Activation of $G_{\text{src}}$ by the Epidermal Growth Factor Receptor Involves Phosphorylation*

(Received for publication, August 1, 1995, and in revised form, November 20, 1995)

Helen Poppleton, Hui Sun, David Fulghamt, Paul Berticst, and Tarun B. Patels
From the Department of Pharmacology, the Center for Health Sciences, University of Tennessee, Memphis, Tennessee 38163 and the Department of Biomolecular Chemistry, University of Wisconsin, Madison, Wisconsin 53706-1532

Previous studies from our laboratory have shown that epidermal growth factor (EGF) stimulates cAMP accumulation in the heart via a process involving $G_{s\alpha}$, and the EGF receptor (EGFR) protein tyrosine kinase activity (Nair, B. G., Parikh, B., Milligan, G., and Patel, T. B. (1990) J. Biol. Chem. 265, 21317-21322; Nair, B. G., and Patel, T. B. (1993) Biochem. Pharmacol. 46, 1239-1245). Therefore, studies were performed to investigate the hypothesis that the EGF protein tyrosine kinase phosphorylates $G_{s\alpha}$ and activates this protein. Employing purified EGFR and $G_{s\alpha}$, we have demonstrated that the EGFR kinase phosphorylates $G_{s\alpha}$ in a time-dependent manner with a stoichiometry of 2 mol of phosphate incorporated/mol of $G_{s\alpha}$. As determined by phosphoamino acid analysis, the phosphorylation of $G_{s\alpha}$ by the EGFR kinase was exclusively on tyrosine residues. Interestingly, GDP and guanosine 5'-O-(thio)triphosphate (GTPyS) inhibited the phosphorylation of $G_{s\alpha}$, without affecting EGFR autophosphorylation. However, G protein $\beta_{2}$-subunits protected against GDP- and GTPyS-mediated inhibition of phosphorylation of $G_{s\alpha}$. In functional studies, phospho-$G_{s\alpha}$ demonstrated a greater GTPase activity and also a greater capacity to bind GTPyS as compared to the nonphosphorylated $G_{s\alpha}$. Moreover, the phospho-$G_{s\alpha}$ augmented adenylyl cyclase activity in S49 cysvette membranes to a greater extent than its nonphosphorylated counterpart. Therefore, we conclude that phosphorylation of $G_{s\alpha}$ on tyrosine residues by the EGFR kinase activates this G protein and increases its ability to stimulate adenylyl cyclase.

Epidermal growth factor (EGF)1 exerts a variety of biological actions ranging from increased DNA synthesis, hyperplasia, and increased glucose and fatty acid metabolism, to alterations in muscular function (see Ref. 1 for review). These pleiotropic actions of EGF are mediated via the activation of several second messenger systems. For instance, following binding of EGF to its receptors, the intrinsic protein tyrosine kinase activity of the EGF receptor is increased, resulting in autophosphorylation of the EGF receptor as well as of other cellular proteins (reviewed in Refs. 1 and 2). The autophosphorylation of the EGF receptor serves to recruit proteins containing the Src homology 2 (SH2) domains such as phospholipase Ca (3), the subsequent phosphorylation of which increases phosphatidylinositol metabolism, and the generation of the second messengers inositol 1,4,5-trisphosphate and diacylglycerol (3). Likewise, recruitment of the SH2-containing proteins such as Grb2 and other adaptor proteins to the phosphorytine-containing domains on the EGF receptor also leads to the activation of serine/threonine phosphorylation cascades such as the mitogen-activated protein kinase cascade (4). In addition, EGF has also been documented to modulate the cAMP second messenger system. Studies from our laboratory have shown that EGF increases contractility, beating rate, and cAMP accumulation in the heart (5) by stimulating adenylyl cyclase via a process involving $G_{s\alpha}$ (6, 7). Moreover, the protein tyrosine kinase activity of the EGF receptor is important for EGF-mediated stimulation of cardiac adenylyl cyclase (8). One implication of this latter finding is that one, or more, of the signaling elements involved in stimulation of adenylyl cyclase by the activated EGF receptor is phosphorylated. Therefore, we have proposed the hypothesis that EGF phosphorylates $G_{s\alpha}$ and activates this G protein to stimulate adenylyl cyclase activity. This hypothesis incorporates the requirement for $G_{s\alpha}$ and the EGF receptor protein tyrosine kinase, the two components which our previous studies have determined to be required for EGF-elicted stimulation of the effector, adenylyl cyclase (7, 8).

