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Role of c-Src Tyrosine Kinase in G Protein-coupled Receptor- and Gβγ Subunit-mediated Activation of Mitogen-activated Protein Kinases*

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Several G protein-coupled receptors that interact with pertussis toxin-sensitive heterotrimeric G proteins mediate Ras-dependent activation of mitogen-activated protein (MAP) kinases. The mechanism involves Gβγ subunit-mediated increases in tyrosine phosphorylation of the Shc adapter protein, Shc·Grb2 complex formation, and recruitment of Ras guanine nucleotide exchange factor activity. We have investigated the role of the ubiquitous nonreceptor tyrosine kinase c-Src in activation of the MAP kinase pathway via endogenous G protein-coupled lysophosphatidic acid (LPA) receptors or by transient expression of Gβγ subunits in COS-7 cells. *In vitro* kinase assays of Shc immunoprecipitates following LPA stimulation demonstrated rapid, transient recruitment of tyrosine kinase activity into Shc immune complexes. Recruitment of tyrosine kinase activity was pertussis toxin-sensitive and mimicked by cellular expression of Gβγ subunits. Immunoblots for coprecipitated proteins in Shc immunoprecipitates revealed a transient association of Shc and c-Src following LPA stimulation, which coincided with increases in Shc-associated tyrosine kinase activity and Shc tyrosine phosphorylation. LPA stimulation or expression of Gβγ subunits resulted in c-Src activation, as assessed by increased c-Src autophosphorylation. Overexpression of wild-type or constitutively active mutant c-Src, but not kinase inactive mutant c-Src, lead to increased tyrosine kinase activity in Shc immunoprecipitates, increased Shc tyrosine phosphorylation, and Shc·Grb2 complex formation. MAP kinase activation resulting from LPA receptor stimulation, expression of Gβγ subunits, or expression of c-Src was sensitive to dominant negatives of mSos, Ras, and Raf. Coexpression of Csk, which inactivates Src family kinases by phosphorylating the regulatory C-terminal tyrosine residue, inhibited LPA stimulation of Shc tyrosine phosphorylation, Shc·Grb2 complex formation, and MAP kinase activation. These data suggest that Gβγ subunit-mediated formation of Shc·c-Src complexes and c-Src kinase activation are early events in Ras-dependent activation of MAP kinase via pertussis toxin-sensitive G protein-coupled receptors.

Many receptors that couple to heterotrimeric G proteins have been shown to mediate the rapid activation of MAP[1] kinases. Among these are receptors for several substances either present in the general circulation, released as neurotransmitters, or produced locally by vascular endothelium or activated platelets. These include catecholamines, acetylcholine, pituitary glycopeptide hormones, adenosine, angiotensins, bombesin, endothelins, LPA, and α-thrombin (1). Receptors for these substances, activated in response to systemic or locally generated ligands, may in turn play significant roles in the endocrine or paracrine regulation of cell proliferation.

Heterogeneity exists in the mechanisms whereby G protein-coupled receptors activate MAP kinases. Depending upon receptor and cell type, MAP kinase activation may be mediated by pertussis toxin-sensitive or -insensitive G proteins and be either PKC- or Ras-dependent. In COS-7 cells, for example, activation of MAP kinase via the pertussis toxin-insensitive, Gq-coupled, α1B adrenergic and M1 muscarinic acetylcholine receptors is significantly inhibited by PKC depletion but insensitive to expression of a dominant-negative mutant of Ras. In contrast, activation of MAP kinase via the pertussis toxin-sensitive Gi-coupled α2A adrenergic and M2 muscarinic acetylcholine receptors is PKC-independent but requires Ras activation and is sensitive to inhibitors of tyrosine protein kinases (2). Similarly, LPA, a potent stimulator of mitogenesis in quiescent fibroblasts that signals via a G protein-coupled receptor coupling to both pertussis toxin-sensitive and -insensitive G proteins (3–5), activates MAP kinase via a pertussis toxin-sensitive pathway involving Ras and Raf activation (6, 7). LPA-mediated MAP kinase activation is sensitive to tyrosine kinase inhibitors (7, 8) but independent of its effects on phosphatidylinositol hydrolysis and its ability to inhibit adenylyl cyclase (4, 8). In COS-7 cells, Ras-dependent MAP kinase activation via α2A adrenergic (9), M2 muscarinic acetylcholine, D2 dopamine, and A1 adenosine receptors (10) is mediated largely by Gβγ subunits derived from pertussis toxin-sensitive G proteins. Indeed, overexpression of Gβγ subunits, but not constitutively activated Gαi1 or Gαi2 mutants, is sufficient to activate MAP kinase (9–11) in these cells.

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[1] The abbreviations used are: MAP, mitogen-activated protein; LPA, lysophosphatidic acid; PKC, protein kinase C; βARK, β adrenergic receptor kinase; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; MBP, myelin basic protein; EGF, epidermal growth factor; PI3K, phosphatidylinositol 3-kinase; Btk, Bruton's tyrosine kinase.

We have shown previously that Ras-dependent MAP kinase activation via LPA and α2A adrenergic receptors in COS-7 cells is associated with increased tyrosine phosphorylation of the Shc adapter protein and is dependent upon Grb2-mediated recruitment of the Ras guanine nucleotide exchange factor mSos-1 (9). Stimulation of LPA, α2A adrenergic (9), thyrotropin-releasing hormone (12), endothelin 1 (13), and formyl methionyl peptide receptors (14) has been reported to cause rapid and transient increases in Shc tyrosine phosphorylation and Shc · Grb2 complex formation. Thyrotropin-releasing hormone and formyl methionyl peptide receptor-mediated Shc phosphorylation is not mimicked by Ca^{2+} ionophore, suggesting that the signal is not PKC-dependent. The α2A adrenergic and formyl methionyl peptide receptor-mediated Shc phosphorylation is pertussis toxin-sensitive and can be mimicked by transient expression of Gβγ subunits (9, 14, 15). Furthermore, cellular expression of a specific Gβγ subunit sequestrant peptide derived from the carboxyl-terminal Gβγ subunit-binding domain of the β adrenergic receptor kinase 1 (βARK1) (16, 17) inhibits LPA and α2A adrenergic receptor-mediated Shc phosphorylation in COS-7 cells (9), indicating that the phosphorylation is mediated largely via Gβγ subunits derived from pertussis toxin-sensitive G proteins. These data suggest that Gβγ subunit-mediated formation of tyrosine-phosphorylated intermediates is one of the earliest events in a MAP kinase activation pathway cascade used by a significant subset of G protein-coupled receptors.

