Gβγ Subunits Mediate Src-dependent Phosphorylation of the Epidermal Growth Factor Receptor

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In many cells, stimulation of mitogen-activated protein kinases by both receptor tyrosine kinases and receptors that couple to pertussis toxin-sensitive heterotrimeric G proteins proceed via convergent signaling pathways. Both signals are sensitive to inhibitors of tyrosine protein kinases and require Ras activation via phosphorylasedependent recruitment of Ras guanine nucleotide exchange factors. Receptor tyrosine kinase stimulation mediates ligand-induced receptor autophosphorylation, which creates the initial binding sites for SH2 domain-containing docking proteins. However, the mechanism whereby G protein-coupled receptors mediate the phosphorytyrosine-dependent assembly of a mitogenic signaling complex is poorly understood. We have studied the role of Src family nonreceptor tyrosine kinases in G protein-coupled receptor-mediated tyrosine phosphorylation in a transiently transfected COS-7 cell system. Stimulation of Gbg-coupled lysophosphatidic acid and α2A adrenergic receptors or overexpression of Gb1γ2 subunits leads to tyrosine phosphorylation of the Shc adapter protein, which then associates with tyrosine phosphoproteins of approximately 130 and 180 kDa, as well as Grb2. The 180-kDa Shc-associated tyrosine phosphoprotein band contains both epidermal growth factor (EGF) receptor and p185

The low molecular weight G protein Rac functions as a signaling intermediate in many pathways involved in the regulation of cellular mitogenesis and differentiation. Rac activation by growth factor receptors that possess intrinsic tyrosine kinase activity follows ligand-induced phosphorylation of specific docking sites on the receptor itself or adapter proteins, such as Shc and insulin receptor substrate-1, which serve to recruit Ras guanine nucleotide exchange factors to the plasma membrane (1, 2). Recently, several receptors that couple to heterotrimeric G proteins, including the lysophosphatidic acid (LPA)1 (3, 4), α-thrombin (5), angiotensin II (6, 7), α2A adrenergic (AR) (8, 9), M2 muscarinic acetylcholine, D2 dopamine, and A1 adenosine receptors (10), have been shown to mediate Ras-dependent mitogenic signals. In COS-7 cells, Ras-dependent activation of mitogen-activated protein kinases via the α2A AR, M2 muscarinic acetylcholine, D2 dopamine, and A1 adenosine receptors is mediated largely by Gβγ subunits released from pertussis toxin-sensitive G proteins (8, 9). These Gβγ subunit-mediated signals are sensitive to inhibitors of tyrosine protein kinases (8), associated with increased tyrosine protein phosphorylation, and dependent upon recruitment of Ras guanine nucleotide exchange factors to the membrane (9), indicating that the pathway converges with the receptor tyrosine kinase pathway at an early point.

G protein-coupled receptors have been shown to mediate rapid tyrosine phosphorylation of several proteins that participate in mitogenic signal transduction. The thyrotropin-releasing hormone (11), endothelin 1 (12), LPA, and α2A AR receptors (9) stimulate tyrosine phosphorylation of the Shc adapter protein. This effect can be mimicked by the transient overexpression of Gβγ subunits (9, 13) and correlates with Shc-Grb2 complex formation (9, 12) and the recruitment of Ras guanine nucleotide exchange factor activity (9). In addition, recent re-

1 The abbreviations used are: LPA, lysophosphatidic acid; AR, adrenergic receptor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; MAP, mitogen-activated protein; PI3K, phosphatidylinositol 3-kinase.

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ports have described G protein-coupled receptor-mediated tyrosine phosphorylation of insulin receptor substrate-1 (14), focal adhesion kinase (4, 15), and several receptor tyrosine kinases, including the platelet-derived growth factor (PDGF) receptor (16), EGF receptor, p185\textsuperscript{neu} (17), and insulin-like growth factor-1 receptor (14).

The mechanism whereby G protein-coupled receptors stimulate tyrosine protein phosphorylation is poorly understood. The observation that the receptors for PDGF (16) and EGF (17) undergo tyrosine phosphorylation following G protein-coupled receptor activation has led to the hypothesis that the intrinsic tyrosine kinase activity of these receptors becomes activated by an unknown mechanism. G protein-coupled receptor-mediated activation of nonreceptor tyrosine kinases has also been reported. Recently, activation of Src family kinases by the a-thrombin (18), LPA (19), angiotensin II (20), N-formyl methionyl peptide chemotractant (21), \(\alpha\)2A AR (18, 19), and M1 muscarinic acetylcholine (18) receptors has been reported. Furthermore, inhibition of Src family kinases has been shown to inhibit angiotensin II-stimulated Ras (22) and phospholipase C-y1 (23) activation in rat aortic smooth muscle cells, LPA and \(\alpha\)2A AR-stimulated MAP kinase activation in COS-7 cells (19), M1 and M2 muscarinic acetylcholine-stimulated MAP kinase activation in avian B cells (24), and endothelin-1-stimulated transcriptional activation in rat glomerular mesangial cells (25).

