Previously, we have demonstrated that epidermal growth factor (EGF) can stimulate adenylyl cyclase activity via activation of Gs in the heart. Moreover, we have recently shown that Gs is phosphorylated by the EGF receptor protein tyrosine kinase and that the juxtamembrane region of the EGF receptor can stimulate Gs directly. Therefore, employing isolated cardiac membranes, the two-hybrid assay, and in vitro association studies with purified EGF receptor and Gs, we have investigated Gs complex formation with the EGF receptor and elucidated the region in the receptor involved in this interaction. In isolated cardiac membranes, immunoprecipitation of EGF receptor was accompanied by co-immunoprecipitation of Gs. In the yeast two-hybrid assay, the cytosolic domain of the EGF receptor and the N-terminal 64 amino acids of this region (Met644–Trp707) associated with Gs. However, interactions of these regions of the EGF receptor with constitutively active Gs were diminished in the two-hybrid assay. Employing purified proteins, our studies demonstrate that the EGF receptor, directly and stoichiometrically, associates with Gs (1 mol of Gs/mol of EGF receptor). This association was not altered in the presence or absence of ATP and therefore, was independent of tyrosine phosphorylation of either of the proteins. Peptides corresponding to the juxtamembrane region of the receptor decreased association of the EGF receptor with Gs. However, neither the C-terminally truncated EGF receptor (Δ1022–1186) nor a peptide corresponding to residues 985–996 of the receptor altered association with Gs, thus indicating the selectivity of the G protein interaction with the juxtamembrane region. Interestingly, peptides corresponding to N and C termini of Gs did not alter the association of Gs with the EGF receptor. Consistent with the findings from the two-hybrid assay where constitutively active Gs poorly associated with the EGF receptor, in vitro experiments with purified proteins also demonstrated that activation of Gs by guanosine 5’-3-O-(thio)triphosphate decreased the association of G protein with the EGF receptor. Thus, we conclude that the juxtamembrane region of the EGF receptor, directly and stoichiometrically, associates with Gs and that upon activation of Gs this association is decreased.

The pleiotropic actions of epidermal growth factor (EGF) are elicited by stimulation of a number of second messenger systems by the ligand-activated EGF receptor (1, 2). In addition to its well-documented effects on the mitogen-activated protein kinase cascade (3) and phospholipase Cγ (4), EGF has also been demonstrated to regulate the cAMP second messenger system (5). We have previously demonstrated that in cardiac myocytes EGF elevates cAMP accumulation (6) by augmenting the activity of adenylyl cyclase (7, 8) and that this increase in cAMP accumulation also augments the beating rate and contractility in intact hearts (9). EGF stimulates cardiac adenylyl cyclase by activation of the α-subunit of the stimulatory GTP-binding protein, Gαs (8), and the protein tyrosine kinase activity of the EGF receptor is important in this modulation (10). More recently, we have demonstrated that a 13-amino acid sequence in the cytosolic, juxtamembrane, region of the EGF receptor is important for activation of Gs and stimulation of adenylyl cyclase (11). In addition to the activation of Gs by the juxtamembrane region of the receptor (11), we have also shown that the EGF receptor protein tyrosine kinase can phosphorylate Gs on tyrosine residues and that this phosphorylation of Gs increases its ability to stimulate adenylyl cyclase (12). Thus, the combined actions of the juxtamembrane region of the activated EGF receptor on Gs (11) and phosphorylation of Gs by the EGF receptor protein tyrosine kinase (12), may, in a mutually reinforcing manner, amplify the signaling events leading to adenylyl cyclase stimulation.

The juxtamembrane region of the EGF receptor has also been shown to be important for determining specificity of mitogenic signaling as well as determining substrate specificity. Thus, the deletion of 8 amino acids in the juxtamembrane region of the EGF receptor (amino acids 660–667) alters the mitogenic activity of EGF (13). In addition, the mutation of one amino acid residue in this region (Arg562) of the EGF receptor alters the mitogenic signaling and pattern of protein phosphorylations within the cells without altering the protein tyrosine kinase activity of the receptor (14). Likewise, the juxtamem-