Among the $\alpha$ subunits of heterotrimeric G proteins, $G_{s\alpha}$ has been demonstrated to be phosphorylated and inactivated by protein kinase C (9–11). The studies of Hausdorff et al. (12) have shown that in vitro, pp60src phosphorylates $G_{s\alpha}$ and that in reconstitution experiments with the $\beta$-adrenergic receptor, $\beta$-adrenoreceptor agonists such as isoproterenol augment GTPyS binding to phosphorylated $G_{s\alpha}$, to a greater extent than to nonphosphorylated $G_{s\alpha}$. The tyrosine kinase pp60src phosphorylates $G_{s\alpha}$ on tyrosine residues 37 and 377 (13) with a stoichiometry between 0.4 and 0.9 mol of phosphate/mol of $G_{s\alpha}$ (12). To date, phosphorylation of $G_{s\alpha}$ by tyrosine kinases other than pp60src, and in particular receptor protein tyrosine kinases, has not been reported. Therefore, to address our aforementioned hypothesis we have investigated whether or not the EGF receptor protein tyrosine kinase phosphorylates $G_{s\alpha}$ and modulates its activity. Our data demonstrate that the activated EGF receptor (EGFR) stoichiometrically phosphorylates $G_{s\alpha}$ and activates this protein as monitored by its ability to bind GTPyS and activate adenylyl cyclase. To our knowledge, this is the first demonstration of phosphorylation of $G_{s\alpha}$ by the EGF receptor with a concomitant increase in activity of this protein.

MATERIALS AND METHODS

Purification of $G_{s\alpha}$ and Bovine Brain $\beta_{2}$-Subunits—The BL21(DE3) strain of Escherichia coli transformed with the plasmid pQE-60, containing cDNA encoding the 45-kDa form of bovine $G_{s\alpha}$, was obtained

---

*This work was supported in part by National Institutes of Health Grant HL 48308 and a grant-in-aid from the American Heart Association, National Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Dept. of Pharmacology, University of Tennessee, 874 Union Ave., University of Tennessee, Memphis, TN 38163. Tel.: 901-448-6006; Fax: 901-448-7300.

‡The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; G protein, GTP-binding regulatory protein; $G_{s\alpha}$, stimulatory GTP binding regulatory protein of adenyl cyclase; $G_{s\alpha}$, $\alpha$ subunit of $G_{s\alpha}$; GTPyS, guanosine 5'-O-(thio)triphosphate; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidene fluoride).
from Alfred Gilman (University of Texas Southwestern Medical Center). Expression of Gsμ was induced with isopropyl β-D-thiogalactopyranoside and the protein was purified essentially as described by Graziolo et al. (14). Botine brain β subunits of heterotrimeric G proteins were purified to homogeneity as described by Mumby et al. (15) and Neer et al. (16). Heterotrimeric Gsμ was reconstituted by mixing active Gμ and β subunits (active Gμ: β ratio 1:1) and incubating for 30 min at 4°C; amount of active Gsμ was determined from maximal GTPγS binding (described below).

Phosphorylation of Gsμ. By Purified EGF-EGFR—EGFR was purified from A431 cells as described previously (17). Monomeric Gsμ (500 ng, equivalent to 110 pmol) or Gsμ:β heterotrimer (1:1) were phosphorylated in 20 mM Hepes, pH 7.4, 5 mM MgSO4, 2 mM MnCl2, 1 mM dithiothreitol, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 10 μM ATP and 100 nM EGF. Sodium vanadate (50 μM), GDP or GTPγS (3-10 μM), and [γ-32P]ATP (5 μCi); 6000 Ci/mmol) were added where indicated. Phosphorylation was initiated by addition of 0.21 pmol (33 ng) of purified EGFR and continued at 25°C for 60 min. Phosphoproteins were separated by SDS-PAGE (12% acrylamide gels) and visualized by autoradiography. Wherever necessary, phosphorylated proteins were excised from the gels and quantitated by scintillation counting.