The identity of the tyrosine kinase(s) and their mechanism of activation by G protein-coupled receptors remains unclear. Several cell surface receptors that lack intrinsic tyrosine kinase activity, including the antigen receptors on T and B cells as well as the receptors for growth hormone, erythropoietin and several cytokines, stimulate tyrosine phosphorylation through association with Src family tyrosine kinases such as Lck, Lyn, and Fyn (18). Similar recruitment of nonreceptor tyrosine kinases might play a role in G protein-coupled receptor signaling. To test this possibility, we have investigated the role of Src kinases in LPA receptor and Gβγ subunit-mediated, Ras-dependent MAP kinase activation in COS-7 cells.

EXPERIMENTAL PROCEDURES

DNA Constructs—The cDNAs encoding Gβ1 (19) and Gγ2 (20) were provided by M. Simon. The cDNA encoding human $p60^{c\text{-}src}$ (21) was provided by D. Fujita, and the cDNA encoding $p50^{csk}$ (22) was provided by H. Hanafusa. The constitutively activated Y530F $p60^{c\text{-}src}$ (TAC(Y) → TTC(F); Refs. 23–25) and kinase inactive K298M $p60^{c\text{-}src}$ (AAA(K) → ATG(M); Ref. 26) mutants were constructed by oligonucleotide-directed mutagenesis using a Sculptor kit (Amersham Corp.). The cDNA encoding mSos1 was provided by M. Sakaue. The dominant-negative Sos-Pro construct, encompassing the proline-rich carboxyl-terminal fragment of mSos1, was prepared as described (9). The cDNAs encoding constitutively activated T24 $p21^{ras}$ (27) and dominant-negative N17 $p21^{ras}$ (28) were provided by D. Altschuler and M. Ostrowski. The cDNA encoding the $p74^{raf\text{-}1}$ (29) dominant-negative mutant was provided by L. T. Williams. The cDNA encoding hemagglutinin-tagged $p44^{mapk}$ (30) was provided by J. Pouyssegur. All cDNAs were subcloned into pRK5 or pcDNA eukaryotic 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 μg/ml gentamicin at 37 °C in a humidified 5% CO_2 atmosphere. Transfections were performed on 80–90% confluent monolayers in 100-mm dishes for immunoprecipitation and c-Src kinase assays or in 6-well tissue culture plates for MAP kinase assays. For transient transfection, cells were incubated at 37 °C in serum-free Dulbecco's modified Eagle's medium (4 ml containing 6–10 μg of DNA/100-mm dish or 1 ml containing 1–2 μg of DNA/well) plus 6 μl of LipofectAMINE reagent (Life Technologies, Inc.)/μg 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 refed with growth medium and incubated overnight. 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 Gβ1 and Gγ2 subunits, Csk, wild-type and mutant c-Src proteins, Sos-Pro, N17 Ras and T24Ras, and ΔNRaf were confirmed by immunoblotting of transfected whole-cell lysates using commercially available antisera. 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.

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 once with ice-cold phosphate-buffered saline, lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 1 mM $NaVO_4$, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotonin, and 10 μg/ml leupeptin), sonicated briefly, clarified by centrifugation, and diluted with RIPA buffer to a protein concentration of 2 mg/ml. Endogenous Shc was immunoprecipitated from 1 ml of lysate using 4 μg/sample of polyclonal anti-Shc antibody (Transduction Laboratories) plus 50 μl 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 and denatured in Laemmeli sample buffer. Following resolution by SDS-polyacrylamide gel electrophoresis (PAGE) and transfer to nitrocellulose membranes, immunoblots were performed to detect Shc phosphotyrosine or the presence of coprecipitated proteins. Shc phosphotyrosine was detected using a 1:1000 dilution of horseradish peroxidase-conjugated antiphosphotyrosine monoclonal antibody (Transduction Laboratories). Shc protein was detected using a 1:1000 dilution of rabbit polyclonal anti-Shc IgG (Transduction Laboratories), and Grb2 was detected using a 1:1000 dilution of rabbit polyclonal anti-Grb2 IgG (Santa Cruz Biotechnology), each with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) as secondary antibody. Wild-type and mutant c-Src were detected using a 1:100 dilution of mAb 327 anti-Src monoclonal antibody (31) with horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson Laboratories) as secondary antibody. Fyn and c-Yes immunoblots were performed using rabbit polyclonal anti-Fyn and anti-Yes antibodies (Santa Cruz Biotechnology). Immune complexes on nitrocellulose were visualized by enzyme-linked chemiluminescence (Amersham) and quantified by scanning laser densitometry.

Detection of Shc-associated Tyrosine Kinase Activity—Shc immune complexes on agarose beads were prepared from RIPA lysates of appropriately stimulated cells as described. To detect coprecipitated tyrosine kinase activity, washed pellets were incubated for 15 min at 20 °C in 30 μl of reaction mix (10 mM PIPES, pH 7.0, 10 mM $MnCl_2$, 5 mM Val^5-angiotensin II (Sigma), 10 mM ATP, and 10 μCi [γ-^{32}P]ATP). Reactions were terminated by the addition of 10 μl of stop solution (6 mg/ml bovine serum albumin and 200 mM EDTA) and briefly centrifuged. Twenty-μl aliquots of each supernatant were added to 40 μl of ice cold 10% trichloroacetic acid, precipitated for 20 min, and centrifuged. Forty-μl aliquots of each clarified supernatant were spotted onto P81 paper and washed three times in 0.425% phosphoric acid and once in acetone; then Val^5-angiotensin II phosphorylation was quantified by scintillation counting.

Measurement of c-Src Autophosphorylation—Rabbit antiserum specific for Y^{416}-phosphorylated Src was the generous gift of M. Weber. To detect endogenously autophosphorylated c-Src, clarified RIPA whole-cell lysates of appropriately stimulated or transfected cells (50 μg of whole-cell protein/lane) were resolved by SDS-PAGE and transferred to nitrocellulose. Y^{416}-phosphorylated c-Src was detected using a 1:500 dilution of anti-pY^{416}-Src antibody, with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) as secondary antibody. Identical samples were immunoblotted with mAb 327 as controls. Immune complexes on nitrocellulose were visualized by enzyme-linked chemiluminescence (Amersham) and quantified by scanning laser densitometry.