We have previously shown in transiently transfected COS-7 cells that pertussis toxin-sensitive G protein-coupled receptors mediate G\(\beta\gamma\) subunit-dependent activation of c-Src and that inhibition of Src family kinases by cellular expression of Csk antagonizes G protein-coupled receptor-mediated MAP kinase activation (19). Here, we examine the role of Src family nonreceptor tyrosine kinases in mediating G\(\beta\gamma\) subunit-dependent tyrosine phosphorylation of receptor tyrosine kinases and Shc. Our data suggest that activation of Src family kinases by G protein-coupled receptors can account for the G-coupled receptor-mediated tyrosine phosphorylation events that direct recruitment of the Shc and Grb2 adapter proteins to the membrane using the EGF receptor as a scaffold.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The cDNA encoding the \(\alpha\)2A AR was cloned in our laboratory. The cDNAs encoding G\(\alpha\)1 and G\(\gamma\)2 were provided by M. Simon (California Institute of Technology, Pasadena, CA). The cDNA encoding human p60\textsuperscript{src} was provided by D. Fujita (University of California, Albany, Canada), and the cDNA encoding p60\textsuperscript{bg} was provided by H. Hanafusa (Rockefeller University, New York, NY). The constitutively activated Y530F p60\textsuperscript{src} (TAC(Y) \(\rightarrow\) TTC(F)), in which the regulatory carboxy-terminal tyrosine residue has been mutated, and catalytically inactive K298M p60\textsuperscript{src} (AA(A) \(\rightarrow\) ATG(M)) mutants were prepared as described (19). All cDNAs were subcloned into pRK5 or pEUK enveloping expression vectors for transient transfection.

**Cell Culture and Transfection—**COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 \(\mu\)g/ml gentamicin at 37 °C in a humidified 5% CO\textsubscript{2} atmosphere. Transfectants were performed in 100-mm dishes. For transient transfection, cells were incubated at 37 °C in 4 ml serum-free Dulbecco’s modified Eagle’s medium containing 6–10 \(\mu\)g of DNA/100-mm dish plus 6 \(\mu\)l of LipofectAMINE reagent (Life Technologies, Inc. \(\beta\)ug/ml of DNA. Empty pRK5 vector was added to transfections as needed to keep the total mass of DNA added per dish constant within an experiment. After 3–5 h of exposure to the transfection medium, monolayers were fed with growth medium and incubated overnight. Transfected monolayers were serum starved in Dulbecco’s modified Eagle’s medium supplemented with 0.1% bovine serum albumin and 10 mm Hepes, pH 7.4, for 16–20 h prior to stimulation. Assays were performed 48 h after transfection. LipofectAMINE transfection of COS-7 cells consistently resulted in transfection efficiencies of greater than 80% (data not shown). Transient expression of G\(\alpha\)1 and G\(\gamma\)2 subunits, Csk, and mutant c-Src proteins was confirmed by immunoblotting of transfected whole cell lysates using commercially available antisera.

**Immunoprecipitation and Immunoblotting**—Stimulations were carried out at 37 °C in serum-free medium as described in the figure legends. After stimulation, monolayers were washed with ice-cold phosphate-buffered saline and lysed in RIPA buffer (150 mm NaCl, 50 mm Tris-Cl, pH 7.5, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 100 \(\mu\)g/ml NaVO\textsubscript{4}, 1 mm NaF, 1 mm phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml leupeptin) for immunoprecipitation under nondenaturing conditions or RIPA/SDS buffer (RIPA buffer containing 0.1% SDS) for immunoprecipitation under denaturing conditions. Cell lysates were sonicated briefly, clarified by centrifugation, and diluted to a protein concentration of 1 mg/ml. Immunoprecipitations from 1 ml of lysate were performed using the appropriate primary antibody plus 50 \(\mu\)l of a 50% slurry of protein G plus/protein A agarose (Oncogene Science) agitated for 1 h at 4 °C. Immune complexes were washed twice with ice-cold RIPA buffer and once with phosphate-buffered saline, denatured in Laemmli sample buffer, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Shc immunoprecipitations were performed using rabbit polyclonal anti-Shc antibody (Transduction Laboratories) and p185\textsuperscript{neu} was immunoprecipitated using rabbit polyclonal anti-HER2 (Santa Cruz Biotechnology).