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1 The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EGFR(JM), juxtamembrane region of the EGFR (amino acids 644–1186); EGFR(JM), juxtamembrane region of the EGFR (amino acids 63–707); EGFR(JM), juxtamembrane region of the EGFR encompassing residues 695–1186 in which juxtamembrane 50 amino acids (residues 645–694) are deleted; EGFR-13, peptide corresponding to amino acids 645–657 of the EGFR; EGF-13, EGFR-13 in which residue corresponding to Thr654 in EGFR is phosphorylated; EGFR-14, peptide corresponding to amino acids 679–692 of the EGF receptor; G-protein, GTP-binding regulatory protein; Gs, stimulatory GTP binding regulatory protein of adenylyl cyclase; Gs, light subunit of Gs; Gs, constitutively active Q213L mutant of Gs; Gs, 15–29, Gs, 354–372, peptides corresponding to amino acids indicated by numbers in parentheses, GTP-S, guanosine 5’-3-O-(thio)triphosphate; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; APP, amyloid precursor protein.
brane region (Met644–GlY666) of the EGF receptor is also important for the association of phosphatidylinositol-4 kinase and phosphatidylinositol 4-phosphate 5-kinase (15). This latter region encompasses the 13-amino acid region that activates Gsz (11). Because the juxtamembrane region of the EGF receptor has been demonstrated to be important for activation of Gsz (11), as well as binding of proteins, and determining substrate specificity for the EGF receptor protein tyrosine kinase (13–15), the studies described herein were performed to characterize the association of EGF receptor with Gsz, and to elucidate region(s) in the receptor that are important for such an association. Our studies demonstrate that in cardiac membranes, the activated EGF receptor associates with Gsz. Moreover, our data from yeast two-hybrid assays, as well as from in vitro association studies with purified proteins (EGF receptor and Gsz), demonstrate that the juxtamembrane region of the EGF receptor is important for association with Gsz, and that the GDP-ligated Gsz, not GTP-Gsz, is the preferred form of the G protein which physically interacts with the EGF receptor. Interestingly, the association between EGF receptor and Gsz is stoichiometric, direct (not involving adapter protein(s)), and does not involve the major phosphorylation sites on the EGF receptor.

MATERIALS AND METHODS
Isolation of Cardiac Membranes and Incubations—Hearts excised from male rats of the Harlan Sprague Dawley strain (180–200-g body weight) were homogenized in medium containing 5 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 1 mM EDTA. Cardiac membranes were isolated from the homogenate by the methods previously described (7, 8). The cardiac membranes (300 μg of protein) were then incubated in adenyly cyclase assay mixture described previously (7, 8) in the presence and absence of EGF (100 nm). Following incubation for 30 min at room temperature, the reactions were terminated by addition of lysis buffer described below and immunoprecipitations performed (see below).

Construction of Plasmids Encoding ChimERIC Proteins—Essentially, this assay was performed using the plasmids and yeast strains provided in the Matchmaker™ kit (Clontech Laboratories Inc.). Employing the full-length human EGF receptor cDNA as template (gift from Dr. Gordon D. Anderson, University of California at San Diego), and primers corresponding to nucleotides 1932–1953 (sense strand; primer sequence: 5′-GGGAATTCATGCGAAGGCGCCACATCGTTCGG-3′) and 3538–3561 (complementary strand; primer sequence: 5′-AGGTGCGACG-GATTCCTACGTGCCTCAATAATACTACTTGCT-3′), the complete cytosolic region of the EGF receptor (amino acids 644–1186; nucleotides 2188–3816) was generated by PCR. The 5′ primer introduced an EcoRI site (underlined above) and the 3′ primer was tagged with BamHI and SaI site downstream (underlined above). The addition of the unique EcoRI site at the 5′ end facilitated the in-frame cloning of the cDNA corresponding to the cytosolic region of EGF receptor into the plasmids pGAD424 and pGBT9; these constructs are referred to as pGAD424-EGFRc and pGBT9-EGFRc, respectively.

The plasmids pGAD424 and pGBT9 contain the activating domain of the yeast alcohol dehydrogenase promoter. The BamHI site at the 3′ end (introduced by PCR) along with an internal BamHI site at nucleotide 2121 in the EGF receptor cDNA facilitated the truncation of the chimeric constructs in plasmids pGAD424-EGFRc and pGBT9-EGFRc, so that only N-terminal 64 amino acids (Met644–Trp707) in the juxtamembrane region of the cytosolic domain of the EGF receptor were expressed as fusion proteins with the activating domain and binding domain of Gsz, respectively. These chimeric constructs in the plasmids expressing the short form of the EGF receptor cytosolic domain are referred to as pGAD424-EGFR△53 and pGBT9-EGFR△53. In addition, using a 5′ primer corresponding to amino acids Gly848–Pheno (sequence: 5′-CAAATCGCTGCG-3′) tagged with a SacI site (underlined) and a 3′ complementary primer corresponding to nucleotides 3796–3816 described above, constructs pGAD424-EGFR△3 and pGBT9-EGFR△3 were also generated. These latter constructs encoded all of the cytoplasmic region (amino acids 694–1186) of the EGF receptor devoid of the juxtamembrane region (amino acids 645–694).

