

Src Tyrosine Kinase Is a Novel Direct Effector of G Proteins

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Summary

Heterotrimeric G proteins transduce signals from cell surface receptors to modulate the activity of cellular effectors. Src, the product of the first characterized proto-oncogene and the first identified protein tyrosine kinase, plays a critical role in the signal transduction of G protein-coupled receptors. However, the mechanism of biochemical regulation of Src by G proteins is not known. Here we demonstrate that $G_{\alpha s}$ and $G_{\alpha i}$, but neither $G_{\alpha q}$, $G_{\alpha 12}$ nor $G\beta\gamma$, directly stimulate the kinase activity of downregulated c-Src. $G_{\alpha s}$ and $G_{\alpha i}$ similarly modulate Hck, another member of Src-family tyrosine kinases. $G_{\alpha s}$ and $G_{\alpha i}$ bind to the catalytic domain and change the conformation of Src, leading to increased accessibility of the active site to substrates. These data demonstrate that the Src family tyrosine kinases are direct effectors of G proteins.

Introduction

Heterotrimeric guanine nucleotide binding regulatory proteins (G proteins) transduce a variety of receptor signals to modulate diverse cellular responses (Gilman, 1987; Morris and Malbon, 1999). G proteins are composed of α , β , and γ subunits. The β and γ subunits are tightly associated and can be regarded as one functional unit (Clapham and Neer, 1997). Based on their sequence and functional similarities, the α subunits can be grouped into four families: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12}$ (Simon et al., 1991). G proteins function as molecular binary switches with their biological activity determined by the bound nucleotide (Bourne et al., 1990). Ligand-bound receptors activate G proteins by catalyzing the exchange of GDP bound to the α subunit (the inactive state) with GTP (the active state), resulting in dissociation of G_{α} -GTP from the $\beta\gamma$ subunits. The system is returned to its resting state by hydrolysis of the bound GTP to GDP by the intrinsic GTPase activity of the G_{α} subunit. Mutations that lock G proteins in GTP-bound forms are dominant and in some tissues may lead to changes in growth control and oncogenesis. The oncogenes *gsp* and *gip2* are GTPase deficient mutants of $G_{\alpha s}$ and $G_{\alpha i}$ genes (Landis et al., 1989; Lyons et al., 1990).

To understand G protein signaling and its biological functions, it is essential to identify the direct targets of G proteins. The direct effectors of G proteins consist of the signaling molecules that are activated by their

physical interaction with a G_{α} or $G\beta\gamma$ subunit. To date, the molecular components directly regulated by activated G proteins remain largely elusive. The direct targets of some of these have already been identified. For example, the α subunit of G_s family proteins can stimulate adenylyl cyclase (Taussig and Gilman, 1995). The α subunits of some members of G_i family proteins are able to inhibit certain types of adenylyl cyclases (Taussig et al., 1993). Phospholipase $C\beta$ and Bruton's tyrosine kinase (Btk) can be stimulated by the α subunit of G_q family proteins (Smrcka et al., 1991; Taylor et al., 1991; Bence et al., 1997). Btk and a Ras-GTPase-activating-protein (Gap1^m) can be stimulated by $G_{\alpha 12}$ (Jiang et al., 1998). $G_{\alpha 13}$ can increase the activity of a Rho-GEF (guanine nucleotide exchange factor), p115 RhoGEF/Lsc (Hart et al., 1998). The $\beta\gamma$ subunits of G proteins can also regulate downstream targets (Clapham and Neer, 1997). Based on the diversity of signaling through G proteins, many additional direct effectors (or targets) remain to be identified.

