were used for the anti-PLC immunoprecipitations; 2.5×10^5 cells were used in the mAb108 (108) immunoprecipitations. Immunoblots were exposed for 12 h.

cells. Only the faster-migrating form of PLC- γ was immunoprecipitated by mAb108 (Fig. 6). This result was confirmed in the experiment shown in Fig. 7, in which the forms of PLC- γ immunoprecipitated by mAb108 or antiphosphotyrosine antibodies were compared; antiphosphotyrosine antibodies immunoprecipitated only the slower-migrating species. This result indicates that anti-EGFR antibodies immunoprecipitate a species of PLC- γ that migrates in a position similar to that of the non-tyrosine-phosphorylated form of PLC- γ and faster than the form immunoprecipitated by antiphosphotyrosine antibodies. This finding demonstrates that EGFR does not associate with heavily tyrosine phosphorylated PLC- γ and suggests that the bulk of PLC- γ associated with EGFR is not tyrosine phosphorylated or only weakly phosphorylated. However, the tyrosine phosphorylation state of the associated PLC- γ cannot be exactly determined, since even the fast-migrating species of PLC- γ contains some phosphotyrosine (17).

An additional approach which demonstrates that either kinase activity or autophosphorylation is essential for enhanced association of PLC- γ with EGFR is shown in Fig. 8. In this experiment, EGFR was immunoprecipitated from unstimulated HER14 cells and phosphorylated *in vitro* with ATP and Mn^{2+} ; lysates from nonstimulated parental NIH 3T3 clone 2.2 cells were then added to the phosphorylated EGFR in the immunoprecipitates. PLC- γ from these EGFR-deficient NIH 3T3 clone 2.2 lysates bound to the immunoprecipitated EGFR but only when the receptor was activated and phosphorylated by the addition of Mn^{2+} and ATP (Fig. 8). Treatment of the phosphorylated EGFR with potato acid phosphatase (5) reversed receptor autophosphorylation and

FIG. 5. Effects of PDGF-induced PLC- γ tyrosine phosphorylation on the coimmunoprecipitation of EGFR by anti-PLC- γ antibodies. HER14 cells were treated with 40 nM EGF or 1.5 nM porcine platelet PDGF for 2 min at 37°C. After immunoprecipitation with anti-PLC- γ antibodies, immunoblotting was performed with antiphosphotyrosine or anti-EGFR antibodies. Immunoblots were exposed for 14 h.

association with PLC- γ (results not shown). This result provides further evidence that either kinase activation *per se* or receptor autophosphorylation is a critical event mediating EGF-enhanced association of PLC- γ with EGFR.

DISCUSSION

Studies of EGF-induced tyrosine phosphorylation of PLC- γ , using either $^{32}P_i$ labeling or antiphosphotyrosine immunoblotting, demonstrated coimmunoprecipitation of a tyrosine-phosphorylated 170-kDa molecule by anti-PLC- γ antibodies (16, 28). In our initial studies, we demonstrated that this 170-kDa molecule was EGFR itself but were not able to clearly assess the effect of EGF on this observed association. The more detailed study of this question presented in this report demonstrates that EGF treatment of cells results in enhanced association of the two molecules and in tyrosine phosphorylation of PLC- γ . These data are in agreement with the finding of Meisenhelder et al. (17) that PDGF promotes the association of the PDGF receptor and PLC- γ and tyrosine phosphorylation of PLC- γ . We further show that EGF enhances the association between PLC- γ and EGFR in a concentration-dependent manner and that this effect of EGF is dependent on EGFR kinase activity. This was demonstrated by the lack of EGF-induced association in cells expressing the kinase-negative EGFR mutant and the inhibition of association mediated by phorbol esters. Further proof came from our previous studies, which demonstrated that tyrphostins, inhibitors of EGFR tyrosine kinase activity, also blocked association (16). Oligomerization of EGFR is not sufficient to induce the association of EGFR with PLC- γ , since the kinase-negative receptor undergoes normal dimerization in response to EGF yet does not associate with PLC- γ .

FIG. 6. Immunoprecipitation of PLC- γ by anti-EGFR antibodies. HER14 cells were stimulated with 40 nM EGF for 2 min at 37°C, and then the lysates were subjected to immunoprecipitation with anti-PLC- γ antibodies or mAb108 (108). After immunoprecipitation and SDS-PAGE, anti-PLC- γ was used for immunoblotting. For the immunoprecipitation with anti-PLC- γ , 3.5×10^5 cells were used; for the mAb108 immunoprecipitation, 25×10^6 cells were used. Immunoblots were exposed for 20 h.

This work further demonstrates that the kinase activity is probably not mediating the EGF enhancement of association by phosphorylating PLC- γ . In fact, it was found that PLC- γ associated with EGFR is a faster-migrating species that is not immunoprecipitated by antiphosphotyrosine antibodies and that anti-EGFR antibodies cannot immunoprecipitate the heavily tyrosine phosphorylated form of PLC- γ . We also demonstrate (Fig. 8) that non-tyrosine-phosphorylated PLC- γ can associate with prephosphorylated EGFR. It has also been demonstrated that the PLC activity immunoprecipitated from EGF-stimulated A431 cells by antiphosphotyrosine antibodies is cytosolic and not membrane bound (29), suggesting that the tyrosine-phosphorylated PLC- γ is not associated with the membrane-bound EGFR. It is possible, therefore, that the association represents an enzyme-substrate intermediate in which the non-tyrosine-phosphorylated PLC- γ is complexed at the substrate-binding site of EGFR and that once the kinase reaction is completed, the phosphorylated PLC- γ product is released. This is consistent with the observation that at any time, only a small fraction of the cellular EGFR is associated with PLC- γ (16, 17). Indeed, in preliminary experiments, we found that the PLC- γ associated with the anti-EGFR immunoprecipitates (Fig. 6) could be partially washed from the EGFR if ATP and Mn^{2+} were added to the immunoprecipitate. In contrast, if only Mn^{2+} was added to the EGFR-PLC- γ complex, then PLC- γ could not be removed by washing with HNTG. However, the tight association of PLC- γ and EGFR may indicate that PLC- γ interacts with sites on EGFR in addition to the substrate-binding site.