Employing the full-length Gsz cDNA as template (obtained from Dr. Alfred Gilman, University of Texas Southwestern Medical Center) and primers corresponding to nucleotides 1–27 (sense strand; sequence: 5′-ATTTCAAGCGCAGGCTGCATACTGCTGCTGAAAGCGC-3′) and 1122–1143 (complementary strand; sequence: GAGGTTGTCGAGCCTCTATTAGCGAAGCTCATACTGAGG 3′), SaI restriction endonuclease sites underlined in sequence above were introduced on the 5′ and 3′ ends, respectively. The SaI site at the 5′ end facilitated the in-frame cloning of the Gsz cDNA into the plasmids pGAD424 and pGBT9 to generate plasmids pGAD424-Gsz+ and pGBT9-Gsz+, respectively; numbering system for nucleotides and amino acids used here are those for the short form of Gsz (16). The constitutively active form of Gsz+ (Q213L) (17) was obtained by a two-step PCR. First, using Gsz+ cDNA as template along with 5′ sense primer mentioned above and a 3′ complementary primer corresponding to nucleotides 628–648 of Gsz+ with a T→A substitution at nucleotide 638 (to substitute glutamine for leucine; sequence: TTCGCGCCAGGCGCCGCCCAC), a PCR product was generated. Similarly, using a sense primer corresponding to nucleotides 628–650 of Gsz+ with an A→T substitution at nucleotide 638 and the 3′ complementary primer to Gsz+ mentioned above, another PCR product was generated. Using the PCR products of Gsz+, with the A→T and T→A substitutions in the coding and noncoding strands, respectively, and using the 5′-most sense and 3′-most primers mentioned above, the full length Gsz+ cDNA coding for the constitutively active form of the protein was generated by a second round of PCR. This latter cDNA encoding the constitutively active form of Gsz+ (Gsz+*) was then cloned to the SaI site of pGAD424 and pGBT9 vectors to generate plasmids pGAD424-Gsz+* and pGBT9-Gsz+*+, respectively. The mutation of Gsz+ at nucleotide 638 was confirmed by di-deoxynucleotide sequencing method (18). Likewise, all of the plasmid constructs were sequenced to confirm that the cloning of the appropriate cDNAs was in-frame for transcription and devoid of any mistakes resulting from PCR.

Two-hybrid Assay—The two-hybrid assay was performed employing the HF7c strain of yeast (Clontech Laboratories Inc.) transformed with the various constructs in plasmids pGAD424 and pGBT9. Transformation of cells was performed as described by Clontech Laboratories Inc. in the Matchmaker™ kit. The transformed yeast cells were grown on plates containing either medium devoid of L-leucine (Leu−) and L-trypophan (Trp−) or medium in which both L-leucine and L-trypophan were omitted. The plates were incubated at 30°C for 3 days. Several of the colonies from transformants were then individually streaked out onto new plates containing the corresponding media.

To monitor β-galactosidase activity in transformants growing on Leu−/Tryp−/His− medium, colonies were individually grown in liquid medium containing 5 mM Tris-HCl, 4 mM MgCl2, 0.5 mM MnCl2, 40 mM NaHPO4, 40 mM NaH2PO4, 1 mM KCl, 1 mM MgCl2, pH 7.4, and then lysed in the same buffer supplemented with Triton X-100 (final concentration, 0.01%). The cell lysates were subjected to two cycles with liquid nitrogen and assayed for β-galactosidase activity employing the chromiluminescence kit obtained from Clontech Laboratories Inc.

Purification of Gsz and EGF Receptor—The BL21(DE-3) strain of Escherichia coli transformed with the plasmid pQE-60, containing cDNA encoding the 45-kDa form of bovine Gsz was obtained from Dr. Alfred Gilman (University of Texas Southwestern Medical Center, Dallas, TX). Expression of Gsz+ was induced with isopropyl-β-D-thiogalactopyranoside, and the protein was purified essentially as described by Graziano et al. (19). EGF receptor was purified from A431 cells as described previously (20).

In Vitro Association of EGF Receptor and Gsz+—Purified EGF receptor (35 ng) and recombinant Gsz+ (25 ng) were incubated in a medium (final volume = 10 μl) containing 5 mM HEPES-NaOH, pH 7.4, 5 mM MgCl2, 2 mM MnCl2, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 μM dithiothreitol, and 1 μM EGF with or without 10 μM ATP at room temperature for 60 min. The mixture was supplemented with 500 μl of immunoprecipitation buffer containing the following (final concentration): 25 mM Tris, pH 7.4, 0.5% Nonidet P-40, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 0.05% SDS, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. The immunoprecipitate of the EGF receptor, monoclonal anti-EGF receptor antibody (EGF1, American Shap Corp.) was added to the above mixture to reach a final concentration of 2–3 μg/ml. To immunoprecipitate Gsz+, 5 μl of CS1 antiserum raised against the C terminus decapetide (8) were added. As a control for EGF1, an irrelevant mAb BBC-4 against adenyl cyclase (provided by Dr. Thomas Pfeuffer, University of Wurzburg, Germany) was
used. Likewise, nonimmune rabbit serum was employed as a control for CS1 antiserum. The mixture was then incubated at 4 °C overnight with constant rolling. Pansporin suspension (20 μl of 10% suspension, Calbiochem), which had been prewashed with immunoprecipitation buffer, was then added, and the mixture was further incubated at 4 °C for 1 h with constant rolling. The samples were centrifuged at 14,000 × g for 1 min, and the resulting pellets were washed once each in 500 μl of buffer containing high salt (25 mM Tris, pH 7.4, 500 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), medium salt (25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), and no salt (25 mM Tris, pH 7.4, 0.1% Nonidet P-40). The final pellet was resuspended in 30 μl of 2 × Laemmli sample buffer, heated, and subjected to SDS-polyacrylamide gel electrophoresis. The association between \( G_{sa} \) and EGF receptor was detected by employing two different approaches. First, since we have shown that the EGF receptor can phosphorylate \( G_{sa} \) (12), the incubation was performed in the presence of 10 μM [γ-32P]ATP, 2 mM MnCl₂, and 5 mM MgCl₂. Following incubation for 1 h at room temperature, the respective proteins were immunoprecipitated with either the EGFRI mAb or CS1 antiserum, separated by SDS-PAGE, and the phosphorylated \( G_{sa} \) and EGF receptors were detected by autoradiography on Kodak X-Omat film. In the second approach, following separation of proteins in the immunoprecipitate by SDS-PAGE and after transfer onto nitrocellulose, the proteins were detected by Western analyses with either EGFRI mAb (CS1 immunoprecipitations) or CS1 antiserum (EGFRI immunoprecipitates) employing the Amersham ECL system. Whenever peptides were employed, these were added in the initial incubation.