Measurement of MAP Kinase Activation—Activation of epitope-tagged $p44^{HA\text{-}mapk}$ was determined using myelin basic protein (MBP) as substrate (30). Appropriately transfected, serum-starved cells in 6-well plates were stimulated as described in the figure legends, lysed in 200 μl of ice-cold RIPA/SDS lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 10 mM NaF, 10 mM sodium pyrophosphate, and 0.1 mM phenylmethylsulfonyl fluoride) and clarified by centrifugation. Immunoprecipitation of $p44^{HA\text{-}mapk}$ from the clarified supernatants was performed using 6.5 μg of anti-HA 12CA5 antibody (Boehringer Mannheim) plus 25 μl of a 50% slurry of protein A-agarose (Oncogene Science) for 1 h at 4 °C. Immune com-

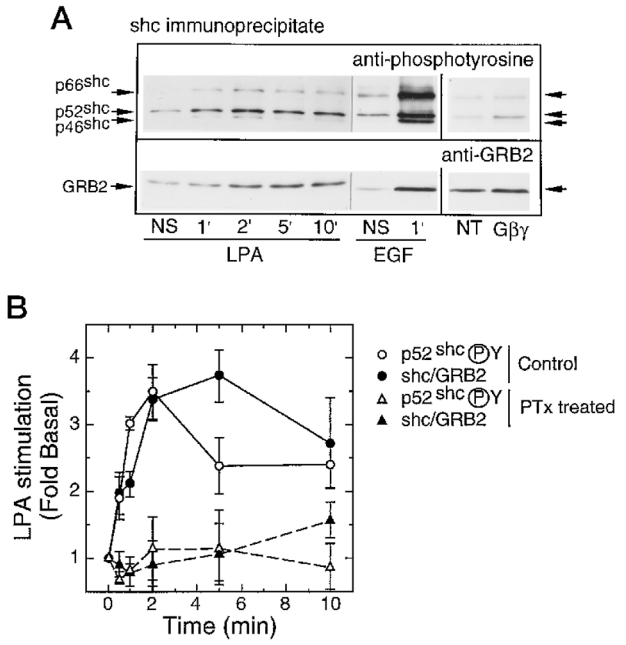

FIG. 1. **Stimulation of Shc tyrosine phosphorylation and Shc·Grb2 complex formation in COS-7 cells following endogenous LPA receptor activation or transient overexpression of Gβγ subunits.** *A*, immunoblots of Shc phosphotyrosine and Grb2 from Shc immunoprecipitates following LPA or EGF stimulation or transient overexpression of Gβγ subunits. Serum-starved cells were stimulated for the indicated times with LPA (10 μM) or EGF (10 ng/ml) (*left panel*) or transiently cotransfected with empty pRK5 vector (*NT*) or Gβ1 and Gγ2 expression plasmids (*right panel*). Immunoprecipitates of Shc from nondenatured RIPA buffer lysates were resolved by SDS-PAGE and immunoblotted with antiphosphotyrosine (*upper panel*) or anti-Grb2 (*lower panel*) as described. The position of tyrosine phosphorylated Shc isoforms and Grb2 are as indicated. *B*, time course of LPA-mediated $p52^{shc}$ tyrosine phosphorylation and Shc·Grb2 complex formation. Cells were serum-starved overnight in the presence or absence of pertussis toxin (*PTx*) (List Biological Labs; 100 ng/ml) prior to stimulation for the indicated times with LPA. Shc phosphotyrosine and Shc·Grb2 complex formation were determined as described. Data are presented as fold increase over nonstimulated controls and represent the means (*bars*, S.E.) for three separate experiments.

plexes were washed twice with lysis buffer and twice with kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, and 1 mM dithiothreitol). MBP phosphorylation was performed at 20 °C for 30 min in 40 μl of kinase buffer containing 250 μg/ml MBP, 20 μM ATP, and 4 μCi [γ-^{32}P]ATP. Reactions were terminated by the addition of 2 × Laemmli sample buffer, and labeled MBP was resolved by SDS-PAGE. Quantitation of labeled MBP was performed using a Molecular Dynamics PhosphorImager. Equal expression of $p44^{HA\text{-}mapk}$ in cotransfected cells was confirmed by immunoblotting with anti-erk1 following immunoprecipitation of $p44^{HA\text{-}mapk}$ from whole-cell lysates using 12CA5 monoclonal antibody.

RESULTS

Activation and Recruitment of c-Src into Shc Adapter Protein Signaling Complexes following LPA Receptor Stimulation or Transient Expression of Gβγ Subunits—Fig. 1*A* depicts the effects of endogenous LPA or epidermal growth factor (EGF) receptor stimulation, or transient coexpression of Gβ1 and Gγ2 subunits, on Shc tyrosine phosphorylation and Shc·Grb2 complex formation in COS-7 cells. LPA stimulation resulted in a transient 3–4-fold increase in Shc phosphotyrosine and Shc·Grb2 complex formation, compared with a 10–12-fold increase resulting from stimulation of the endogenous EGF receptor tyrosine kinase. Overexpression of Gβ1γ2 subunits resulted in a sustained 2-fold increase. As shown in Fig. 1*B*, LPA receptor-mediated Shc tyrosine phosphorylation and Shc·Grb2 complex formation were maximal after 2–5 min of stimulation. The responses were inhibited by pretreatment of cells with