Tyrosine phosphorylation or the presence of coprecipitated proteins was detected by protein immunoblotting. Phosphotyrosine was detected using a 1:1,000 dilution of horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody (Transduction Laboratories). Shc protein was detected using a 1:1,000 dilution of rabbit polyclonal anti-Shc IgG (Transduction Laboratories); p185\textsuperscript{neu} was detected using a 1:1,000 dilution of mouse monoclonal anti-c-Src antibody 327 with horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson Laboratories) as secondary antibody. C-Src was detected using 1:500 dilution of mouse monoclonal anti-c-Src antibody 327 with horseradish peroxidase-conjugated donkey anti-mouse IgG (Amersham Corp.) as secondary antibody. MAP kinase activation was detected using rabbit polyclonal anti-HER2 IgG (Santa Cruz Biotechnology), and Grb2 was detected using a 1:1,000 dilution of rabbit polyclonal anti-Grb2 IgG (Santa Cruz Biotechnology), each with horseradish peroxidase-conjugated donkey anti-rabbit antibody (Jackson Laboratories) as secondary antibody. Immunoabsorbs for autophosphorylated EGF receptor were performed using mouse monoclonal anti-activated EGF receptor IgG (25) with horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson Laboratories) as secondary antibody. Immune complexes on nitrocellulose were visualized by enzyme-linked chemiluminescence (Amersham Corp.) and quantified by scanning laser densitometry.

**GST Fusion Proteins Containing the c-Src SH2 and SH3 Domains—**GST fusion proteins containing the human c-Src SH2 (amino acids 144–249), SH3 (amino acids 87–143), or SH2 and SH3 (amino acids 87–249) domains were prepared as GST Sepharose conjugates as described previously (20). For the detection of c-Src SH2 or SH3 domain-binding proteins, appropriately transfected and stimulated COS-7 cells were lysed in RIPA/SDS buffer containing 5 mM dithiothreitol, sonicated briefly, clarified by centrifugation, precleared with 6 \(\mu\)g/ml GST Sepharose for 1 h and incubated with 6 \(\mu\)g/ml of the GST fusion protein Sepharose for 3 h at 4 °C. After incubation, fusion protein complexes were washed twice with ice-cold RIPA buffer and once with phosphate-buffered saline, denatured in Laemmli sample buffer, and resolved by SDS-PAGE. Coprecipitated tyrosine phosphoproteins and EGF receptor were detected by protein immunoblotting as described.

**RESULTS**

**G-coupled Receptors and G\(\beta\gamma\) Subunits Mediate Formation of a Mitogenic Signaling Complex Containing EGF Receptor, Shc, and Grb-2—**As shown in Fig. 1A, stimulation of endogenous LPA receptors in COS-7 cells leads to a rapid 3–5-fold increase in tyrosine phosphorylation of each of the three Shc isoforms. The phosphorylation is maximal within 2 min of stimulation and declines slowly thereafter (9, 19). Under non-denaturing conditions, Shc coprecipitates with two major tyrosine phosphoprotein bands of approximately 130 and 180 kDa and with the adapter protein Grb2. The association of Shc with the p130 and p180 phosphoproteins is modulated with a time course that parallels the time course of Shc phosphorylation and Shc-Grb2 complex formation, suggesting that LPA stimu-
lulation induces association of these proteins. As shown in Fig. 1B, similar increases in Shc phosphorylation and Shc-p180 association result from transient expression of G\(\beta y\) subunits of Gi-coupled receptor-mediated Shc phosphorylation and Shc-p180 association are pertussis toxin-sensitive in these cells. Because G protein-coupled receptor-mediated tyrosine phosphorylation of PDGF receptor (16), EGF receptor, and p185 neu tyrosine kinases are present in the Shc-associated p180 phosphotyrosine band. COS-7 cells lack detectable expression of PDGF receptor (16). As shown in Fig. 2A, EGF receptor is not detectable in Shc immunoprecipitates from nonstimulated cells, but stimulation of either G\(\alpha\)-coupled or G\(\beta y\)-coupled receptor coprecipitation. In contrast, Shc-p185 neu complexes are present in nonstimulated cells and do not increase detectably following LPA or EGF receptor stimulation. As expected, EGF receptor phosphorylation results in both Shc-EGF receptor and Shc-p185 neuro association, which may reflect heterodimerization and transphosphorylation of the two related receptor tyrosine kinases (32). As shown in Fig. 2B, the tyrosine phosphorylation states of Shc, EGF receptor, and p185 neu, determined following direct immunoprecipitation of each protein, reflect the changes in Shc-receptor tyrosine kinase association. Shc and EGF receptor phosphorylation is increased following LPA, EGF receptor stimulation. P185 neu exhibits significant basal tyrosine phosphorylation, consistent with the detection of Shc-p185 neu complexes in nonstimulated cells, which detects only following EGF receptor stimulation.