**Materials—**The monoclonal anti-EGF receptor antibody was purchased from Amersham Corp. Antiserum against the C terminus decapeptide of \( G_{sa} \) was the generous gift of Dr. Graeme Milligan, University of Glasgow. Peptides EGFRI-13, EGFRI-14, and \( G_{sa} \) 371–380 were synthesized as described previously (11). Threonine phosphorylated EGFRI-13 (P-EGFRI-13) was custom synthesized by Genosys Biotechnologies Inc. (Woodlands, TX). Peptide EGFRI 985–996 was purchased from Bacham Laboratories (Torrance, CA). Peptides corresponding to amino acids 15–29 (\( G_{sa} \) 15–29) and 354–372 (\( G_{sa} \) 354–372) were gifts from Dr. Heidi Hamm (University of Illinois College of Medicine, Chicago). All of the other chemicals employed were of the highest grade commercially available.

**RESULTS AND DISCUSSION**

Our initial studies to characterize the association between EGF receptor and \( G_{sa} \) were performed in isolated membranes derived from rat hearts. This system was selected because we have previously demonstrated that, in membranes isolated from rat hearts, EGF stimulates adenyl cyclase activity via activation of \( G_{sa} \) (7, 8). Essentially, rat heart membranes were incubated under the conditions of adenyl cyclase activity assay in the presence or absence of ATP, with and without EGF. Reactions were terminated by addition of lysis buffer and the EGF receptor was immunoprecipitated. Following separation of proteins by SDS-PAGE, Western analysis with anti-\( G_{sa} \) antiserum, CS1, demonstrated the presence of \( G_{sa} \) in the EGF receptor immunoprecipitates of incubations performed in the presence of EGF and ATP, but not in EGF receptor immunoprecipitates of incubations conducted in the absence of EGF (Fig. 1). Moreover, ATP was also required to observe association of the EGF receptor with \( G_{sa} \) (Fig. 1). The presence or absence of EGF and/or ATP in the incubation did not alter immunoprecipitation of the EGF receptor (Fig. 1, bottom panel). We have previously demonstrated that the EGF receptor protein tyrosine kinase activity is important for EGF to stimulate adenyl cyclase activity in cardiac membranes (10). These earlier findings, along with the requirement for ATP and EGF to observe EGF receptor association with \( G_{sa} \) (Fig. 1), suggest that the activation of the EGF receptor and perhaps autophosphorylation of the receptor alter its conformation to make sites accessible for association with \( G_{sa} \). Indeed, Cadena et al. (21) have shown that, upon activation and autophosphorylation of the EGF receptor, the receptor undergoes a conformational change from a compact to a more extended form.

To further characterize the association between EGF recep-
growth on Leu⁻/Trp⁻/His⁻ medium was observed in any of the combinations of the plasmids, except the positive control with pGBT9-EGFRc and pGAD424-Gsa. That these cells were successfully transformed with the various constructs is demonstrated by growth of all transformants on Leu⁻/Trp⁻/His⁻ medium (Fig. 2C, right panel).

The differences in activity of β-galactosidase in the various transformants were also consistent with the findings described above with growth in medium lacking histidine (Fig. 2, panel D). Hence, both the entire cytosolic domain of the EGF receptor (EGFRc) and the N-terminal 64 amino acids of this region (EGFRCJM) demonstrated greater expression of β-galactosidase activity in the presence of Gsa as compared to Gsa⁺ (Fig. 2D). Moreover, as observed for cell growth, transformation of cells with Gsa⁺ (wild type and constitutively active) in plasmid pGAD424 and EGF receptor constructs in pGBT9 yielded higher β-galactosidase activities than expression of these proteins in the reciprocal vectors (Fig. 2D). Since controls performed with either EGFR cDNA constructs (EGFRc and EGFRCJM) or Gsa⁻ constructs paired with the empty plasmids did not grow on Leu⁻/Trp⁻/His⁻ medium, β-galactosidase ac-