Src-family tyrosine kinases are another major group of cellular signal transducers (Thomas and Brugge, 1997). c-Src was the first identified protein tyrosine kinase (Bishop, 1983; Hunter and Cooper, 1985). These tyrosine kinases can be activated by various extracellular signals, and can modulate a variety of cellular functions including proliferation, survival, adhesion and migration (Thomas and Brugge, 1997). G protein-coupled receptors can stimulate tyrosine phosphorylation of cellular proteins (Thomas and Brugge, 1997). Many G protein-mediated physiological functions are sensitive to tyrosine kinase inhibitors. Activation of many G protein-coupled receptors have been shown to increase the activity of Src-family tyrosine kinases (Chen et al., 1994; Ishida et al., 1995; Ptaszniak et al., 1995; Wan et al., 1996; Luttrell et al., 1996; Rodriguez-Fernandez and Rozengurt, 1996; Schieffer et al., 1996, 1997; Simonson et al., 1996; Postma et al., 1998). Some documented G protein-coupled receptor-induced events that involve tyrosine kinases include: activation of mitogen-activated protein kinase cascades, chemotaxis, neuronal growth cone collapse, cardiovascular hypertrophy/hyperplasia in hypertension, platelet aggregation, focal adhesion assembly and stress fiber formation, and smooth muscle contraction (Chen et al., 1994; Wan et al., 1996, 1997; Luttrell et al., 1996; Dikic et al., 1996; Schieffer et al., 1996; Simonson et al., 1996). However, the mechanisms by which these G protein-coupled receptors activate c-Src are not clear. Despite some previous reports suggesting a possible direct association of Src protein with G proteins (Neer and Lok, 1985; Bushman et al., 1990; Hausdorff et al., 1992; Torti et al., 1992; Akiho et al., 1993; Moyers et al., 1995), direct G protein regulation of Src kinase activity has not been established. Here, we provide evidence that $G_{\alpha s}$ and $G_{\alpha i}$ proteins, but neither $G_{\alpha q}$, $G_{\alpha 12}$, nor $G\beta\gamma$ proteins, directly stimulate the kinase activity of Src and Hck, two members of the Src-family tyrosine kinases. These results establish Src-family tyrosine kinases as novel direct effectors of $G_{\alpha s}$ and $G_{\alpha i}$ proteins. These novel findings also reveal a new mechanism for the action of hormones.

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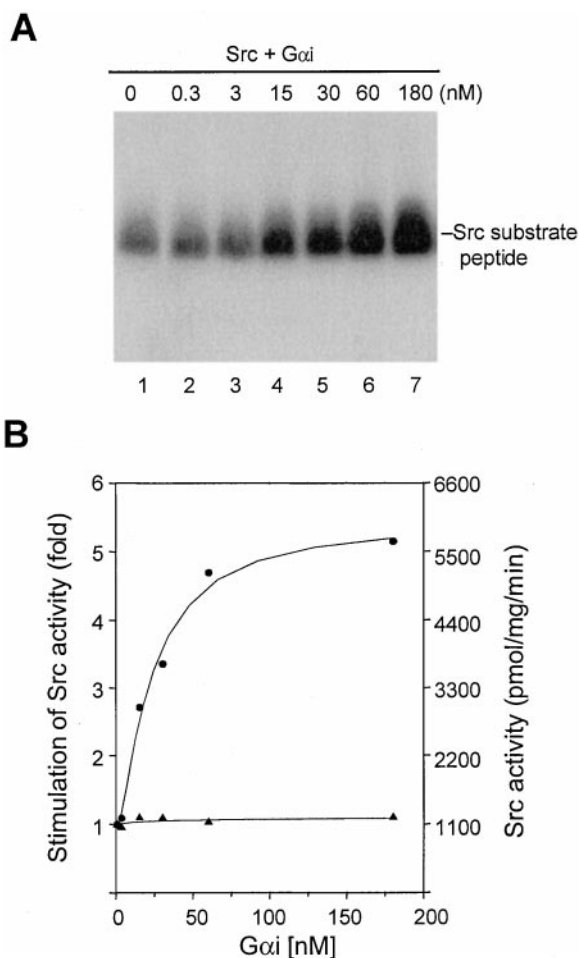


Figure 1. Stimulation of c-Src Kinase Activity by Gαi
(A) Stimulation of c-Src kinase activity by different concentrations of Gαi1-GTPγS.
(B) The corresponding bands on the gel were cut out and phosphorylation was quantified by scintillation counting. Recombinant, Csk-phosphorylated c-Src was incubated with indicated concentrations of Gαi1-GTPγS (circles) or Gαi1-GDP (triangles). The phosphorylation of a Src peptide substrate was measured. Src activity was expressed as pmoles PO₄ incorporated into peptide substrate per mg Src per min. Data shown are representative of nine (for Gαi1-GTPγS) or five (for Gαi1-GDP) similar experiments.

Results

Direct Stimulation of the Kinase Activity of c-Src by Gαi-GTPγS

To investigate the possible direct regulation of Src by G proteins, we used an *in vitro* reconstitution assay with purified G proteins and c-Src. Intensive studies, corroborated by crystallographic structures (Xu et al., 1997; Sicheri et al., 1997; Williams et al., 1997), reveal that the kinase activity of c-Src is maintained at a low basal level by two major intramolecular interactions. One is the binding of the SH3 domain to the linker between the SH2 domain and the kinase domain. The other is the binding of the SH2 domain to the phosphorylated tyrosine residue 527 (Tyr527) in the carboxyl-terminal tail. A specific tyrosine kinase, Csk (for C-terminal Src Kinase), phosphorylates Tyr527 of c-Src and represses its kinase

activity, generating a downregulated state from an activated (unphosphorylated) state (Nada et al., 1991). This negative regulation of Src activity by Csk has been confirmed genetically; cells from Csk-knockout mice have elevated Src specific activity (Imamoto and Soriano, 1993; Nada et al., 1993).