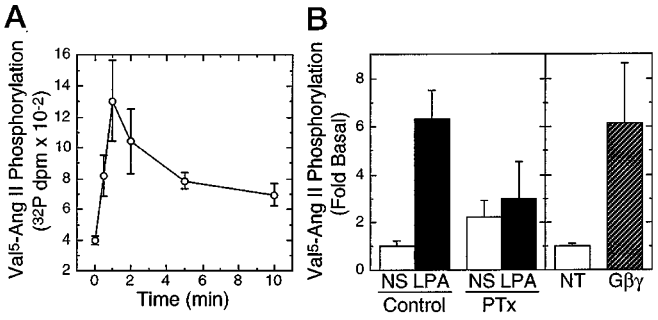

FIG. 2. **Detection of tyrosine kinase activity in Shc immunoprecipitates of COS-7 cells following endogenous LPA receptor stimulation or transient overexpression of Gβγ subunits.** *A*, time course of recruitment of tyrosine kinase activity into Shc immunoprecipitates following stimulation of endogenous LPA receptors. Serum-starved cells were stimulated for the indicated times with LPA (10 μM), and Shc immunoprecipitates from nondenatured RIPA buffer lysates were prepared as described. Shc-containing immune complexes were assayed *in vitro* for the presence of coprecipitated tyrosine kinase activity using Val^5-angiotensin II as substrate as described. Data are presented net [^{32}P] dpm incorporated into Val^5-angiotensin II and represent the means (*bars*, S.E.) for duplicate determinations in one of three separate experiments. *B*, pertussis toxin-sensitive recruitment of tyrosine kinase activity into Shc immunoprecipitates following endogenous LPA receptor stimulation or transient overexpression of Gβγ subunits. Cells were serum-starved in the presence or absence of pertussis toxin (100 ng/ml) and stimulated for 1 min with LPA (10 μM) (*left panel*) or transiently cotransfected with empty pRK5 vector (*NT*) or Gβ1 and Gγ2 expression plasmids (*right panel*). Shc immunoprecipitates were prepared and assayed *in vitro* for coprecipitated tyrosine kinase activity as described. Data are presented as fold increase over nonstimulated or vector-only transfected controls. Data shown represent the means (*bars*, S.E.) for four separate experiments.

pertussis toxin, as is LPA receptor-mediated MAP kinase activation in these cells (6).

To determine whether stimulation of endogenous LPA receptors or transient expression of Gβγ subunits lead to direct recruitment of a tyrosine kinase into Shc-containing signaling complexes, we assayed for tyrosine kinase activity in Shc immunoprecipitates from COS-7 cells stimulated with LPA or transiently cotransfected with Gβ1γ2 subunits. As shown in Fig. 2*A*, LPA stimulation resulted in the rapid appearance of tyrosine kinase activity in Shc immunoprecipitates assessed by an *in vitro* kinase assay using Val^5-angiotensin II as exogenous substrate. The kinase activity was maximal 1–2 min after stimulation and declined subsequently. As shown in Fig. 2*B*, the LPA-induced recruitment of tyrosine kinase activity was pertussis toxin-sensitive. Cells transiently expressing Gβγ subunits showed a similar 6–8-fold increase in Shc-associated tyrosine kinase activity, suggesting that the presence of free Gβγ subunits alone was sufficient for kinase recruitment.

The Src family kinases Src, Fyn, and Yes are expressed in COS-7 cells (data not shown). To determine whether recruitment of Src family kinases could account for the increase in Shc-associated tyrosine kinase activity, Shc immunoprecipitates were immunoblotted with antibodies specific for Src, Fyn, or Yes. Only c-Src was detected in Shc immunoprecipitates from cells following LPA or EGF stimulation, as shown in Fig. 3*A*. Coprecipitation of a c-Src with Shc was also observed in cells transiently expressing a constitutively activated human c-Src mutant (Y530F) (23–25). As shown in Fig. 3*B*, the LPA-stimulated association between c-Src and Shc was rapid and transient, reaching a maximum within 1–2 min of stimulation. Thus, the time course of c-Src·Shc protein complex formation paralleled the time course of Shc tyrosine phosphorylation and recruitment of tyrosine kinase activity into Shc immunoprecipitates.

Two- to 3-fold increases in c-Src autophosphorylation and

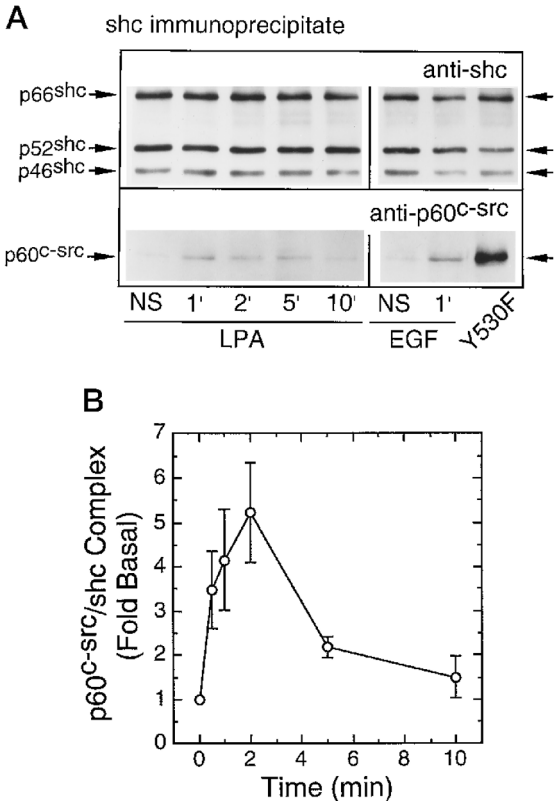

FIG. 3. **Recruitment of c-Src into Shc immunoprecipitates of COS-7 cells following stimulation of endogenous LPA or EGF receptors.** *A*, detection of c-Src in Shc immunoprecipitates by immunoblotting. Serum-starved cells were stimulated for the indicated times with LPA (10 μM) or EGF (10 ng/ml) or transiently transfected with the constitutively active c-Src mutant, Y530F. Immunoprecipitates of Shc from nondenatured RIPA buffer lysates were resolved by SDS-PAGE and immunoblotted with anti-Shc (*upper panel*) or anti-Src monoclonal antibody (*lower panel*) as described. The position of Shc isoforms and c-Src are as indicated. *B*, time course of recruitment of c-Src into Shc immunoprecipitates following stimulation of endogenous LPA receptors. Serum-starved cells were stimulated for the indicated times with LPA (10 μM), and Shc immunoprecipitates were assayed for the presence of coprecipitated $p60^{c\text{-}src}$ as described. Data are presented as fold increase over nonstimulated controls and represent the means (*bars*, S.E.) for three separate experiments.