Phosphoamino Acid Analysis of Phospho-Gsμ—Following phosphorylation of Gsμ and SDS-PAGE as described above, the proteins were electrophoretically transferred to PVDF membranes (Bio-Rad). Following autoradiography to locate the phosphoproteins on the PVDF membrane, the portions of the PVDF containing the proteins of interest were excised and subjected to phosphoamino acid analysis exactly as described by Martensen (18). Briefly, PVDF was rinsed with 2 × 1 ml of water and hydrolyzed for 60 min at 110°C in 6 n HCl, followed by evaporation to dryness in a vacuum desiccator (Speedvac, Savant). Hydroydysis was washed with 1.0 ml of H2O and 1.0 ml of 0.1 n HCl/formic acid (HCOOH) prior to application to an column of Dowex AG 50W-X2 (H+), 100-200 mesh (Bio-Rad) in 0.2 ml of 0.1 n HCOOH. Phosphoamino acids were eluted with 2 × 0.2 ml of 0.1 n HCOOH and 1 × 0.6 ml of 0.1 n HCOOH and separated by thin layer electrophoresis on cellulose acetate-coated plates (Merck, Darmstadt, Germany) in pyridine/glacial acetic acid/H2O (10:100:1890), pH 3.5 at 750 V for 90 min. Phosphoamino acids were located by autoradiography and identified by comigration with phosphoamino acid standards visualized with ninhydrin.

Detection of Phosphorylated Gsμ by Western Blotting—After incubation with or without EGF or ATP, Gsμ was separated from EGFR by SDS-PAGE (12% gel) and electrophoretically transferred to nitrocellulose membrane. Membrane was blocked in 10% newborn calf serum in phosphate-buffered saline and exposed to polyclonal anti-phosphotyrosine antibody (Zymed; Inc.: 1:10000 dilution). After washing, the membranes were exposed to goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10000 dilution). The Western blot was developed with the Amersham Corp. ECL system.

GTPase Activity Measurements—GTPase activity was measured as described by Brandt et al. (19) and modified by Okamoto et al. (20). Briefly, 500 ng of Gsμ (monomeric or heterotrimeric) were phosphorylated in the presence of 3 μM GDP. GTPase activity was then monitored in medium containing the following at final concentration: 25 mM Hepes- NaOH, pH 8.0, 110 μM EDTA, 200 μM MgSO4, 1 mM dithiorthetol, and 100 μM [γ-32P]GTP (total volume, 1 ml). Aliquots (100 μl) were withdrawn at various times and transferred to tubes containing ice-cold 5% (w/v) Norit A in 50 mM NaH2PO4. Following centrifugation, the 32P content of the supernatants was measured by scintillation counting.

GTPγS Binding Studies—GTPγS binding was measured by the method of Northup et al. (21) as modified by Sun et al. (22). Essentially, heterotrimeric Gsμ (500 ng of Gsμ) were phosphorylated in the presence of 10 μM GDP as described above. GTPγS binding was initiated by addition of 0.98 ml of a buffer containing the following at final concentration: 50 μM Hepes, pH 8.0, 120 μM MgSO4, 100 μM EDTA, 1 mM dithiorthetol, and 100 nM GTPγS. Aliquots (0.1 ml) were withdrawn at various times and transferred to tubes containing 2.0 ml of ice-cold 25 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 25 mM MgSO4 to terminate the binding reaction. Bound and unbound GTPγS were separated by rapid filtration through BAB5 nitrocellulose filters (0.45 μm; Schleicher & Schuell). Non-specific binding was measured in the presence of excess (100 μM) unlabeled GTPγS, and maximal binding was measured in the presence of 1 μM GTPγS and 25 mM MgSO4.

Adenylyl Cyclase Assays with S49 cytosol—Cell Membranes—The ability of heterotrimeric nonphosphorylated Gsμ and phospho-Gsμ to activate adenylyl cyclase activity in mouse lymphoma S49 cytosol cell membranes was measured as described by Sun et al. (22). Briefly, heterotrimeric Gsμ was phosphorylated in the presence of 10 μM GDP; nonphosphorylated Gsμ was similarly treated in the absence of EGF. Both forms of Gsμ were then precipitated for 60 min at 25°C with GTPγS (100 nM) in the GTPγS-binding buffer described above except that the MgSO4 concentration was 500 μM. The phosphorylated and nonphosphorylated Gsμ (1.1 pmol) were then reconstituted with 10 μg of S49 cytosol membrane protein for 5 min at 0°C. Adenylyl cyclase reactions were performed in quadruplicate as described by Sun et al. (22).