To confirm that Shc, Grb2, and EGF receptor directly associate following G\(\alpha\)-coupled receptor stimulation, EGF receptor immunoprecipitates were assayed for the presence of Shc and Grb2 following LPA stimulation. As shown in Fig. 2C, stimulation with either LPA or EGF resulted in the association of Shc and Grb2 with EGF receptor. G\(\alpha\)-coupled receptor-induced association of Src family nonreceptor tyrosine kinases with Shc has been reported (19, 21). As shown, c-Src can also be detected in EGF receptor immunoprecipitates from LPA- or EGF-stimulated cells, but stimulation of either G\(\alpha\)-coupled or G\(\beta y\)-coupled receptor coprecipitation. In contrast, Shc-p185 neu complexes are present in nonstimulated cells and do not increase detectably following LPA or EGF receptor stimulation. As expected, EGF receptor phosphorylation results in both Shc-EGF receptor and Shc-p185 neu association, which may reflect heterodimerization and transphosphorylation of the two related receptor tyrosine kinases (32).
**Fig. 2. Correlation between Gi-coupled receptor-stimulated EGF receptor and p185^{new} tyrosine phosphorylation with Shc complex formation.** A, coprecipitation of endogenous EGF receptor and p185^{new} with Shc following Gi-coupled receptor or EGF receptor stimulation. Serum-starved cells, transiently transfected with empty pRK5 vector or α2A AR, were stimulated with 2 min with UK14304 (UK), LPA, or EGF as indicated. Immunoprecipitates of Shc from nondenatured RIPA buffer lysates were resolved by SDS-PAGE and immunoblotted with anti-EGF receptor (top panel), anti-p185^{new} (HER2, center panel), or anti-Shc (bottom panel) as described. The position of EGF receptor, p185^{new}, and Shc isoforms are as indicated. B, tyrosine phosphorylation of endogenous EGF receptor and p185^{new} following Gi-coupled receptor or EGF receptor stimulation. Serum-starved cells, transiently transfected with empty pRK5 vector or α2A AR, were stimulated with UK14304, LPA, or EGF as indicated. Immunoprecipitates of EGF receptor (top panel), p185^{new} (center panel), or Shc (bottom panel) from RIPA/SDS buffer lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine as described. C, coprecipitation of Grb2, Shc, and c-Src with endogenous EGF receptor following LPA receptor or EGF receptor stimulation. Serum-starved cells were stimulated for 2 min with LPA or EGF as indicated. Immunoprecipitates of EGF receptor from nondenatured RIPA buffer lysates were resolved by SDS-PAGE and immunoblotted with anti-EGF receptor, anti-Grb2, anti-Shc, or anti-c-Src as described. The position of EGF receptor, Grb2, Shc isoforms, and c-Src are as indicated. NS, nonstimulated.

regulated cell lysates, suggesting that activation of the Gi-coupled receptor results in association of EGF receptor, c-Src, Shc, and Grb2 in a multiprotein complex.

Src Family Kinase Activity Is Required for Both Gi-coupled Receptor and Gβγ Subunit-mediated Tyrosine Phosphorylation of EGF Receptor and Shc—Gi-coupled receptor-mediated increases in EGF receptor phosphorylation might result from ligand-independent activation of the receptor tyrosine kinase, phosphorylation by an activated nonreceptor tyrosine kinase, or inhibition of a phosphotyrosine phosphatase. To distinguish between these alternative mechanisms, we employed a monoclonal anti-EGF receptor antibody specific for autophosphorylated EGF receptor. This antibody selectively recognizes activated EGF receptor via an epitope distal to amino acid 1052 (26), which is distinct from the major in vitro c-Src phosphorylation sites (33). As shown in Fig. 3 (A and B), antiphosphotyrosine immunoblots of EGF receptor immunoprecipitated from EGF-stimulated cells, from cells transiently expressing Y530F p60^{src}, and from cells in which phosphotyrosine phosphatase activity is inhibited by incubation with sodium orthovanadate, each exhibit increased total receptor phosphorylation. Identical immunoblots probed with the anti-activated EGF receptor antibody give increased signals from EGF-stimulated and sodium orthovanadate-treated cells but not from Y530F p60^{src}-transfected cells. Thus, the anti-activated EGF receptor antibody is able to discriminate between increased autophosphorylation resulting from activation of the intrinsic tyrosine kinase activity of the EGF receptor or inhibition of a phosphotyrosine phosphatase versus phosphorylation of the EGF receptor mediated by the c-Src nonreceptor tyrosine kinase. As shown, this antibody does not detect EGF receptor autophosphorylation following stimulation of LPA or α2A AR receptors, despite a 3–5-fold increase in total EGF receptor phosphorytrosine, suggesting that the increase in receptor tyrosine phosphorylation does not reflect activation of the intrinsic tyrosine kinase.