**Fig. 2. Yeast two-hybrid analyses of EGF receptor and Gsa interaction.** HF7c strain of yeast were transformed with plasmids pGAD424 and pGBT9 containing the various cDNAs indicated. Controls performed with one of the plasmids not containing cDNA encoding either portions of the EGF receptor and Gsa are denoted by dashes (---). Panel A, interactions between complete cytosolic region of the EGF receptor (amino acids 645–1186, EGFRc) and Gsa or Gsa⁺ (constitutively active). Transformants were grown on medium lacking leucine, tryptophan, and histidine (Leu⁻, Trp⁻, His⁻) (left) or medium devoid of leucine and tryptophan (Leu⁻, Trp⁺) (right). Growth of three individual colonies transformed with the various plasmid constructs on day 3 is shown. Panel B, interactions between the N terminus 64 amino acids of the cytosolic domain of the EGF receptor (EGFRCJM, juxtamembrane region) with Gsa or Gsa⁺. Other conditions are identical to those for panel A. Panel C, interactions of the EGF receptor devoid of juxtamembrane region (amino acids 695–1186) (EGFRCJM) with Gsa and Gsa⁺. Other conditions were same as described for panel A. Panel D, β-galactosidase activity in various transformed cells grown in medium lacking Leu, Trp, and His (panels A and B). Individual colonies (six each) of yeast transformed with the various constructs were grown overnight in liquid medium lacking the three amino acids. Growth of cells was then continued in complete medium for 3 h and β-galactosidase activity was monitored as described under "Materials and Methods" section. Data are presented as mean ± S.E. of six determinations. Student's unpaired t test analysis was performed to determine the significance of change between Gsa (wild type) versus Gsa⁺ (active) interactions with the entire cytosolic region of the EGF receptor and its N terminus 64-amino acid constructs.
activity in these cells could not be monitored. Likewise, since transformants with plasmids constructs encoding the cytosolic region of EGF receptor without the N terminus 50 amino acids (deletion of residues 645–694; EGFR a) also did not grow in the absence of histidine, β-galactosidase activity could not be monitored. These findings in the two-hybrid system therefore suggest that the EGF receptor and Gsa interact with each other and at least a portion of the 64 amino acids (residues 644–707) in the juxtamembrane region of the receptor are required for this interaction. Additionally, the data from the yeast two-hybrid experiments (Fig. 2, B and D) demonstrate that the juxtamembrane region of the EGF receptor interacts better with the wild type Gsa as compared to the constitutively active form of this protein, Gsa a.

In order to determine whether the association between the EGF receptor and Gsa is direct (i.e. not involving other proteins) and to further delineate the region(s) on the EGF receptor which associate with Gsa, in vitro association experiments were performed employing the purified EGF receptor and Gsa. Since Gsa is phosphorylated by the EGF receptor (12), experiments were performed wherein, after phosphorylation of Gsa either the EGF receptor or the Gsa were immunoprecipitated, and 32P-labeled proteins in the immunoprecipitate were detected by autoradiography following separation by SDS-PAGE. As demonstrated by the data in Fig. 3, immunoprecipitation of the EGF receptor resulted in co-immunoprecipitation of Gsa. Likewise immunoprecipitation of Gsa was accompanied by the presence of EGF receptor. In controls performed with either an irrelevant monoclonal antibody (BBC-4) or nonimmune serum, neither EGF receptor nor the Gsa was immunoprecipitated (Fig. 3A). Similarly, in additional control experiments with EGF receptor or Gsa alone in the incubation mixture, the anti-EGF receptor antibody (EGFR1) did not immunoprecipitate Gsa, and the anti-Gsa antiserum (CS1) did not immunoprecipitate EGF receptor (see e.g. Fig. 3B). The requirement for ATP to observe association between the EGF receptor and Gsa in experiments with cardiac membranes (Fig. 1) would suggest that autophosphorylation of the EGF receptor is important for interaction with the G protein. Therefore, to further elucidate the role of phosphorylation of EGF receptor and/or Gsa, if any, in association of the two proteins, incubations were performed in the presence or absence of unlabeled ATP. Following immunoprecipitation of EGF receptor, the Gsa in the immunoprecipitate was detected by Western analysis with CS1 antiserum. The data in Fig. 3B demonstrate that the amount of Gsa co-immunoprecipitated with EGF receptor was the same whether or not ATP was present; the phosphorylation states of Gsa and EGF receptor in these experiments were confirmed by probing the Western blot with anti-phosphotyrosine antibodies (not shown). Thus, the data in Fig. 3B demonstrate that the association of the EGF receptor with Gsa is independent of ATP and the phosphorylation state of the EGF receptor and Gsa. Notably, the purified EGF receptor preparation contains EGF (~5–8 μM), and therefore, experiments in the absence of the growth factor are not possible. However, since the purified EGF receptor which is not tyrosine phosphorylated does not require ATP to associate with Gsa, it would appear that the ligand bound, nonphosphorylated, pure EGF receptor is already in a conformation in which the site(s) on the receptor are accessible for binding the G protein. Conversely, in intact cardiac membranes, the presence of ATP is required to observe EGF-dependent association of the G protein with the receptor (Fig. 1). These latter data suggest that in its native membrane environment, as demonstrated by Cadena et al. (21), the EGF receptor changes its conformation upon autophosphorylation and assumes a structure in which the Gsa binding sites are accessible.