We have used a form of c-Src with lower basal activity, i.e., the Csk-phosphorylated downregulated form, to examine the activation of c-Src kinase by purified G proteins. When recombinant c-Src (human c-Src(ΔN85)) purified from Sf9 cells was assayed by phosphorylation of a peptide substrate, the specific activity of this C-terminal, unphosphorylated c-Src is 5.5 nmol/mg/min, similar to previously published data (Boerner et al., 1996). Phosphorylation of this preparation of c-Src by purified Csk reduces its specific activity to 1.1 nmol/mg/min (Figure 1). Remarkably, as shown in Figure 1, Gαi-GTPγS stimulated the kinase activity of C-terminal phosphorylated c-Src in a concentration-dependent manner. In the presence of 180 nM Gαi-GTPγS, the specific activity of phosphorylated c-Src was increased ~5-fold, to 5.7 nmol/mg/min. Gαi-GDP had no effect on c-Src activity, indicating that activation of Gαi is required (Figure 1). In addition, heat-inactivated Gαi-GTPγS had no effect (data not shown). Hence, Gαi-GTPγS directly increases the kinase activity of C-terminal phosphorylated c-Src.

Direct Stimulation of c-Src Activity by Gαs, but Not by Gαq, Gα12, and Gβγ

To investigate whether this direct stimulation of c-Src is unique to the Gi family of G proteins, we studied regulation of c-Src by other G proteins. As shown in Figure 2, purified Gαs-GTPγS also directly increased the kinase activity of C-terminal phosphorylated c-Src by ~5-fold (Figure 2). The efficacy of Gαs-GTPγS here ($EC_{50} \approx 30$ nM) is similar to the stimulation of adenylyl cyclase by Gαs-GTPγS (Kleuss and Gilman, 1997). Neither Gαs-GDP nor heat-inactivated Gαs-GTPγS had any effect (Figure 2 and data not shown). In contrast to Gαi and Gαs, purified Gαq-GTPγS, Gα12-GTPγS, and Gβγ had no direct effect on the activity of c-Src even though these G proteins could stimulate another tyrosine kinase, Btk (Table 1) (Langhans-Rajasekaran et al., 1995; Bence et al., 1997; Jiang et al., 1998). AlF₄⁻ activated Gαi-GDP and Gαs-GDP have the same effect as GTPγS bound Gαi and Gαs (Table 1). Therefore, both Gαs and Gαi can directly stimulate the kinase activity of c-Src.

Stimulation of Hck Activity by Gαi and Gαs

To examine whether this direct G protein regulation is a general feature of the Src-family of tyrosine kinases, we tested G protein modulation of Hck, another member of the Src-family tyrosine kinases. As shown in Figure 3A, the specific activity of Csk-phosphorylated Hck (human Hck(ΔN77)) was increased from 1.9 nmol/mg/min to 5.2 nmol/mg/min by 180 nM Gαi-GTPγS. The specific activity of C-terminal unphosphorylated Hck purified from Sf9 cells was 5.72 nmol/mg/min. The higher basal activity seen with Csk-phosphorylated Hck may reflect the reported weak interaction of the SH2 domain and the phosphorylated tail (Schindler et al., 1999); nevertheless, the specific activity of Hck after stimulation is similar to that of c-Src after stimulation. Gαs-GTPγS similarly increased the activity of Csk-phosphorylated Hck (Figure 3B). Gαq, Gα12, and Gβγ had no direct effect on the activity of Hck (data not shown). Thus, like c-Src,