RESULTS AND DISCUSSION

Previous data from this laboratory have demonstrated that EGF stimulates adenylyl cyclase activity in cardiac membranes via activation of Gsμ (6, 7) and that the EGF receptor tyrosine kinase activity is necessary for this effect (8). Therefore, in order to determine whether or not the EGF protein tyrosine kinase phosphorylates Gsμ and to evaluate the functional significance of such a phosphorylation, experiments were performed with the purified EGFR and pure Gsμ. Initially, the ability of the EGFR kinase to phosphorylate Gsμ as a function of time and the dependence of this phosphorylation on the presence of EGF and ATP were determined. As illustrated by the data in Fig. 1, the purified EGFR receptor increased phosphorylation of Gsμ in a time-dependent manner (Fig. 1A); maximal phosphorylation of the Gsμ was obtained 60 min after initiation of the phosphorylation reaction at 25°C. Moreover, as demonstrated by the data in Fig. 1B, the phosphorylation of Gsμ was dependent upon the presence of the EGFR and ATP. In studies similar to those depicted in Fig. 1, the stoichiometry of phosphorylation of Gsμ by EGF was determined to be 1.73 ± 0.3 (n = 4) mol of P, incorporated/mol of Gsμ. As a control, the stoichiometry of autophosphorylation of the EGFR receptor was monitored in parallel and found to be 4.5 ± 0.5 (n = 5) sites phosphorylated per mol of the EGFR. While the latter data...
Interestingly, in the absence of GDP or GTP, the protein bands corresponding to EGFR and Gs were excised from the gels and counted for 32P content. The data in Fig. 4 demonstrate that the GTPase activity of the phospho-Gs was 2-fold greater than that of its nonphosphorylated counterpart; in these experiments, controls with βγ subunits plus EGFR alone were found to have negligible activity (not shown). This increase in GTPase activity of the phosphorylated Gs also suggests that the EGFR kinase phosphorylates Gs on tyrosine residues which may be different to those phosphorylated by the pp60-src (13) since phosphorylation of Gs by the latter kinase does not alter its GTPase activity (12). As a second approach to assess functional significance, experiments were also performed to determine if phosphorylation of Gs by EGFR kinase altered the ability of the protein to bind GTPγS. As demonstrated by the data in Fig. 4B, phospho-Gs bound GTPγS at a greater rate and to a larger extent as...
ence of guanine nucleotides as described under “Materials and Methods.” Addition of phospho-Gs subunits markedly activated this G protein. Therefore, to determine whether or not the phosphorylated Gs subunits (Gsφ) were different from the nonphosphorylated form (Gsα), we performed experiments to assess the activatory ability of each form. The data (Fig. 3) indicate that the GTP-bound form of Gsα subunits were different from the GDP-bound form in essentially three regions (switch I, switch II, and switch III) none of which contains tyrosine residues (26). However, on Gsα, tyrosine residues (Tyr-176 and -239) are located proximal to switch I and switch III regions and it is plausible that phosphorylation of one or both of these residues by the EGFR receptor increases the rate of GTP hydrolysis binding that is observed (Fig. 4B). Likewise, since tyrosine residues (Tyr-325, -344, and -346) are also located in the proximity of adenyl cyclase interacting regions on Gsα (see e.g. Ref. 26), it is tempting to speculate that phosphorylation of one of these residues increases the interaction of Gsα with adenyl cyclase, thereby augmenting activity as observed in Fig. 4C. Interestingly, phosphorylation of the heterotrimeric form of Gsα by the EGFR receptor was not altered by either GDP (Fig. 3B) or GTP-γS (not shown) suggesting that the tyrosine residues on Gsα that are phosphorylated reside in region(s) whose conformation is stabilized by the βγ subunits. Additional studies which will identify the sites on Gsα that are phosphorylated by the EGFR receptor will provide more mechanistic information in the light of the crystal structure of Gtα (26). Presently, from the data presented in Fig. 3, we can conclude that guanine nucleotide binding (either GDP or GTP) inhibits the phosphorylation of Gsα by the EGFR receptor and that βγ subunits protect against such inhibition of phosphorylation.