Because expression of activated mutant c-Src is sufficient to mediate EGF receptor phosphorylation in the absence of ligand, we tested the hypothesis that Gi-coupled receptor-mediated activation of Src family kinases can account for the observed tyrosine phosphorylation of Shc and EGF receptor. To inhibit endogenous Src family kinases, cells were transiently transfected with cDNA encoding either Csk or a kinase-inactive dominant negative mutant c-Src (K298M p60^{src}; Ref. 34). Csk is a cytoplasmic tyrosine protein kinase (35) that inactivates Src family kinases by phosphorylating the regulatory carboxyl-terminal tyrosine residue. Csk overexpression has been shown to impair G protein-coupled receptor-mediated MAP kinase activation in COS-7 cells (19) and c-fos transcription in rat glomerular mesangial cells (25). As shown in Fig. 4 (A and B), coexpression of either Csk or K298M p60^{src} markedly inhibits Gβ1y2 subunit-, α2A AR-, and LPA receptor-
mediated tyrosine phosphorylation of both Shc and EGF receptor. EGF-stimulated Shc and EGF receptor phosphorylation were less dramatically effected. Shc and EGF receptor phosphorylation mediated by Y530F, which is not a substrate for Src kinase rather than the intrinsic receptor tyrosine kinase (33). To determine if phosphorylation of this site is mediated by endogenous Src kinases in the intact cell following Gi-coupled receptor stimulation, we tested the effect of Csk overexpression on LPA-stimulated phosphorylation of the c-Src SH2 domain binding site. As shown in Fig. 5C, the ability of the c-Src SH2 domain GST fusion protein to precipitate EGF receptor following LPA stimulation is markedly attenuated in Csk-expressing cells. Because Src kinases also mediate phosphorylation of this site following receptor tyrosine kinase activation, EGF receptor phosphorylation mediated by Y530F, which is not a substrate for Src kinase rather than the intrinsic receptor tyrosine kinase (33).

**DISCUSSION**

Several lines of evidence suggest that Src family kinases play a key role in the transduction of mitogenic signals by G protein-coupled receptors. Pertussis toxin-sensitive activation of the Src family kinases Src, Fyn, Yes, and Lyn (18–21) in various cell types has been reported, and inhibition of Src family kinases has been shown to block G protein-coupled receptor-mediated Ras and phospholipase C-γ1 activation, MAP kinase activation, and c-fos transcription (19, 22–24). Our data demonstrate that in COS-7 cells, G1-coupled receptor-stimulated tyrosine phosphorylation of the EGF receptor results in formation of a complex between the membrane-associated EGF receptor and the cytosolic adapter proteins Shc and Grb2, thus providing a scaffold for the assembly of a mitogenic signaling complex. The G1-coupled receptor effects can be mimicked by cellular overexpression of Gβγ subunits, suggesting that the process is Gβγ subunit-mediated. Because inhibition of endogenous Src kinases blocks both G protein-coupled receptor-mediated EGF receptor phosphorylation and binding of the EGF receptor to the c-Src SH2 domain, the data also suggest that Src family kinases directly associate with and phosphorylate the EGF receptor following G1-coupled receptor stimulation.

**Fig. 6** depicts a model of Gβγ subunit-mediated, Ras-dependent MAP kinase activation that is consistent with these data. Gβγ subunit-dependent activation of endogenous Src family nonreceptor tyrosine kinases is an early event following G1-coupled receptor stimulation (19). Once activated, the Src kinases mediate phosphorylation of several intracellular targets, including receptor tyrosine kinases, adapter proteins such as Shc and insulin receptor substrate-1, and possibly cytoskeletal-associated Src substrates such as focal adhesion kinase and paxillin. One such phosphorylated, membrane-associated protein such as the receptor tyrosine kinases and focal adhesion kinase would provide docking sites for the SH2 domains of the Shc and Grb2 adapter molecules, resulting in the recruitment of Ras guanine nucleotide exchange factors, and potentially of other components of the mitogenic signaling complex, to the plasma membrane. The ensuing activation of Ras would recruit the Raf kinase to the membrane and initiate the phosphorylation process.
tion cascade leading to MAP kinase activation.