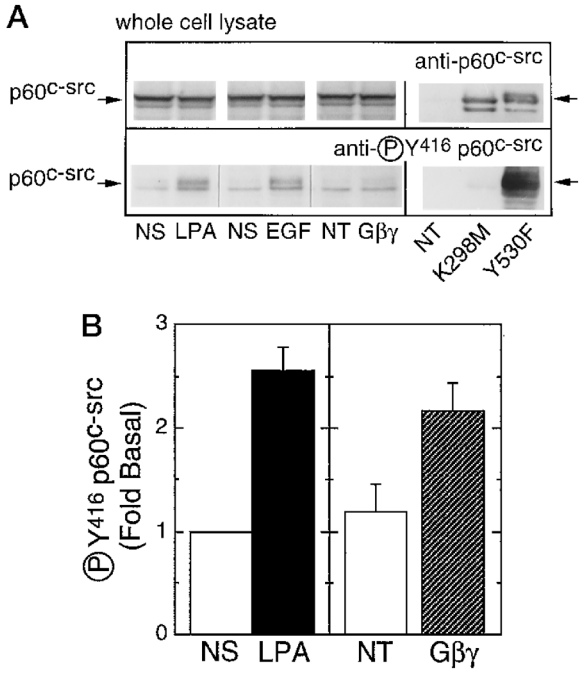

FIG. 4. **Activation of c-Src in COS-7 cells following endogenous LPA or EGF receptor activation or transient overexpression of Gβγ subunits.** *A*, detection of increased c-Src autophosphorylation following LPA or EGF receptor stimulation or Gβγ subunit overexpression using antiserum specific for Y^{416}-phosphorylated Src. Serum-starved cells were stimulated for 1 min with LPA (10 μM) or EGF (10 ng/ml) or transiently cotransfected with empty pRK5 vector (*NT*) or Gβ1 and Gγ2 expression plasmids. Whole cell lysates were resolved by SDS-PAGE, and c-Src was detected by protein immunoblotting using either anti-Src monoclonal antibody 327 (*upper panel*) or polyclonal antiserum specific for Y^{416}-phosphorylated Src (*lower panel*). Control immunoblots for each antibody were performed on lysates prepared from empty pRK5 transfected cells or cells transiently expressing kinase-deficient mutant c-Src (K298M) or constitutively active c-Src (Y530F) (*right panels*). *B*, quantitation of c-Src autophosphorylation following LPA receptor stimulation or Gβγ subunit overexpression. Autophosphorylation of c-Src was determined as described following stimulation with LPA or transient overexpression of Gβγ subunits. Autoradiographs were quantified by scanning laser densitometry, and data are presented as fold increase over nonstimulated or empty pRK5 vector-transfected controls. Data shown represent the means (*bars*, S.E.) for three separate experiments.

kinase activity have been reported following stimulation of LPA (32), α-thrombin, α2A adrenergic, M2 muscarinic (33), and angiotensin II receptors (34). Because there is a correlation between autophosphorylation of Y^{416} and activation of the c-Src kinase (24), whole-cell lysates from stimulated cells were assayed for c-Src activation by immunoblotting using antiserum specific for autophosphorylated c-Src (anti-pY^{416} c-Src) (35). As shown in Fig. 4*A*, in control immunoblots of c-Src from cells expressing kinase-deficient, K298M (26) or constitutively autophosphorylated, Y530F, c-Src mutants, anti-pY^{416} c-Src exhibited high specificity for the autophosphorylated kinase. One min stimulation with LPA or EGF, or transient expression of Gβ1γ2 subunits, resulted in increased Y^{416} phosphorylated c-Src, consistent with c-Src kinase activation in response to stimulation. Both LPA stimulation and expression of Gβ1γ2 subunits resulted in 2–3-fold increases in autophosphorylated c-Src, as shown in Fig. 4*B*. LPA-induced c-Src autophosphorylation, like LPA-stimulated Shc phosphorylation and Shc·Grb2 association, was pertussis toxin-sensitive (data not shown). Thus, LPA- and Gβγ subunit-stimulated c-Src·Shc protein complex formation correlated with activation of the kinase.

Shc Tyrosine Phosphorylation and Ras-dependent MAP Kinase Activation by c-Src—The transforming viral oncogene product v-Src is known to mediate tyrosine phosphorylation of Shc (36), suggesting that the cellular homologue might play a similar role. As shown in Fig. 5*A*, transient overexpression of wild-type c-Src or the Y530F and K298M mutants resulted in increased c-Src·Shc complex formation, detected in c-Src immunoblots performed on Shc immunoprecipitates. As shown in Fig. 5*B*, Shc immunoprecipitates from wild-type c-Src and Y530F-expressing cells contained increased tyrosine kinase activity, whereas cells expressing the kinase inactive K298M mutant exhibited less than basal levels of Shc-associated tyrosine kinase activity, suggesting that the overexpressed kinase inactive mutant competed with endogenous kinase for Shc binding. Fig. 5*C* depicts the effects of c-Src overexpression on Shc tyrosine phosphorylation and Shc·Grb2 complex formation. Transient expression of wild-type c-Src or the activated Y530F mutant increased Shc phosphorylation and Shc·Grb2 association to a level comparable to that observed following EGF stimulation. The ability of wild-type c-Src to induce Shc phosphorylation comparable to the constitutively active Y530F mutant probably results from the high levels of expression achieved in the transient transfection system.

To determine whether c-Src expression could mimick the effects of LPA stimulation and Gβγ subunit expression on MAP kinases, we determined the effects of each on Ras-dependent