It appears that kinase activation in some fashion alters EGFR, allowing enhanced association with PLC- γ . On the basis of the analysis presented here, it is impossible to distinguish between kinase activation per se and receptor

autophosphorylation as the critical step. It is noteworthy that autophosphorylation is a distinct step that can be separated from kinase activation. Nonetheless, it is interesting to speculate how autophosphorylation of EGFR might mediate this association. In EGFR, all four tyrosine autophosphorylation sites are located in the carboxy-terminal tail of the receptor (3, 15). It has been shown that autophosphorylation does not increase the kinase activity of the receptor, but rather autophosphorylation sites act as competitive inhibitors of exogenous substrate phosphorylation (6, 8). In this manner, the autophosphorylation sites can dampen substrate phosphorylation and finely control cellular responses to EGF. This model predicts that wild-type EGFR will autophosphorylate the receptor carboxy terminus, removing the competitive inhibition of the autophosphorylation sites and allowing PLC- γ to bind. In the kinase-negative mutant, autophosphorylation sites will not be phosphorylated and thus compete with PLC- γ for binding to the receptor substrate-binding site. Because the kinase-negative receptor undergoes normal oligomerization, the local concentration of the autophosphorylation sites may be very high and successfully block the association of PLC- γ with the substrate-binding site. It should be noted that this interpretation relies on the previously reported bi-bi molecular kinetics of the EGFR kinase with substrate binding before ATP binding (4). Obviously, if ATP must bind before the substrate, then this kinase-negative receptor will not associate with substrates because its interaction with ATP is impaired.

Another hypothesis that could explain the results is that the tyrosine autophosphorylation sites of the receptor con-

FIG. 7. Demonstration that anti-EGFR antibody coimmunoprecipitates the non-tyrosine-phosphorylated species of PLC- γ . HER14 cells were stimulated as for Fig. 6, and immunoprecipitation was performed with anti-PLC- γ , antiphosphotyrosine, or anti-EGFR (mAb108) antibodies. After immunoprecipitation and SDS-PAGE, immunoblotting was performed with anti-PLC- γ antibodies. A similar number of cells was used as in Fig. 6; 10×10^6 cells were used in the antiphosphotyrosine immunoprecipitation. Immunoblots were exposed for 16 h.

FIG. 8. Demonstration that cell-free autophosphorylation of EGFR enhances its association with PLC- γ . Immunoprecipitation was carried out with mAb108 on lysates from two unstimulated 15-cm-diameter dishes of HER14 cells. After washing with HNTG, the immunoprecipitates were allowed to autophosphorylate for 10 min at room temperature by addition of 5 mM MnCl₂ and 30 μ M ATP (+) or, as a control, 5 mM MnCl₂ alone (-). After further washes, lysates from two 15-cm-diameter dishes of nontransfected NIH 3T3 clone 2.2 cells were added to the immunoprecipitates for 90 min at 4°C. The immunoprecipitates were further washed with HNTG, and the proteins of the immunoprecipitates separated on a 6% SDS-gel. After transfer to nitrocellulose, anti-PLC- γ antibodies were used for immunoblotting; the blots were exposed for 14 h for autoradiography.

tribute to the PLC- γ -binding site on EGFR. It is possible that phosphotyrosine residues in the carboxy-terminal tail of the receptor are essential for binding or for a conformational change that results in PLC- γ binding. In initial experiments to test this hypothesis, we have found that a mutant EGFR with a 126-amino-acid carboxy-terminal deletion associates poorly with PLC- γ . However, it is difficult to assess how such an extensive deletion affects overall receptor folding. The inability of this mutant receptor to associate with PLC- γ may be due more to an altered tertiary structure than to deletion of the autophosphorylation sites proper. Further experiments will be necessary to resolve this problem. Studies on EGFR with autophosphorylation point mutants (6, 8) may be helpful in defining the importance of autophosphorylation versus kinase activation in mediating enhanced association. Additional information may be obtained by studying the ability of the kinase-negative receptor to associate with PLC- γ after it has been phosphorylated in an intermolecular process by active EGFR (9).

The actual relationship between the association of PLC- γ with growth factor receptors and the activation of PLC- γ is not known. If tyrosine phosphorylation of PLC- γ is the activating event, then the association may merely represent the substrate-enzyme interaction that is necessary for phosphorylation. The stability of the association between EGFR and PLC- γ might appear to be greater than can be explained by a pure substrate-enzyme interaction. However, very similar results have recently been obtained with a substrate of the PDGF receptor, raf-1, which also forms a stable complex with the PDGF receptor (19). As more substrates of

growth factor receptors are identified, it may be determined that this type of association is essential for phosphorylation. However, it is possible that the association of PLC- γ with these receptors is different from the association with other substrates. According to this scenario, the association might play a role in activation, such as by allowing better access to membrane phospholipids. Obviously, further studies on the exact mechanism of activation will be necessary to answer these questions. These studies will certainly lead to greater insights into signal transduction by growth factor receptors.

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