Depending upon cell type, the G protein-coupled receptors for angiotensin II, LPA, and α-thrombin have been shown to stimulate ligand-independent tyrosine phosphorylation of PDGF receptor (16), insulin-like growth factor-1 receptor

Fig. 4. Effect of Csk and K298M p60Src expression on Gβγ-coupled receptor-mediated Shc and EGF receptor tyrosine phosphorylation. A. Immunoblots of Shc phosphoryrosine following α2A AR, LPA or EGF receptor stimulation and Gβγ subunit or Y530F p60Src expression. Cells were transiently cotransfected with empty vector (Control) or expression plasmid encoding Csk or K298M p60Src, plus empty pRK5 vector, Gβγ1 and Gγ2, α2A AR, or Y530F p60Src. Serum-starved cells were stimulated for 2 min with UK14304 (UK), LPA, or EGF as indicated, and immunoprecipitates of Shc from RIPA/SDS buffer lysates were immunoblotted with anti-phosphotyrosine as described. The position of tyrosine phosphorylated Shc isoforms is as indicated. B, immunoblots of EGF receptor phosphoryrosine following α2A AR, LPA, or EGF receptor stimulation and Gβγ subunit or Y530F p60Src expression. Serum-starved, transiently cotransfected cells were stimulated as described and immunoprecipitates of EGF receptor from RIAPA/SDS buffer lysates were immunoblotted with anti-phosphotyrosine as described. The position of tyrosine phosphorylated EGF receptor is as indicated. C, quantitation of the effects of Csk and K298M p60Src coexpression on Gβγ subunit-, α2A AR-, LPA-, EGF-, and Y530F p60Src-stimulated Shc and EGF receptor tyrosine phosphorylation. Shc and EGF receptor phosphoryrosine were determined as described following α2A AR, LPA, or EGF receptor stimulation and Gβγ subunit or Y530F p60Src expression. Autoradiographs were quantified by scanning laser densitometry, and the data were presented as fold increase over nonstimulated or empty pRK5 vector transfected controls. The data shown represent the means ± S.E. for three separate experiments. NS, nonstimulated.

Fig. 5. Src kinase-dependent association of EGF receptor with c-Src SH2 domain GST fusion proteins. A, association of p180 with c-Src SH2 domain and SH2-SH3 domain GST fusion proteins following LPA and EGF receptor stimulation. RIPA/SDS lysates of serum-starved cells, stimulated for 2 min with LPA or EGF, were incubated with GST-Sepharose (GST) or GST-Src SH2, SH3, or SH2-SH3-Sepharose as described. Precipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine monoclonal antibody. The position of the c-Src SH2 domain binding p180 protein is as indicated. B, association of EGF receptor with the c-Src SH2 domain GST fusion protein following LPA and EGF receptor stimulation. RIPA/SDS lysates of stimulated serum-starved cells were incubated with GST-Src SH2 sepharose as described. Precipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-EGF receptor. The position of the EGF receptor from GST-Src SH2 Sepharose precipitates and COS-7 whole cell lysates is as indicated. C, quantitation of the effect of Csk expression on LPA- and EGF-stimulated association of EGF receptor with the c-Src SH2 domain GST fusion protein. Cells were transiently cotransfected with empty vector (Control) or expression plasmid encoding Csk (CSK), and LPA- and EGF-stimulated association of EGF receptor with the c-Src SH2 domain GST fusion protein was determined. Autoradiographs were quantified by scanning laser densitometry, and the data were presented as fold increase over nonstimulated or empty pRK5 vector transfected controls. The data shown represent the means ± S.E. for three to five separate experiments. NS, nonstimulated.
subunit (14), EGF receptor, and p185 neu (17). The finding that several receptor tyrosine kinases undergo G protein-coupled receptor-mediated phosphorylation suggests the existence of a common mechanism that is not receptor tyrosine kinase-specific, such as activation of a nonreceptor tyrosine kinase or inhibition of a phosphotyrosine phosphatase. Our data, demonstrating inhibition of G\(_i\)-coupled receptor-mediated tyrosine phosphorylation of the EGF receptor by specific inhibitors of Src family kinases, support the hypothesis that activation of Src kinases can account for the observed receptor tyrosine kinase phosphorylation.