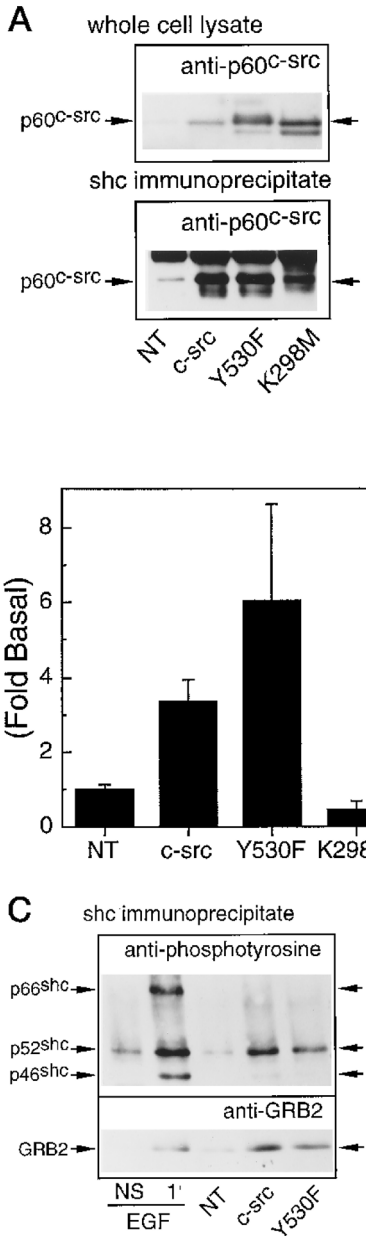

FIG. 5. **Recruitment of c-Src into Shc immunoprecipitates and enhanced tyrosine phosphorylation of Shc in COS-7 cells transiently expressing wild-type and mutant c-Src.** *A*, detection of c-Src in Shc immunoprecipitates of cells transiently expressing wild-type and mutant c-Src. Cells were transiently transfected with empty pRK5 vector (*NT*) or expression plasmids encoding wild-type (c-Src) constitutively active mutant (Y530F) or kinase-inactive (K298M) c-Src. Whole-cell lysates (*upper panel*) and Shc immunoprecipitates from nondenatured RIPA buffer lysates (*lower panel*) were resolved by SDS-PAGE and immunoblotted with anti-p60$^{c\text{-}src}$ monoclonal antibody as described. *B*, detection of tyrosine kinase activity in Shc immunoprecipitates of cells transiently expressing wild-type and mutant c-Src. Immunoprecipitates of Shc from cells transiently expressing wild-type (c-Src), constitutively active mutant (Y530F), or kinase-inactive mutant (K298M) c-Src were prepared as described. Shc-containing immune complexes were assayed *in vitro* for the presence of coprecipitated tyrosine kinase activity using Val^5-angiotensin II as substrate. Data are presented as fold increase over empty pRK5 vector-transfected controls. Data shown represent the means (*bars*, S.E.) for three separate experiments. *C*, immunoblots of Shc phosphotyrosine and Grb2 from Shc immunoprecipitates following transient overexpression of wild-type and mutant c-Src. Immunoprecipitates of Shc from cells transiently expressing wild-type (c-Src) or constitutively active mutant (Y530F) c-Src were resolved by SDS-PAGE and immunoblotted with antiphosphotyrosine (*upper panel*) or anti-Grb2 (*lower panel*) as described. Nonstimulated and EGF-stimulated lanes are shown as controls. The position of tyrosine-phosphorylated Shc isoforms and Grb2 are as indicated.

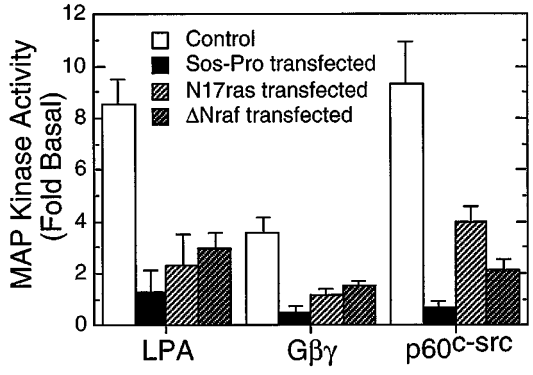

FIG. 6. **Effects of dominant-negative Sos, Ras, and Raf proteins on LPA, Gβγ, and c-Src-mediated MAP kinase activation.** COS-7 cells were transiently cotransfected with hemagglutinin-tagged p44Mapk (p44$^{HA\text{-}mapk}$) and either empty vector (*Control*) or dominant-negative mutants of mSos (*Sos-Pro*), p21^{ras} (N17ras) or p74$^{raf\text{-}1}$ (ΔNraf) plus expression plasmids encoding Gβ1 and Gγ2 or wild-type c-Src as indicated. Basal or 5-min LPA-stimulated (10 μM) p44$^{HA\text{-}mapk}$ activity was determined following immunoprecipitation of p44$^{HA\text{-}mapk}$ using MBP as substrate as described. Expression of p44$^{HA\text{-}mapk}$ was not significantly affected by coexpression of Sos-Pro, N17ras, or DNraf, as determined in anti-p44^{mapk} immunoblots from cotransfected cells (data not shown). Data are presented as fold increase in p44$^{HA\text{-}mapk}$ activity over nonstimulated, empty pRK5 vector cotransfected controls (*NS*). Data shown represent the means (*bars*, S.E.) of duplicate determinations in one of three separate experiments.

MAP kinase activation. As shown in Fig. 6, stimulation of endogenous LPA receptors or transient overexpression of either Gβ1γ2 subunits or c-Src resulted in MAP kinase activation, as determined by an *in vitro* kinase assay following immunoprecipitation of coexpressed epitope-tagged p44^{MapK} (30). In each case, MAP kinase activation was inhibited by coexpression of dominant negatives of mSos1 (9, 37), p21^{ras} (28), and p74$^{raf\text{-}1}$ (29), indicating that the activation was Ras-dependent. Thus, overexpression of c-Src mimicked the effects of LPA receptor activation and Gβγ subunit expression, resulting in tyrosine phosphorylation of Shc, Shc·Grb2 complex formation, and Ras-dependent activation of MAP kinase.

Inhibition of LPA Receptor- and Gβγ subunit-mediated Shc Phosphorylation and MAP Kinase Activation by Csk Overexpression—To directly determine whether Src family kinase activity is necessary for LPA receptor- and Gβγ subunit-mediated signaling, we determined the effects of cellular expression of the c-Src kinase, Csk, on Shc phosphorylation and MAP kinase activation. Csk is a cytoplasmic protein tyrosine kinase (22) that inactivates Src family kinases by phosphorylating a carboxyl-terminal regulatory tyrosine residue. Mouse embryos lacking *csk* exhibit increased c-Src, Fyn, and Lyn activity and increased levels of tyrosine protein phosphorylation (38). Overexpression of wild-type Csk suppresses endogenous c-Src activity (39) and, in opossum kidney cells, blocks acid-induced activation of Na^+/H^+ antiporter, a process associated with p60$^{c\text{-}src}$ activation (40).

Fig. 7*A* depicts the effects of Csk overexpression on LPA- and EGF-stimulated Shc tyrosine phosphorylation and Shc·Grb2 association in transfected COS-7 cells. EGF-induced Shc phosphorylation was reduced by approximately 40%, whereas the LPA-mediated signal was abolished. The inhibition of Shc·Grb2 complex formation paralleled the effects on Shc phosphorylation. As shown in Fig. 7*B*, LPA-stimulated Shc tyrosine phosphorylation and Shc·Grb2 complex formation were reduced to levels not significantly different from basal in cells overexpressing Csk.