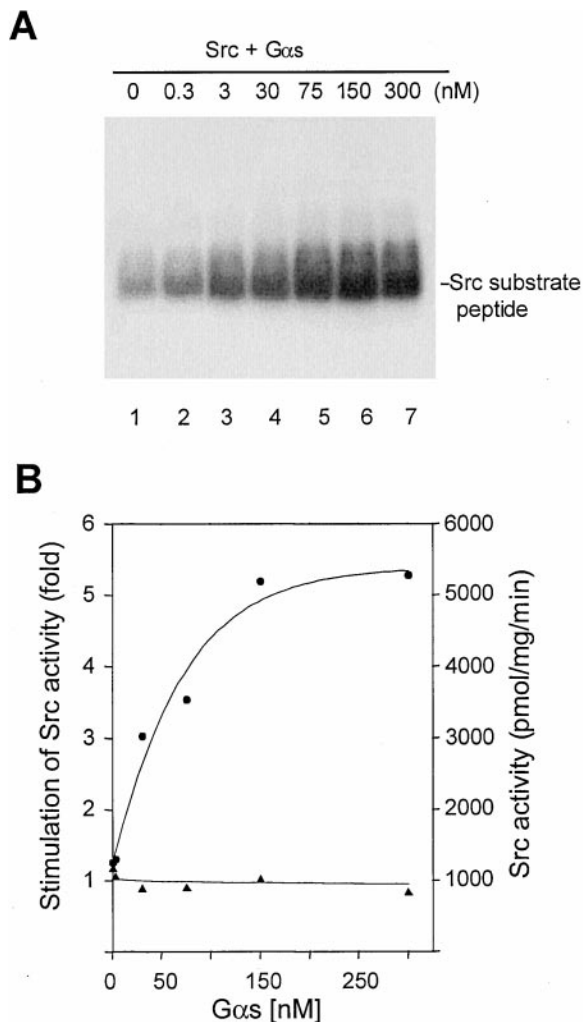


Figure 2. Stimulation of c-Src Kinase Activity by Gαs
(A) Stimulation of c-Src kinase activity by different concentrations of Gαs-GTPγS.
(B) The corresponding bands on the gel were cut out and phosphorylation was quantified by scintillation counting. Increasing amounts of Gαs-GTPγS (circles) stimulated Src activity. Gαs-GDP (triangles) had no effect on Src activity. Data are representative of ten similar experiments.

Hck can be directly stimulated by G proteins, suggesting that direct modulation of Src-family tyrosine kinases by G proteins is likely to be a general phenomenon.

Direct Interaction of c-Src with Gαi and Gαs In Vitro

To further examine the direct interaction of c-Src with Gαi-GTPγS or Gαs-GTPγS, we tested purified c-Src and Gαi-GTPγS or Gαs-GTPγS for binding in vitro. We found that purified GTPγS-bound Gαi and Gαs readily bound purified c-Src, whereas GDP-bound Gαi and Gαs did not (Figures 4A and 4B). As additional controls, neither Gαi-GTPγS nor Gαs-GTPγS bound to Btk, a tyrosine kinase from a different family (data not shown). These results suggest that Gαi and Gαs specifically and directly interact with c-Src.

We next determined the interacting sites on c-Src for

Table 1. Effect on Src Kinase Activity by G Proteins

Treatment	Src activity (pmol min ⁻¹ mg ⁻¹)
Downregulated c-Src	1100
+180 nM Gαi-GDP	1100
+180 nM Gαi-GDP + AIF ₄ ⁻	5500
+180 nM Gαi-GTPγS	5700
+300 nM Gαs-GDP	1000
+300 nM Gαs-GDP + AIF ₄ ⁻	5000
+300 nM Gαs-GTPγS	5200
+1 μM Gβγ	1000
+300 nM Gαq-GTPγS	1300
+300 nM Gα12-GTPγS	1200

AIF₄⁻: 10 mM NaF plus 30 μM AlCl₃. Src activity is expressed as pmol phosphate incorporated into peptide substrate per min per mg of Src. Data shown are from a representative experiment of three or four similar experiments.

Gαi and Gαs. We made GST-fusion proteins GST-c-Src, GST-SH3SH2, and GST-kinase, and examined their relative binding to Gαi and Gαs (Figures 4C–4E). We found that the catalytic domain, but not the SH3SH2, is required for binding to Gαi and Gαs (Figure 4E). This is reminiscent of the interaction of Gαs and Gαi with the catalytic core of adenylyl cyclase (Tesmer et al., 1997). Similarly, cyclin A binds to the catalytic domain of cyclin-dependent kinase-2, leading to the increase of kinase activity (Jeffrey et al., 1995).

Association and Stimulation of c-Src by Gαi and Gαs in Cells

To assess the in vivo interaction, we assayed the physical association between endogenous c-Src and Gαs or Gαi by coimmunoprecipitation from mouse neuronal NG108 cells transfected with constitutively active mutants of Gαs (GαsQ227L) and Gαi (GαiQ205L) (Figure 5A). c-Src was specifically coimmunoprecipitated with Gαs and Gαi, indicating that these proteins physically interact in mammalian cells. c-Src was not coprecipitated by a control membrane potassium channel protein EAG or anti-Gαq antibody under the same conditions. Furthermore, using an immunoprecipitation-kinase assay, we found that the activity of c-Src was increased by expression of these constitutively active mutants GαsQ227L and GαiQ205L in NG108 cells (Figure 5B). These data implicate c-Src as a downstream target of Gαs and Gαi in cells, consistent with the above in vitro data.