The role of the intrinsic tyrosine kinase activity of receptor tyrosine kinases in G\(_i\)-coupled receptor-mediated tyrosine phosphorylation is unclear. Daub et al. (17) have reported that inhibition of EGF receptor function in Rat1 cells, by either an EGF receptor-selective tyrphostin, AG1478, or expression of a dominant negative mutant EGF receptor, blocks endothelin-1, EGF receptor-selective tyrphostin, AG1478, or G\(_i\)-coupled receptor stimulation.

The mechanism whereby effectors of activated G protein-coupled receptors stimulate Src family kinases is unknown. Stimulation of phosphatidylinositol 3-kinase activity may play a role in Ras-dependent MAP kinase activation in some cells. G\(\beta\gamma\) subunit-mediated PI3K activity has been described in neutrophils and platelets (36, 37), a G\(\beta\gamma\) subunit-regulated isoform of p110 PI3K has been cloned, and G\(\beta\gamma\) subunits may contribute to the regulation of the conventional p85/p110 PI3K (38). We have previously reported that G\(_i\)-coupled receptor- and G\(\beta\gamma\) subunit-mediated MAP kinase activation in COS-7 and CHO cells is sensitive to the PI3K inhibitors wortmannin and LY294002 and to expression of a dominant negative form of the p85 regulatory subunit of PI3K (39). Interestingly, MAP kinase activation by transiently expressed Y530F p60\(^\text{src}\)-mSos, and constitutively activated mutants of Ras and MAP kinase ERK (39) is wortmannin-insensitive, suggesting that the PI3K-dependent step in the pathway may lie upstream of Src kinase activation. The recent report that the c-Src SH2 domain can bind with high affinity to phosphatidylinositol 3,4,5-trisphosphate, the product of PI3K (40), may provide an explanation for this phenomenon.

Interaction between Src kinases and novel G\(\beta\gamma\) subunit-regulated nonreceptor tyrosine kinases might also contribute to the regulation of Src kinase activity. In neuronal cells, G\(_q\)-coupled receptors have been shown to stimulate the Ca\(^{2+}\) and protein kinase C dependent tyrosine protein kinase, PYK2 (41). PYK2 is a member of the focal adhesion kinase family of integrin receptor-associated tyrosine kinases and like p125\(^\text{FAK}\) (42) can complex with activated c-Src upon stimulation (43). However, phospholipase C activation and Ca\(^{2+}\) mobilization are apparently unable to account for G protein-coupled receptor-mediated tyrosine phosphorylation in many noneuronal cells (4, 44, 45). Bruten’s tyrosine kinase (Btk) and Tsk, two members of a family of pleckstrin homology domain-containing tyrosine protein kinases that includes Btk, Itk, Tsk and Tec A, are reportedly regulated by G\(\beta\gamma\) subunits (46). In hematopoietic cells, Btk interacts with the Src family kinases Fyn, Lyn, and Hck (47), and Src-Btk interaction is associated with Btk autoactivation (48). This is unlikely to be a general mechanism for G protein-coupled receptor regulation of Src kinases, however, because the pleckstrin homology domain-containing tyrosine kinases appear to have limited tissue distribution and are not known to be involved in the regulation of Ras.

The data presented in this report suggest that both Src family kinases and receptor tyrosine kinases play central roles in directing the assembly of membrane-associated mitogenic signaling complexes in response to G\(_i\)-coupled receptor activation in some cells. An understanding of the mechanisms whereby G protein-coupled receptors regulate tyrosine protein phosphorylation and of the basis for cross-talk between G protein-coupled receptor and receptor tyrosine kinase signaling pathways may ultimately provide strategies for selective activation or inhibition of cellular proliferation.