The experimental strategy described in Fig. 3A also allowed the determination of stoichiometry of the EGF receptor and Gsa association. In these studies, the EGF receptor (35 ng) and Gsa (500 ng) were incubated in the presence of [γ-32P]ATP, and phosphorylation of both proteins was allowed to reach stoichiometry as described previously (12). An aliquot of the incubations was directly applied to SDS-PAGE, and following separation of known amounts of proteins, the stoichiometry of phosphorylation of each protein was calculated. This also allowed the determination of specific radioactivity of each protein. Consistent with our previous report (12) the stoichiometry of phosphorylation of Gsa was 2 mol of P/mol of Gsa, and that of the EGF receptor was 4.5 mol of P/mol of receptor. The remainder of the incubation was immunoprecipitated with the anti-EGF receptor antibody, and following separation of proteins by SDS-PAGE, the radioactivity associated with EGF receptor and Gsa in the immunoprecipitate by the specific radioactivity of each protein (obtained from the stoichiometry of phosphorylation), the amount of each protein in the immunoprecipitate was calculated. By this method, the stoichiometry of association between the two proteins was determined to be 0.9 mol of Gsa associated with 1 mol of EGF receptor, i.e. the association between the receptor and Gsa is stoichiometric. Because the phosphorylation states of the EGF receptor and Gsa were not important for association of these proteins (Fig. 3B), all subsequent studies were performed in the absence of ATP.
The juxtamembrane region of the EGF receptor activates G\(_s\) (11) and this region of the receptor has also been reported to bind proteins and determine substrate specificity with respect to the proteins that are phosphorylated (13–15). Moreover, our data from the two-hybrid assay suggested that the juxtamembrane region of the EGF receptor is important for association with G\(_s\) (Fig. 2). Therefore, additional in vitro association studies were aimed at elucidating whether or not the juxtamembrane region of the EGF receptor is important for physical interactions with G\(_s\). In our approach we employed peptides EGFR-13 and EGFR-14, which correspond to amino acids 645–657 and 679–692 in the juxtamembrane domain of the EGF receptor (11). We have previously demonstrated that EGFR-13 is a potent activator of G\(_s\) (11). As demonstrated by the data in Fig. 4A, in the presence of EGFR-13 and EGFR-14 the amount of G\(_s\) which co-immunoprecipitated with the EGF receptor was markedly diminished. Densitometric analyses demonstrated a 95% decrease in association in the presence of EGFR-13 and EGFR-14. This decrease in EGF receptor-G\(_s\) association was not the result of decreased EGF receptor immunoprecipitation, since the latter remained constant as determined by Western analysis of the immunoprecipitate with the anti-EGF receptor antibody (Fig. 4B). Previously we have shown that phosphorylation of EGFR-13 on threonine residue corresponding to Thr\(^{654}\) in the EGF receptor diminishes its ability to activate G\(_s\) and stimulate adenyl cyclase (11). Likewise, phosphorylation of EGFR-13 (P-EGFR-13) also abolished the ability of the peptide to compete for association of G\(_s\) with the EGF receptor (Fig. 4D). These findings demonstrate that the effects of EGFR-13 are specific. Since both EGFR-13 and EGFR-14 are basic peptides, additional controls were performed with polylysine and polyarginine (Fig. 4D). Neither polyarginine nor polylysine competed for the association between EGF receptor and G\(_s\) (Fig. 4D). These findings, along with the observation that neither a peptide corresponding to amino acids 985–996 of the EGF receptor (Fig. 4C) nor three other peptides corresponding to sequences in G\(_s\) (discussed later) decreased the association of EGF receptor with G\(_s\), demonstrate that the effects of EGFR-13 and EGFR-14 are specific. Thus the data with EGFR-13 and EGFR-14 demonstrate that the juxtamembrane region of the EGF receptor (amino acids 645–692) is important for association with G\(_s\). Previously we demonstrated that micromolar concentrations of EGFR-13 are required to activate G\(_s\) (11). However, in the present study millimolar concentrations of the peptide are required to compete for the association of EGF receptor with G\(_s\) (Fig. 4, A and C). This difference in concentration probably relates to the fact that in the G\(_s\) activation studies (11) only the peptide and G\(_s\) were present. On the other hand, in the present study, the peptide is being utilized to compete for association between the receptor and G\(_s\). Thus it would appear that the affinity of the full-length EGF receptor for G\(_s\) is high, and therefore, high concentrations of a peptide corresponding to a sequence within the receptor are required for competition. Previously we have also shown that the 13-amino acid region (EGFR-13; amino acids 645–657), but not the 14-amino acid region (EGFR-14; amino acids 679–692), is important for activation of G\(_s\) by the EGF receptor (11). This coupled with the competition studies with the peptides EGFR-13 and EGFR-14 (Fig. 4, A and C) suggest that within the juxtamembrane domain of the EGF receptor, regions which activate G\(_s\) (e.g. amino acids 645–657) (11) as well as other regions not involved in