To evaluate the observed c-Src and Gαs or Gαi interaction under more physiological conditions, we examined the association of endogenous c-Src with endogenous Gαs and Gαi. c-Src became associated with Gαs and Gαi, but not Gαq, after incubating NG108 cell extracts with GTPγS to activate endogenous G proteins (Figure 5C). Moreover, we used cholera toxin (CTX), which catalyzes the ADP-ribosylation of Gαs, resulting in the inhibition of Gαs GTPase activity, to activate endogenous Gαs. As shown in Figure 5D, CTX treatment of NG108 cells induced the coimmunoprecipitation of Gαs, but not Gαq, with c-Src. At the same time, the activity of endogenous c-Src was stimulated by CTX activation of endogenous Gαs (Figure 5E). Although the in vivo stimulation of c-Src activity by Gαs and Gαi is consistent with our in vitro data, we should note that it

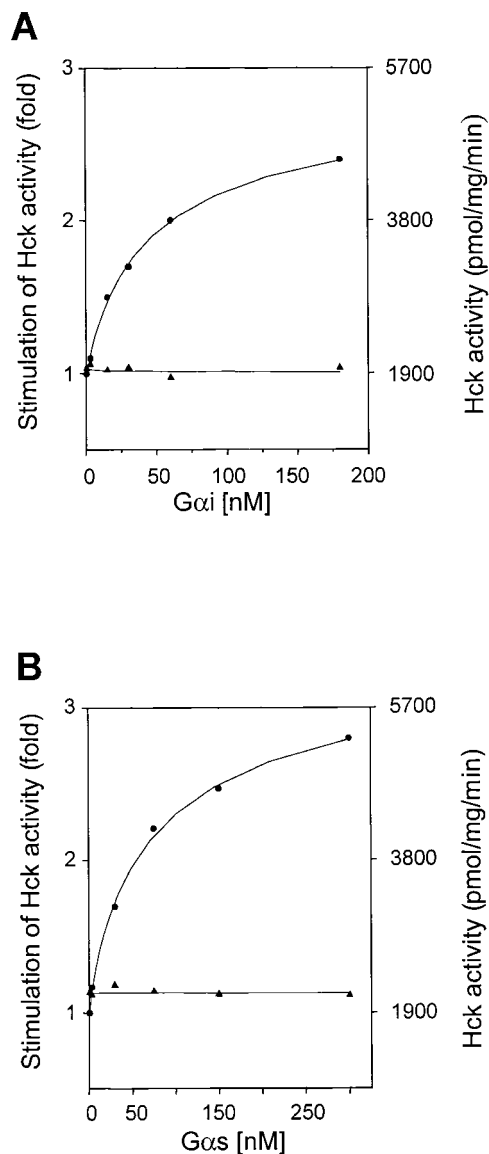


Figure 3. Stimulation of Hck Kinase Activity by Gαi and Gαs (A) Stimulation of Csk-phosphorylated Hck by Gαi1-GTPγS. (B) Stimulation of Csk-phosphorylated Hck by Gαs-GTPγS. Circles: Gαi1-GTPγS or Gαs-GTPγS; triangles: Gαi1-GDP or Gαs-GDP. Data are representative of 3–5 experiments.

has been recently reported that β arrestin brings activated c-Src to the agonist-occupied β₂-adrenergic receptor, and targets both to clathrin-coated pits, leading to ERK activation, when expressed in HEK-293 cells (Luttrell et al., 1999). Whether Gαs and β arrestin act within similar or distinct pathways in cells to activate c-Src needs further investigation. Nevertheless, Gαs and Gαi could stimulate c-Src kinase activity in cells.

Src-Family Tyrosine Kinases Are the Major Mediator of Gαs and Gαi-Induced Cellular Protein Tyrosine Phosphorylation

To further investigate Gαs and Gαi stimulation of Src-family tyrosine kinases in cells, we examined the overall profile of cellular protein tyrosine phosphorylation in

Src-family tyrosine kinase knockout cells (Klinghoffer et al., 1999). Deletion of the ubiquitously expressed Src-family tyrosine kinases c-Src, Yes, and Fyn in mice led to embryonic lethality (Stein et al., 1994). Embryonic fibroblast cells (SYF cells) from these knockout mouse embryos are devoid of all known Src-family tyrosine kinase activity (Klinghoffer et al., 1999), and were used to study the effect of deletion of Src-family kinases on the overall protein tyrosine phosphorylation induced by Gαs and Gαi. Expression of activated mutants of Gαs (GαsQ227L) and Gαi (GαiQ205L) in murine embryonic fibroblast NIH3T3 cells, which express endogenous c-Src, induced enhanced tyrosine phosphorylation of many cellular proteins (Figure 5F). In contrast, in SYF cells, GαsQ227L- and GαiQ205L-induced tyrosine phosphorylation of many of these cellular proteins was severely reduced (Figure 5F). An exception was the two proteins (~35 kDa) whose tyrosine phosphorylation was still induced by GαsQ227L and GαiQ205L in SYF cells (Figure 5F). Reintroduction of c-Src into SYF cells rescued GαsQ227L and GαiQ205L induced protein tyrosine phosphorylation (Figure 5F). These data suggest that Src-family tyrosine kinases play a prominent role in mediating Gαs- and Gαi-induced protein tyrosine phosphorylation events in cells.