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REFERENCES
1. Medema, R. H., and Bos, J. L. (1993) Crit. Rev. Oncog. 4, 615–661
2. Boguski, M. S., and McCormick, F. (1993) Nature 361, 463–465
3. Howe, L. R., and Marshall, C. J. (1993) J. Biol. Chem. 268, 20717–20720
4. B. E. Hawes, T. van Biesen, and R. J. Lefkowitz, unpublished observations.
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4. Hordijk, P. L., Verlaan, I., van Corven, E. J., and Moenaar, W. H. (1994) J. Biol. Chem. 269, 645–651
5. Lathe, R. J., Kennedy, E. D., Collins, L. R., Goldstein, D., Harootunian, A. T., Brown, J. H., and Feramisco, J. R. (1995) J. Biol. Chem. 268, 19411–19415
6. Duff, D. R., Berk, B. C., and Corson, M. A. (1992) Biochem. Biophys. Res. Commun. 180, 257–264
7. Ishida, Y., Kawahara, Y., Tsuda, T., Koida, M., and Yokoyama, M. (1992) FEBS Lett. 310, 41–45
8. Hawes, B. E., van Ienzen, T., Koz, W. J., Luttrell, L. M., and Lefkowitz, R. J. (1995) J. Biol. Chem. 270, 17148–17153
9. van Ienzen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Parfeti, E., Sakai, M., Luttrell, L. M., and Lefkowitz, R. J. (1995) Nature 376, 781–784
10. Faure, M., Vojno-Yasenenetskaya, T. A., and Bourne, H. R. (1995) J. Biol. Chem. 270, 7851–7854
11. Ohmichi, M., Sawada, T., Kanda, Y., Koike, K., Hirota, K., Miyake, A., and Saitlie, A. R. (1994) J. Biol. Chem. 269, 3783–3788
12. Cazaubon, S. M., Ramos-Morales, F., Fischer, S., Schweighoffer, F., Strosberg, A. D., and Couraud, P. O. (1994) J. Biol. Chem. 269, 24805–24809
13. Touhara, K., Hawes, B. E., van Ienzen, T., and Lefkowitz, R. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9284–9287
14. Linseman, D. A., Benjamin, C. W., and Jones, D. A. (1995) J. Biol. Chem. 270, 12563–12568
15. Daub, H., Weiss, F. U., Wallasch, C., and Ullrich, A. (1996) Nature 379, 592–597
16. Hordijk, P. L., Verlaan, I., van Corven, E. J., and Moolenaar, W. H. (1994) FEBS Lett. 350, 240–244
17. Ishida, Y., Kawahara, Y., Tsuda, T., Koida, M., and Yokoyama, M. (1992) FEBS Lett. 310, 41–45
18. LaMorte, V. J., Kennedy, E. D., Collins, L. R., Goldstein, D., Harootunian, A. T., Brown, J. H., and Feramisco, J. R. (1993) J. Biol. Chem. 268, 19411–19415
19. Samson, M. S., Wang, Y., and Herman, W. H. (1996) J. Biol. Chem. 271, 10329–10333
20. Polte, T. R., Naftilan, A. J., and Hanks, S. K. (1994) J. Biol. Chem. 269, 10334–10339
21. Naka, T., Kida, Y., and Hori, T. (1993) Biochem. Biophys. Res. Commun. 193, 645–651
22. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, J. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5192–5196
23. Stover, D. R., Becker, M., Liebentanz, J., and Lydon, N. B. (1995) J. Biol. Chem. 270, 15591–15597
24. Snyder, M. A., Bishop, J. M., McGrath, J. P., and Levinson, A. D. (1985) Mol. Cell. Biol. 5, 1772–1779
25. Matsui, T., Heidaran, M., Miki, T., Popescu, N., La Rochelle, W., Kraus, M., Pierce, J., and Aaronson, S. (1989) Science 243, 800–804
26. Saltiel, A. R. (1994) Science 269, 24805–24809
27. Raleigh, A. R., Chen, C.-S., and Cantley, L. C. (1995) J. Biol. Chem. 270, 19443–19450
28. Cartwright, C. A., Eckhart, W., Simon, S., and Kaplan, P. L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 9284–9287
29. Plowman, G. D., Rudy, B., and Schlessinger, J. (1995) Nature 379, 737–745
30. Stoyanov, B., Volinia, S., Hanek, T., Rubio, I., Loubtchenkov, M., Malek, D., and Stuyven, S., Vanhaesebroeck, B., Dhand, R., and Nurnberg, B. (1995) Science 269, 680–693
31. Rameh, L. E., Chen, C.-S., and Cantley, L. C. (1995) Cell 83, 821–830
32. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rody, B., and Schlessinger, J. (1996) Nature 379, 737–745
33. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., and Schlessinger, J. (1996) Nature 383, 547–550
34. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., and Schlessinger, J. (1996) Nature 383, 547–550
35. Allias, J., van Corven, E. J., Hordijk, P. L., Milligan, G., and Moenaar, W. H. (1993) J. Biol. Chem. 268, 22235–22238
36. Crespo, P., Xu, X., Daniotti, J. L., Troppmair, J., Rapp, U. R., and Gutkind, J. S. (1994) J. Biol. Chem. 269, 21103–21109
37. Langhansrajasekaran, S. A., Wan, Y., and Huang, X. Y. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8601–8605
38. Cheng, G., Ye, Z. S., and Baltimore, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8152–8155
39. Mahajan, S., Fargnoli, J., Martini, S. P., and Aaronson, S. A. (1995) Mol. Cell. Biol. 15, 5304–5311