**Fig. 4. Juxtamembrane region of the EGF receptor is important for association of EGF receptor with G\(_s\).** Panel A, purified EGF receptor and G\(_s\) were incubated in the absence of ATP and with or without the peptides EGFR-13 and EGFR-14. Proteins were immunoprecipitated with the anti-EGF receptor mAb EGFR1. Proteins in the immunoprecipitate were detected by anti-G\(_s\) antiserum CS1. Panel B, the Western blot in panel A was probed with mAb EGFR1 to ensure equal immunoprecipitation of the EGF receptor. Panel C, experiments were performed as described for panel A, except the ability of the peptide corresponding to the residues 985–996 of the EGF receptor to decrease association of EGF receptor and G\(_s\) was also tested. Additionally, the ability of the C terminus truncated EGF receptor (Δ1022–1186) to co-immunoprecipitate G\(_s\) was also compared with the full-length EGF receptor. Panel D, the ability of 1 μM each of peptides EGFR-13, phospho-EGFR-13 (P-EGFR-13) in which the threonine residue corresponding to Thr\(^{654}\) in the EGF receptor was phosphorylated, polylysine, and polyarginine to compete for EGF receptor-G\(_s\) association was tested as described for experiments in panel A.
lipase C

SH2 domain-containing proteins such as Shc and phospho-

that harbors the autophosphorylation sites (24–26) to which

participation, if any, of the C terminus region of the receptor

EGF receptor (not shown). Notably, the effects of GTP

acids 985–996 of the EGF receptor did not decrease association

between EGF receptor and Gs

The association of single transmembrane protein tyrosine

kinase receptors with adapter proteins that participate in the

activation of Gα (e.g. amino acids 679–692) (11) participate in the

association. Although the studies with peptides corresponding to the

juxtamembrane region of the EGF receptor suggest that this region of the receptor is important for association with Gα (Fig.

4, A and C) these experiments do not completely rule out the participation, if any, of the C terminus region of the receptor that harbors the autophosphorylation sites (24–26) to which SH2 domain-containing proteins such as Shc and phospholipase Cγ bind (3, 4). Therefore, to determine whether or not the C terminus of the EGF receptor is involved in association between EGF receptor and Gα, experiments were performed with the purified full-length and truncated EGF receptor (∆1022–1186). In the truncated receptor all amino acids after threonine 1022 are deleted. In these studies equal amounts of the full-length receptor and truncated receptors were employed. As shown in Fig. 4C, the amount of Gα co-immunoprecipitated with the truncated receptor was the same as that associated with the full-length EGF receptor. Moreover, a peptide corresponding to amino acids 985–996 of the EGF receptor did not decrease association between EGF receptor and Gα (Fig. 4C). Therefore, the data in Fig. 4 demonstrate that the juxtamembrane region, but not the C terminus region, of the EGF receptor is involved in its association with Gα.

The importance of the juxtamembrane region of the EGF receptor in association with Gα (Fig. 4) and activation of Gα by this region (11) would suggest that upon activation of Gα, the α-subunit does not associate with the receptor. Although some support for this contention is provided by the data with yeast two-hybrid assay (Fig. 2, A, B, and D), additional experiments with purified proteins were performed to determine whether or not the active, GTPγS-bound, form of Gα associates with the EGF receptor. As demonstrated by the data in Fig. 5, in immunoprecipitates of the EGF receptor, in the presence of GTPγS (1 μM), the co-immunoprecipitation of Gα was reduced by an average of 85%. GTPγS did not alter the immunoprecipitation of the EGF receptor (not shown). Notably, the effects of GTPγS are specific since neither GDP (1 μM, Fig. 5) nor another nucleotide such as ATP (Fig. 3) affected the co-immunoprecipitation of Gα with the EGF receptor. The data in Fig. 5 are also consistent with the findings from the two-hybrid assay which compared the association of the wild type and constitutively active Gα with EGF receptor (Fig. 2) and demonstrate that upon activation, Gα is no longer associated with the EGF receptor.

Employing chimeric proteins, antibodies, and peptides, several studies have shown that the C terminus region of Gα is important for activation of the G protein by receptors (see e.g. Refs. 8, 24, and 25). Indeed, employing CS1 antisera, which is directed against the C terminus decapptide of Gα, we have previously shown that this region of Gα is important for its activation by the EGF receptor and β-adrenergic receptors (8). More recently, employing peptides, studies from Hamm’s laboratory have shown that the activation of Gα by β-adrenergic receptors can be obliterated by peptides corresponding to the C terminus of Gα. Therefore, to determine whether or not the C terminus regions of Gα is also important for association with EGF receptor, in experiments similar to those described in Fig. 4, the ability of peptides corresponding to amino acid residues 15–29, 354–372, and 371–380 of Gα, to compete for the association of the EGF receptor with Gα was investigated. Although the C terminus region of Gα is important for its activation by EGF receptor in terms of stimulation of adenyl cyclase (8), none of the peptides tested altered the association of Gα with the EGF receptor (data not shown). These data (not shown) indicate that the region of the Gα molecule which is involved in association is not necessarily the same region that is important for activation of this G protein by receptors in terms of mediating a signal. Moreover, our findings that the three peptides corresponding to sequences in Gα at concentrations as high as 1 mM did not alter association of the EGF receptor with Gα (not shown) indicate that the effects of EGFR-13 and EGFR-14 as observed in Fig. 4 are specific. Three-dimensional structure studies of the transducin and Gα proteins have demonstrated that the N terminus of the α-subunits of heterotrimeric G proteins interacts with the βγ-subunits (26, 27). Additionally, the N terminus region of α-subunits of G proteins has been implicated to interact with receptors (26). However, the inability of a peptide corresponding to amino acids 15–29 of Gα to compete for association between the EGF receptor and Gα (not shown) suggests that the region of Gα, which is important in interactions with βγ-subunits (26, 27) and which may interact with receptors, is not the region important in association with the EGF receptor. Given our observation that GTPγS decreases the association of Gα with the EGF receptor and the findings from the three-dimensional structures of G protein α-subunits (28, 29) that GTP binding most markedly changes the conformation in the three switch regions (28, 29), it is tempting to speculate that perhaps one of the switch regions and/or a domain proximal to this switch is involved in association with the EGF receptor. Whatever the case, presently, the precise region of Gα, which associates with EGF receptor remains unknown and forms the subject of further investigations.