The effects of Csk expression on MAP kinase activation are depicted in Fig. 8. In Csk-transfected cells, LPA-stimulated MAP kinase activation was reduced by 60% and Gβγ subunit-

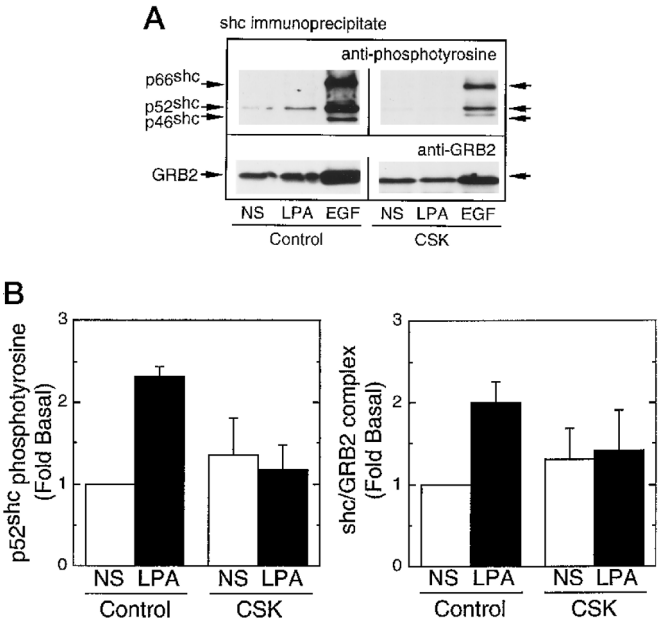

FIG. 7. **Effect of Csk expression on Shc tyrosine phosphorylation and Shc·Grb2 complex formation in COS-7 cells following endogenous LPA receptor activation.** *A*, immunoblots of Shc phosphotyrosine and Grb2 from Shc immunoprecipitates following LPA stimulation. Cells were transiently transfected with empty vector (*Control*) or expression plasmid encoding Csk. Serum-starved cells were stimulated for 2 min with LPA (10 μM) or EGF (10 ng/ml), and immunoprecipitates of Shc from nondenatured RIPA buffer lysates were immunoblotted with antiphosphotyrosine (*upper panel*) or anti-Grb2 (*lower panel*) as described. The position of tyrosine-phosphorylated Shc isoforms and Grb2 are as indicated. *B*, inhibition of LPA-mediated Shc tyrosine phosphorylation and Shc·Grb2 complex formation in cells overexpressing Csk. Serum-starved control and Csk-expressing cells were stimulated for 2 min with LPA. Shc phosphotyrosine and Shc·Grb2 complex formation were determined as described. Data are presented as fold increase over nonstimulated controls and represent the means (*bars*, S.E.) for three separate experiments.

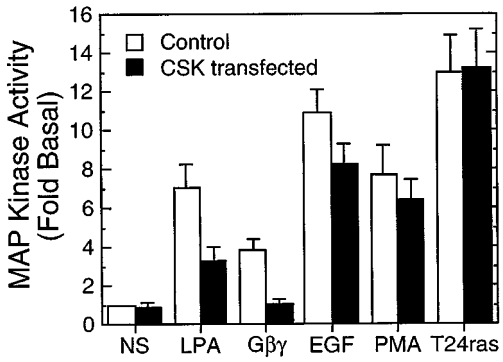

FIG. 8. **Effect of Csk expression on MAP kinase activation in COS-7 cells.** Cells were transiently cotransfected with $p44^{HA\text{-}mapk}$ and either empty vector (Control) or an expression plasmid encoding Csk plus Gβ1 and Gγ2 or constitutively active $p21^{ras}$ (T24ras) as indicated. Basal, LPA (10 μM), EGF (10 ng/ml), or phorbol myristate acetate (*PMA*; 1 μM) stimulated $p44^{HA\text{-}mapk}$ activity was determined following immunoprecipitation of $p44^{HA\text{-}mapk}$ using MBP as substrate as described. Data are presented as fold increase in $p44^{HA\text{-}mapk}$ activity over nonstimulated, empty pRK5 vector cotransfected controls. Data shown represent the means (*bars*, S.E.) for four separate experiments.

mediated activation by greater than 90%, with no significant effect on basal levels of MAP kinase activity. In contrast, EGF-stimulated MAP kinase activation was impaired by only 25%, consistent with the less dramatic effects of Csk expression on EGF-mediated Shc phosphorylation. Phorbol ester-mediated MAP kinase activation and that resulting from overexpression of constitutively activated $p21^{ras}$ (T24ras) (27) were not significantly affected, suggesting that the Csk-sensitive step lies at a point in the pathway upstream of Ras and is not involved with PKC-dependent MAP kinase activation. The partial inhibition of LPA-mediated MAP kinase activation by Csk overexpression, compared to nearly complete inhibition of LPA-stimulated Shc phosphorylation, probably reflects downstream signal amplification occurring in subsequent steps of the pathway. Although LPA receptors have been reported to couple both to Gi and Gq/11 family G proteins, LPA-stimulated MAP kinase activation observed in COS-7 cells was greater than 90% pertussis toxin-sensitive (data not shown). Thus, the alternative pertussis toxin-insensitive, Ras-independent, PKC-mediated MAP kinase activation pathway, used by M1 muscarinic and α1B adrenergic receptors in COS-7 cells (2), probably does not account for the residual signal in the CSK-expressing cells. The ability of Csk expression to inhibit pertussis toxin-sensitive G protein-mediated Shc phosphorylation, Shc·Grb2 complex formation, and MAP kinase activation without affecting PKC- or T24ras-dependent MAP kinase activation suggests that Src family kinases are required for the G protein-coupled, receptor-mediated tyrosine phosphorylation events that precede Ras activation.

DISCUSSION

Gi-coupled receptors transduce intracellular signals via the stimulation or inhibition of several effectors, including phospholipase C and adenylyl cyclase isoforms and some ion channels. Recently, pertussis toxin-sensitive activation of the Src family kinases Src, Fyn, Yes, and Lyn in various cell types has been reported (14, 33, 34), suggesting that these kinases may also function in Gi-coupled receptor signaling. Here, we demonstrate that recruitment and activation of c-Src is involved in Gi-coupled receptor-mediated activation of the Ras/MAP kinase pathway. In COS-7 cells, LPA receptor stimulation leads to the rapid and transient formation of protein complexes containing Shc and c-Src, which parallels the time course of LPA-stimulated Shc tyrosine phosphorylation and Shc·Grb2 association. These events are mimicked both by cellular expression of Gβγ subunits and activated c-Src mutants. Furthermore, expression of Csk, which inactivates Src kinases, inhibits both LPA receptor-mediated Shc tyrosine phosphorylation and MAP kinase activation, indicating that Src family kinase activity is an important intermediate in the signal transduction pathway. These results support a model of MAP kinase activation wherein stimulation of Gi-coupled receptors and release of free Gβγ subunits leads to activation of c-Src- and Src kinase-dependent tyrosine phosphorylation of Shc, followed by Grb2-mediated recruitment of Ras guanine nucleotide exchange factor and Ras activation.