Gαs Mutants, Defective in Stimulating c-Src In Vitro, Failed to Increase c-Src Kinase Activity in Cells

G proteins, including heterotrimeric and Ras-family G proteins, use their effector domains to contact the downstream effectors. The structural comparison of the GDP-bound and GTP-bound states of Ras showed that there are only two regions of structural difference between the inactive and active states. These two regions are named switch I and II. Although the determinants of specificity of interactions of Ras with structurally and functionally distinct effectors are not completely clear, the switch I region is a core Ras effector domain essential for all effector interactions (Wittinghofer and Herrmann, 1995; Vojtek and Der, 1998). Similarly, the switch II region of Gαs and Gαi has been shown to be important in interacting with their common effector adenylyl cyclase (Berlot and Bourne, 1992; Grishina and Berlot, 1997; Tesmer et al., 1997). (There are three switch regions in heterotrimeric G proteins.) To identify the residues on Gαs involved in stimulation of c-Src, we first examined mutants of some residues in the switch II region, based on an assumption that, similar to Ras protein, Gαs could use the switch II region to interact with different effectors. The co-crystal structure of Gαs and the catalytic core of adenylyl cyclase showed that R231, R232, Q236, and N239 residues of the switch II region of Gαs, as well as some residues (including N279, R280, L282, and R283) in the α3/β5 loop, directly interact with adenylyl cyclase (Tesmer et al., 1997). Mutation analyses also showed that mutants of R232 and I235 residues (the switch II region) are defective in stimulating adenylyl cyclase in cells (Grishina and Berlot, 1997). We generated and purified some point mutant (to Ala) proteins of Gαs and tested their ability to stimulate c-Src kinase activity in vitro. All these mutant proteins are capable of adopting active conformations as examined by protection of trypsin digestion by loading of GTPγS (Figure 6A). We found that some of these mutants (such as W234A, Q236A, N279A, and R280A) could still stimulate c-Src (Figure 6B), although they failed to stimulate

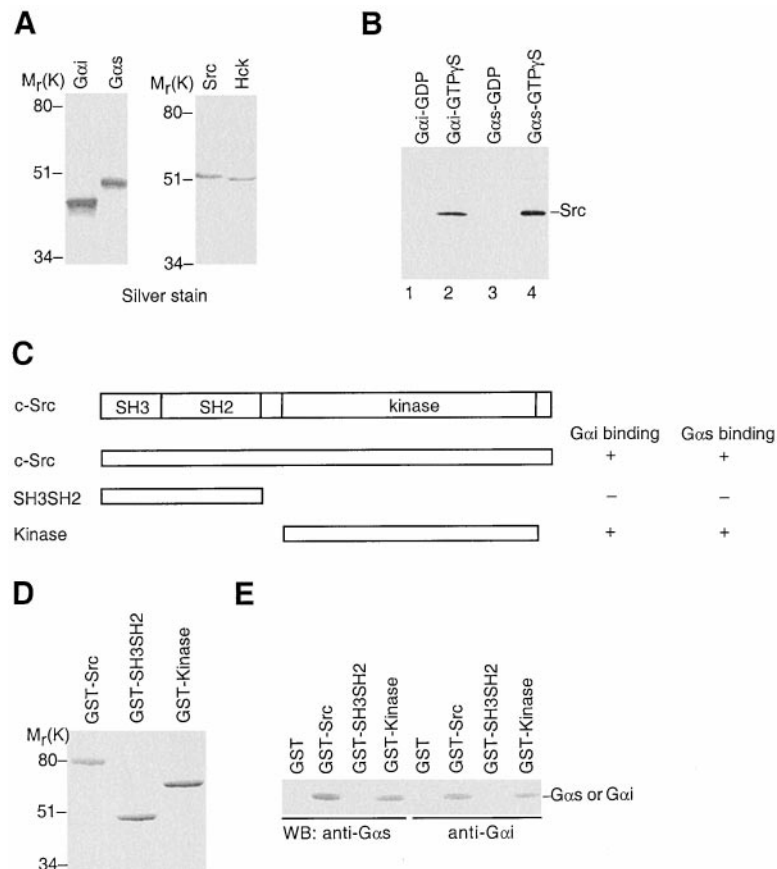


Figure 4. Interaction of Gαi and Gαs with c-Src In Vitro

(A) Silver staining of purified proteins.
(B) Interaction of purified Src with purified Gαi and Gαs.
(C) Representation of GST-fusion protein constructs.
(D) Coomassie staining of SDS/PAGE of purified GST-fusion proteins.
(E) Binding of Gαi and Gαs to c-Src involves the catalytic domain of c-Src. Data are representative of four experiments.