The association of single transmembrane protein tyrosine kinase receptors with adapter proteins that participate in the signaling to serine/threonine kinases has been well documented (see Refs. 30 and 31 for reviews). However, the interactions of this family of receptors with G protein-mediated processes has been less well defined. Likewise, although a large number of studies have demonstrated that regions in G protein α-subunits or domains in heptahelical receptors which activate these G proteins are important for transducing signals (8, 24–29, 32, 33), to date the association of G protein α-subunits with heptahelical receptors has only been demonstrated for the angiotensin AT2 receptor (34). However, even in the case of the angiotensin AT2 receptor, the region of the receptor that asso-

FIG. 5. GTPγS decreases the association of Gα with the EGF receptor. Panel A, purified EGF receptor and Gα were incubated in the presence of GDP or GTPγS (1 μM each) as described in Fig. 3. The EGF receptor was immunoprecipitated with EGFR1 mAb and Gα co-immunoprecipitation was detected with CS1 antisera. Panel B, densitometric analyses of the amount of Gα co-immunoprecipitated with the EGF receptor in the presence and absence of GTPγS. Data are the mean ± S.E. of three determinations.
Association of EGF Receptor with Gsα

ciates with Gsα and Gαi3 remains unknown (34). Moreover, whether or not the association of AT2 receptor with Gαs and Gαi3 is direct or involves another protein also remains to be investigated. In this respect, the data presented in this study are the first to show the direct, and stoichiometric, association of a G protein α-subunit with a receptor which can activate this G protein (8, 11, 12). Although the association of Gs with EGF receptor has been suggested (35), in that study, phospholipase Cγ was shown to be associated with EGF receptor as well as Gsα. Therefore, whether the association of Gsα with the EGF receptor is direct or indirect via phospholipase Cγ remains unknown. Similarly, although Nishimoto et al. (36) have demonstrated the association of Gs with amyloid precursor protein (APP), heterotrimeric Gs was demonstrated to be associated with APP, and therefore, whether or not the Gαs-subunit associates directly with APP is also not known. Since the regions within the EGF receptor and the β-adrenergic receptors that activate Gs are similar (11, 32) and because the region (EGFR-13) in the EGF receptor that activates Gs also modulates association of the receptor with Gsα it is tempting to speculate that the similar motif in the third cytosolic loop of the β-adrenergic receptor would also be involved in association of that receptor with Gsα.

Interestingly, both EGFR-13 and EGFR-14 compete for the association between EGF receptor and Gsα. Since the association of the two proteins is stoichiometric (1 mol of Gsα:1 mol of EGF receptor), it is possible that there are at least two contact sites for Gsα on the EGF receptor; one site at amino acids 645–657 (EGFR-13) and the other at amino acids 679–692 (EGFR-14). Moreover, because the addition of either EGFR-13 or EGFR-14 effectively decreased the association between EGF receptor and Gαs, it would appear that the loss of contact of Gsα at one of the two sites on the EGF receptor diminishes the affinity for the other site. Alternatively, in the presence of one of the peptides corresponding to the EGF receptor juxtamembrane domain, the conformation of the Gsα is altered so that it loses the ability to bind at any point on the juxtamembrane region of the EGF receptor. The identification of the critical amino acid residues involved in association with the Gsα will be facilitated by future experiments involving site-directed mutagenesis of the receptor.

In conclusion, the studies described herein demonstrate that the cytosolic, juxtamembrane region of the EGF receptor encompassing sequences corresponding to EGFR-13 and EGFR-14 (48 amino acids; Arg645-Lys657) is important for the direct, and stoichiometric, association with Gsα. Since the first 13 amino acids in this region (Arg645–657) are also important for activation of Gαi (11) and because the EGF receptor protein tyrosine kinase can phosphorylate Gsα on tyrosyl residues (12), it is possible that association of Gsα with the juxtamembrane region of the EGF receptor is important for its activation. Further support for the latter contention is derived from the observation that expression of the constitutively active form of Gαs in the two-hybrid assay (Fig. 2) or the addition of GTPγS (Fig. 5) decreases the interaction of EGF receptor with Gαsα. Another interesting finding from the experiments described herein is that the region on Gsα that is involved in association with the EGF receptor is different from the region (C termi

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