Several laboratories have reported the phosphorylation of α subunits of G proteins by different kinases as a potential regulatory event in signal transduction. However, functional significance of the phosphorylation has been demonstrated in only a few of the studies. Thus it is clear that phosphorylation of Gα by protein kinase C decreases the activity of this G protein (9–11). On the other hand, the functional consequence of phosphorylation of Gα by protein kinase C (29) remains to be elucidated. Similarly, although the α subunits of transducin (30), Go, and Gi (31) have been shown to be phosphorylated by the insulin receptor protein tyrosine kinase, the functional consequences of these phosphorylations remain unknown. It should be noted that Gα is not phosphorylated to any significant extent by either protein kinase C (29) or the insulin receptor protein tyrosine kinase (31). In this respect, our findings for the first time, demonstrate the phosphorylation of Gα by a receptor protein tyrosine kinase and provide information concerning the alteration in the Gα function due to these phosphorylations. Most importantly, the data presented here provide a possible mechanism for EGF-mediated stimulation of adenyl cyclase activity. Notably, however, this may not be the only mechanism involved in EGF-elicted stimulation of adenyl cyclase activity but may represent one of two different, but not mutually exclusive, manners by which EGFR may augment adenyl cyclase activity. Hence, recently we have shown that a juxtamembrane 13-amino acid sequence in the EGFR can activate Gα and thereby stimulate adenyl cyclase activity (22). This finding is consistent with the hypothesis that upon binding to its receptors EGFR stimulates autophosphorylation of the receptors which results in a change in the conformation of the cytosolic domain of the EGFR receptor from a compact to an extended form (32) and thereby, allows the juxtamembrane

**Fig. 3.** GDP and GTP-γS inhibit the phosphorylation by EGFR kinase of Gsα in its monomeric but not heterotrimeric form. Panel A, inhibition of monomeric Gsα phosphorylation in the presence of varying concentrations of GDP (circles) or GTP-γS (squares). Purified Gsα was phosphorylated by the EGFR kinase in the absence and presence of guanine nucleotides as described under “Materials and Methods.” After separation of proteins by SDS-PAGE and location of the Gsα and EGFR by autoradiography, the two protein bands were excised and counted for 32P content. Data are presented as percent of radioactivity as determined by Coomassie staining of the gel.

Panel B, GDP does not inhibit phosphorylation of the Gsα in its heterotrimeric form. Purified Gsα mixed with G protein βγ subunits (Gsα:βγ = 1:1) were phosphorylated in the absence and presence of GDP (10 μM). Following separation of proteins, the gels were dried and subjected to autoradiography. Lane 1, Gsα + EGFR; lane 2, Gsα + GTP + GDP (10 μM); lane 3, Gsα:βγ + EGFR; lane 4, Gsα:βγ + GDP + GTP (10 μM). The asterisk indicates the migration of β subunit as determined by Coomassie staining of the gel.

compared to the nonphosphorylated Gsα; GTP-γS binding to βγ subunits plus EGFR (control) was negligible (not shown). These data (Figs. 4, A and B) strongly suggested that phosphorylation of Gsα in its heterotrimeric form (i.e. in presence of GDP and βγ subunits) markedly activated this G protein. Therefore, to determine whether or not the phosphorylated Gsα was indeed more effective as an activator of adenyl cyclase, experiments were performed employing 549 cyc−cell membranes which do not contain any endogenous Gsα (25). The data from these experiments demonstrated that upon reconstitution of equal amounts of nonphosphorylated and phosphorylated Gsα with cyc−membranes, the adenyl cyclase activity was 2-fold greater in the presence of the phospho-Gsα than its non-phosphorylated counterpart (Fig. 4C). In controls performed with βγ subunits with the EGFR (control), adenyl cyclase activity was very low and comparable to the activity in the absence of any additions (cf. Fig. 4C and Ref. 22).

Since GDP and GTP-γS inhibit phosphorylation of Gsα similarly (Fig. 3), and because the structures of GDP- and GTP-bound forms of Gsα subunits are different; see e.g. with transducin α subunit (Gsβ) (26) and Gαi (27, 28), our data (Fig. 3A) would suggest that the tyrosine residues whose phosphorylation is altered by the guanine nucleotides is(are) not located in the regions of the molecule that change conformation upon exchange of GDP for GTP. Indeed the crystal structure data of the GDP- and GTP-bound forms of Gsα and Gαi (26–28) indicate that the GTP-bound form of Gsα subunits are different from the GDP-bound form in essentially three regions (switch I, switch II, and switch III) none of which contains tyrosine residues (26). However, on Gsα, tyrosine residues (Tyr-176 and -239) are located proximal to switch I and switch III regions and it is plausible that phosphorylation of one or both of these residues by the EGFR receptor increases the rate of GTP hydrolysis binding that is observed (Fig. 4B). Likewise, since tyrosine residues (Tyr-325, -344, and -346) are also located in the proximity of adenyl cyclase interacting regions on Gsα (see e.g. Ref. 26), it is tempting to speculate that phosphorylation of one of these residues increases the interaction of Gsα with adenyl cyclase, thereby augmenting activity as observed in Fig. 4C. Interestingly, phosphorylation of the heterotrimeric form of Gsα by the EGFR receptor was not altered by either GDP (Fig. 3B) or GTP-γS (not shown) suggesting that the tyrosine residues on Gsα that are phosphorylated reside in region(s) whose conformation is stabilized by the βγ subunits. Additional studies which will identify the sites on Gsα that are phosphorylated by the EGFR receptor will provide more mechanistic information in the light of the crystal structure of Gtα (26). Presently, from the data presented in Fig. 3, we can conclude that guanine nucleotide binding (either GDP or GTP) inhibits the phosphorylation of Gsα by the EGFR receptor and that βγ subunits protect against such inhibition of phosphorylation.
**Activation of Gs by Phosphorylation**