adenylyl cyclase (Berlot and Bourne, 1992; Grishina and Berlot, 1997; Tesmer et al., 1997). One mutant (I235A) failed to increase c-Src activity in vitro (Figure 6B). To investigate the effect of I235A and other mutants in activating c-Src in cells, we transfected these mutants into NG108 cells. These mutant constructs were made in the activated (R201C, GTPase deficient) and the EE epitope-tagged GαsRCEE background to bypass receptor activation, which could activate endogenous Gαs, and for detection (Grishina and Berlot, 1997). As shown in Figure 6C, while the control GαsR201C increased c-Src kinase activity in cells, I235A mutant did not increase c-Src kinase activity in transfected cells. The expression levels of these mutant G proteins in cells were examined by Western blot with an anti-EE epitope antibody (data not shown) (Grishina and Berlot, 1997). These data suggest that I235 is required for Gαs stimulation of c-Src both in vitro and in vivo. Furthermore, the mutants Q236A, N279A, and R280A increased c-Src activity in cells (Figure 6C), despite having failed to interact with adenylyl cyclase (Berlot and Bourne, 1992; Tesmer et al., 1997). These data indicate that stimulation of adenylyl cyclase is not essential for stimulation of c-Src in cells, as our in vitro data suggested.

G Protein Stimulation of c-Src Increases the Autophosphorylation of Tyr416

c-Src kinase activity can be regulated by tyrosine phosphorylation. Phosphorylation of Tyr527 has a profound inhibitory effect on c-Src kinase activity. When Tyr527 is not phosphorylated, c-Src has higher activity. Therefore,

phosphorylation and dephosphorylation of Tyr527 is a regulatory mechanism of c-Src activity. Indeed, some receptors use phosphotyrosine phosphatases to dephosphorylate Tyr527 leading to the activation of c-Src (Thomas and Brugge, 1997). Therefore, we monitored the phosphorylation state of Tyr527 during the stimulation of c-Src by Gαi and Gαs. We used the specific antibody for phospho-Tyr527-containing c-Src. As shown in Figure 6D, we did not observe any changes on the phosphorylation state of Tyr527 of c-Src before and after G protein incubations. Before Csk phosphorylation, phosphorylation on Tyr527 is very low (Figure 6D, lane 1). Csk phosphorylation dramatically increased the Tyr527 phosphorylation of c-Src (Figure 6D, lane 2), which did not change after stimulation by Gαs or Gαi (Figure 6D). We confirmed this observation with phospho-peptide mapping after cyanogen bromide digestion (data not shown). Thus, direct G protein regulation of c-Src does not involve the autodephosphorylation of Tyr527.

It has become clear that autophosphorylation of Tyr416 at the activation loop is a critical step leading to full activation of Src-family tyrosine kinases (Thomas and Brugge, 1997; Xu et al., 1999). In the suppressed form of c-Src, this activation loop adopts an α-helical configuration. In the activated form of c-Src, the activation loop becomes extended (Xu et al., 1999). Phosphorylation of Tyr416 has been proposed to stabilize this extended conformation (Xu et al., 1999). We have examined the phosphorylation state of Tyr416 before and after G protein stimulation using the specific antibody for phospho-Tyr416-containing c-Src. As shown in Figure 6E, Gαs