Our data directly implicate c-Src in Gβγ subunit-mediated MAP kinase activation in COS-7 cells. Although it is likely that other Src family tyrosine kinases, such as Lyn, Fyn and Yes, function in an analogous manner in other cell types, we were able to demonstrate only c-Src in Shc immunoprecipitates from COS-7 cells. Since Csk specifically phosphorylates and inactivates Src family kinases, such as Src, Fyn, and Yes (41), its inhibition of LPA receptor and Gβγ subunit-mediated MAP kinase activation in COS-7 cells supports a requirement for c-Src in the pathway. Since transient overexpression of c-Src was sufficient to cause Shc tyrosine phosphorylation and Grb2 recruitment, LPA receptor-mediated activation of Src kinase is probably sufficient to account for the tyrosine phosphorylation events required for Ras activation. In our system, coexpression of the kinase-deficient K298M c-Src mutant also blocks LPA- and Gβγ subunit-stimulated MAP kinase activation. Unlike CSK expression, however, this construct also strongly inhibits EGF receptor-, T24Ras-, and PMA-stimulated MAP kinase ac-

tivation (data not shown). This apparently nonspecific effect on MAP kinase activation probably results from overexpression of the c-Src SH2 domain, which at high levels of expression could function as a relatively nonspecific phosphotyrosine-binding protein, blocking the MAP kinase signal transduction pathway at some point downstream of the initial c-Src-dependent phosphorylations.

The detection of endogenous or transiently expressed c-Src in Shc immunoprecipitates may reflect either a direct interaction between the two molecules or an association of both with an unknown intermediate. However, in stimulated neutrophils (14), the Lyn kinase can be precipitated by a Shc-SH2 domain containing fusion protein, supporting the hypothesis that the interaction between Shc and this Src family kinase is direct and SH2 domain-mediated.

Activation of c-Src via G protein-coupled receptors may also provide a direct link between this class of receptor and other receptor pathways involved in the regulation of cell growth and differentiation. Src associates with activated platelet-derived growth factor receptor, EGF receptor, and ErbB2 (42) and phosphorylates EGF receptor and ErbB2 on nonautophosphorylation sites required for the binding of Src and possibly other signaling molecules (43). Genistein-sensitive tyrosine phosphorylation of insulin-like growth factor-1 receptor and IRS-1 following thrombin stimulation of rat aortic smooth muscle cells has been reported (44). Thus, Src activation might provide a mechanism for G protein-coupled, receptor-mediated assembly of a mitogenic signaling complex directly on a tyrosine kinase growth factor receptor scaffold. Recent data have suggested such a role for platelet-derived growth factor receptors in vascular smooth muscle cells (45) and for EGF receptor and $p185^{neu}$ in Rat-1 fibroblasts (46). Src is also known to redistribute into a cytoskeletal compartment upon activation, where it associates with integrin-dependent cytoskeletal complexes. Bombesin, vasopressin, endothelin, thrombin, and LPA receptors stimulate tyrosine phosphorylation of focal adhesion kinase through both PKC-dependent and -independent pathways. In addition to focal adhesion kinase and Src, integrin signaling complexes contain Csk, the protein tyrosine phosphatase PTP1B, PI3K, and Grb2/mSos, suggesting that these complexes may regulate intracellular signal transduction pathways as well as integrin-mediated cell adhesive interactions (47).

The focal adhesion kinase-related protein-tyrosine kinase PYK-2, which is highly expressed in brain, has been implicated in Shc·Grb2·Sos complex formation. Activation of PYK-2 is Ca^{2+} and PKC-dependent and occurs following stimulation of the Gq-coupled bradykinin receptor in PC12 cells (48). Thus, in appropriate tissues, G protein-coupled, receptor-mediated phospholipase C activation and Ca^{2+} influx might mediate Ras-dependent MAP kinase activation via PYK-2-induced tyrosine phosphorylation. The mechanism whereby Gβγ subunit-regulated effector(s) promote Src kinase activation remains unclear. Gβγ subunit-mediated phosphatidylinositol hydrolysis and Ca^{2+} mobilization are unable to account for Gβγ subunit-mediated tyrosine phosphorylation in COS-7 cells (4, 8). Gβγ subunit-mediated phosphorylation of $p52^{shc}$ is inhibited by the PI3K inhibitor, wortmannin, (15), suggesting that PI3K activity is required for assembly of the Ras activation complex. Gβγ subunit-sensitive PI3K activity has been described in neutrophils and platelets (49, 50), and the recently cloned p110 PI3Kγ can be activated by Gβγ subunits (51). Association between c-Src and PI3K has been reported in chicken embryo fibroblasts expressing activated c-Src mutants (52). Direct interaction between phosphatidylinositol 3,4,5-trisphosphate and the Src SH2 domain has also been proposed (53) and might contribute to the localization or activation of the kinase.

The Src family tyrosine kinases Fyn, Lyn, and Hck have been reported to interact with the Bruton's tyrosine kinase (Btk) in hematopoietic cells via an SH3 domain-mediated interaction (53). Src/Btk interaction is associated with Btk autoactivation (54). Btk, and the related tyrosine kinases Itk, Tsk, and TecA, like the serine/threonine kinases βARK1 and βARK2, contain pleckstrin homology domains. The pleckstrin homology domain of βARK is required for kinase regulation, because it mediates Gβγ subunit- and phosphatidylinositol-dependent translocation of the kinase from cytosol to membrane (55, 56). The activation of Btk and Tsk by Gβγ subunits has been reported (57). These findings raise the interesting possibility that Gβγ subunits, possibly in conjunction with the products of PI3K, might regulate a class of tyrosine protein kinase in a manner analogous to the βARK kinases and provide the initial signaling events leading to Src family kinase activation and a program of tyrosine protein phosphorylation. The relevance of G protein-coupled, receptor-regulated PI3K and pleckstrin homology domain-containing tyrosine protein kinases to the pathway of c-Src-dependent Ras and MAP kinase activation remains the subject of further study.

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