**FIG. 4.** Phosphorylation of Gs by EGFR kinase increases its functional activity. GsGTP was phosphorylated in the presence of GDP and βγ subunits as described under "Materials and Methods." Control (nonphosphorylated) GsGTP was similarly treated except that the EGFR was not added in the phosphorylation reactions. As additional control, the EGFR and βγ subunits were incubated in the absence of GsGTP. Following the phosphorylation reaction, GTPase activity, GTPγS binding, and the ability of phosphorylated and nonphosphorylated GsGTP to stimulate adenylyl cyclase were monitored. Panel A, GTPase activity of phosphorylated and nonphosphorylated GsGTP. Following the initial incubation for 60 min in the phosphorylation reaction with and without EGFR, the GsGTP(βγ) was transferred into the GTPase reaction mixture, and aliquots were withdrawn at the various times indicated. A representative experiment of three is shown. Panel B, GTPγS binding to phosphorylated and nonphosphorylated GsGTP. The conditions were similar to those described for data in Panel A except that the Gs was transferred into GTPγS binding buffer and aliquots were withdrawn to monitor binding. The precise experimental conditions are described under "Materials and Methods." A representative experiment of three is shown. Panel C, ability of phosphorylated and nonphosphorylated GsGTP to stimulate adenylyl cyclase activity in S49 cell membranes. Following incubation of GsGTP with GDP (10 μM) and βγ subunits in phosphorylation buffer in the presence or absence of EGFR, the GsGTP was incubated in the presence of GTPγS (100 nM) in the binding reaction mixture for 60 min. All aliquots (1.2 pmol) of GsGTP were then reconstituted with 10 μg of GTPγS cell membranes and assayed for adenylyl cyclase activity as described under "Materials and Methods." Data are presented as the mean ± S.E. of four determinations.

In conclusion, we have presented experimental evidence to demonstrate that EGFR receptor protein tyrosine kinase can phosphorylate GsGTP on tyrosine residues and stimulate the functional activity of this G protein. To our knowledge, this is the first demonstration of a receptor protein tyrosine kinase that can phosphorylate and activate Gs. Moreover, these data provide mechanistic insights into EGFR elicited stimulation of adenylyl cyclase activity. Presently, the identity of the tyrosine residues on GsGTP that are phosphorylated by EGFR kinase remain to be elucidated. However, stoichiometry analyses indicate that at least 2 tyrosine residues are phosphorylated. Whether or not the phosphorylation of one of these 2 tyrosine residues alters the GTPase and GTPγS binding activities preferentially or phosphorylation of both tyrosines is required to observe the functional changes is not known. These questions and the possibility that there may be a hierarchy in the phosphorylation of the two tyrosines on GsGTP by the EGFR kinase forms the subject of our future investigations.

Acknowledgments—We are greatly indebted to Dr. A. G. Gilman (University of Texas Southwestern Medical Center, Dallas) for providing us with the BL21-DE3 strain of E. coli transformed to express recombinant GsGTP (45 kDa). We are also grateful to Dr. Ravi Iyengar (Mt. Sinai Medical School, New York) for providing us with S49 GTPγS cell membranes. We thank Dr. James C. Garrison (University of Virginia School of Medicine, Charlottesville, VA) for providing purified recombinant GsGTP.

REFERENCES

1. Carpenter, G., and Wahl, M. I. (1990) Handb. Exp. Pharmacol. **95**, 69–171
2. Carpenter, G., and Cohen, S. (1960) J. Biol. Chem. **265**, 7709–7712
3. Hernandez-Sotomayer, S. M. T., and Carpenter, G. (1992) J. Membr. Biol. **128**, 81–89
4. Marshall, C. J. (1995) Cell **80**, 179–185
5. Nair, B. G., Rashed, H. M., and Patel, T. B. (1993) Growth Factors **8**, 41–48
6. Nair, B. G., Rashed, H. M., and Patel, T. B. (1988) Biochem. J. **264**, 563–571
7. Nair, B. G., Parikh, B., Milligan, G., and Patel, T. B. (1990). J. Biol. Chem. **265**, 21317–21322
8. Nair, B. G., and Patel, T. B. (1993) Biochem. Pharmacol. **46**, 1239–1245
9. Pyne, N. J., Murphy, J. G., Milligan, G., and Houslay, M. D. (1989) FEBS Lett. **243**, 77–82
10. Bushfield, M., Pyne, N. J., and Houslay, M. D. (1991) Eur. J. Biochem. **192**, 537–542
11. Strasheim, D., and Malbon, C. C. (1994) J. Biol. Chem. **269**, 14307–14313
12. Hausdorff, W. P., Pitcher, J. A., Luttrel, D. K., Linder, M. E., Kurose, H., Parsons, S. J., Caron, M. G., and Lefkowitz, R. J. (1992) Proc. Natl. Acad. Sci. U. S. A. **89**, 5720–5724
13. Moyers, J. S., Linder, M. E., Shannon, J. D., and Parsons, S. J. (1995) Biochem. J. **305**, 411–417
14. Graziano, M. P., Freimuth, M., and Gilman, A. G. (1991) Methods Enzymol. **196**, 192–213
15. Mumbay, S., Pang, I.-H., Gilman, A. G., and Sternweis, P. C. (1988) J. Biol. Chem. **263**, 2020–2026
16. Nair, B. G., Lok, J. M., and Wolf, L. G. (1984) J. Biol. Chem. **259**, 14222–14229
17. Hubler, L., Levant, S. P., and Bertics, P. J. (1992) Biochem. J. **281**, 107–114
18. Martenssen, T. (1984) Methods Enzymol. **107**, 3–23
19. Brandt, D. R., Assano, T., Pedersen, S. E., and Ross, E. M. (1983) Biochemistry **22**, 4357–4362
20. Okamoto, T., Murayama, Y., Hayashi, Y., Uii, M., Ogata, E., and Nishimura, T. (1991) Cell **68**, 723–730
21. Northup, J. K., Singel, M. D., and Gilman, A. G. (1982) J. Biol. Chem. **257**, 11416–11423
22. Sun, H., Seyder, J. M., and Patel, T. B. (1995) Proc. Natl. Acad. Sci. U. S. A. **92**, 2229–2233
23. Margolis, B. J., Lax, I., Kris, R., Dombalagian, M., Hongger, A. M., Hawk, R., Givol, D., Ullrich, A., and Schlessinger, J. (1989) J. Biol. Chem. **264**, 10667–10671
24. Hart, M. J., Polakis, P. G., Evans, T., and Cerione, R. A. (1990) J. Biol. Chem. **265**, 5990–6001
25. Sternweis, P. C., and Gilman, A. G. (1979) J. Biol. Chem. **254**, 3333–3340
26. Lambright, D. G., Noël, J. P., Hamm, H. E., and Sigler, P. B. (1994) Nature **369**, 621–628
27. Coleman, D. E., Lee, E.,Mixin, M. B., Linder, M. E., Berghuis, A. M., Gilman, A. G., and Sprang, S. R. (1994) J. Mol. Biol. **238**, 630–634
28. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) Science **265**, 1405–1412
29. Lounsbury, K. M., Casey, P., Brass, L. F., and Manning, D. R. (1991) Biochem. Pharmacol. **46**, 41–48
30. Zick, Y., Sagi-Eisenberg, Pines, M., Gierschik, P., and Spiegel, A. M. (1986) Proc. Natl. Acad. Sci. U. S. A. **83**, 9294–9297
31. Krupinski, J., Rajaram, R., Lakonishok, M., Benovic, J. L., and Cerione, R. A. (1988) J. Biol. Chem. **263**, 12333–12341
32. Cadena, D. L., Chan, C., and Gill, G. N. (1994) J. Biol. Chem. **269**, 260–265