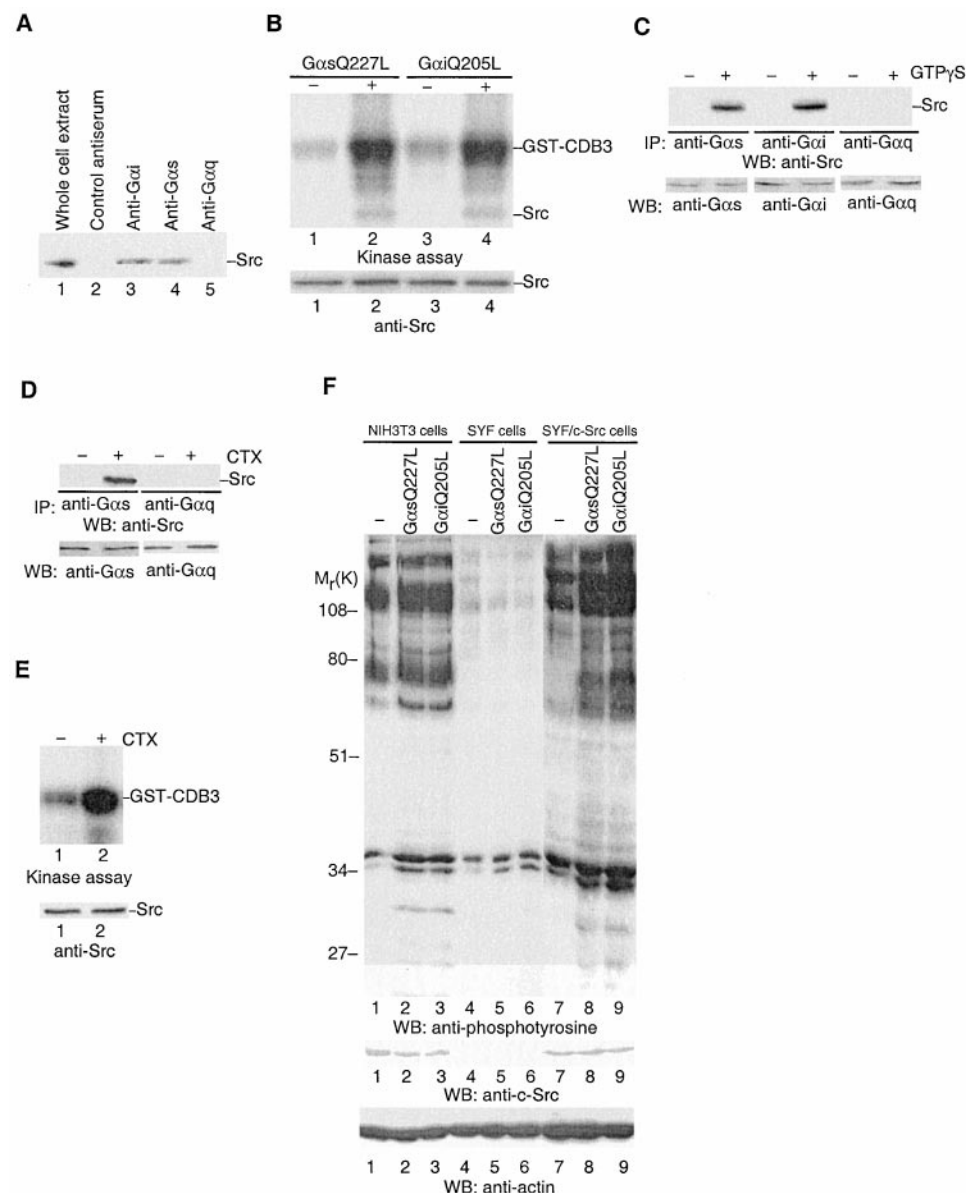


Figure 5. Interaction of Gαi and Gαs with c-Src In Vivo

(A) Coimmunoprecipitation of endogenous c-Src with Gαi or Gαs in GαiQ205L or GαsQ227L transfected NG108 cells. Anti-EAG potassium channel antibody and anti-Gαq antibody were used as negative controls (lanes 2 and 5). Lane 1, 10% of cell lysate used in immunoprecipitation was loaded as control.

(B) Expression of GαsQ227L or GαiQ205L in NG108 cells increased endogenous c-Src kinase activity.

(C) Coimmunoprecipitation of endogenous c-Src with endogenous Gαs and Gαi after stimulation with GTPγS of NG108 cell extracts. An anti-Gαq antibody was used as a control.

(D) Treatment of NG108 cells with CTX-induced coimmunoprecipitation of endogenous c-Src and endogenous Gαs.

(E) Treatment of NG108 cells with CTX increased endogenous c-Src activity.

(F) GαsQ227L and GαiQ205L induced increased cellular protein tyrosine phosphorylation in murine embryonic fibroblast NIH3T3 cells (lanes 1–3). In SYF murine embryonic fibroblast cells, the induction of tyrosine phosphorylation was reduced (lanes 4–6). Reintroduction of c-Src into SYF cells restored the GαsQ227L- and GαiQ205L-induced protein tyrosine phosphorylation. Data are representative of three experiments.

and Gαi increased the autophosphorylation of Tyr416 of c-Src.

Compared to the down-regulated form of c-Src, the activated c-Src has been proposed to have an open structure such that the SH2 domain is available for binding to phosphotyrosine-containing proteins. To investigate whether G protein stimulation of c-Src makes the SH2 domain available for protein–protein interaction, we

examined the binding ability of c-Src to phosphotyrosine-agarose before and after G protein stimulation (Sun et al., 1998). As shown in Figure 6F, downregulated c-Src had no detectable binding to phosphotyrosine agarose, while the active form of c-Src bound readily. After stimulation with either Gαs or Gαi, the binding of c-Src to phosphotyrosine agarose was increased (Figure 6F). These results imply that G protein stimulation of

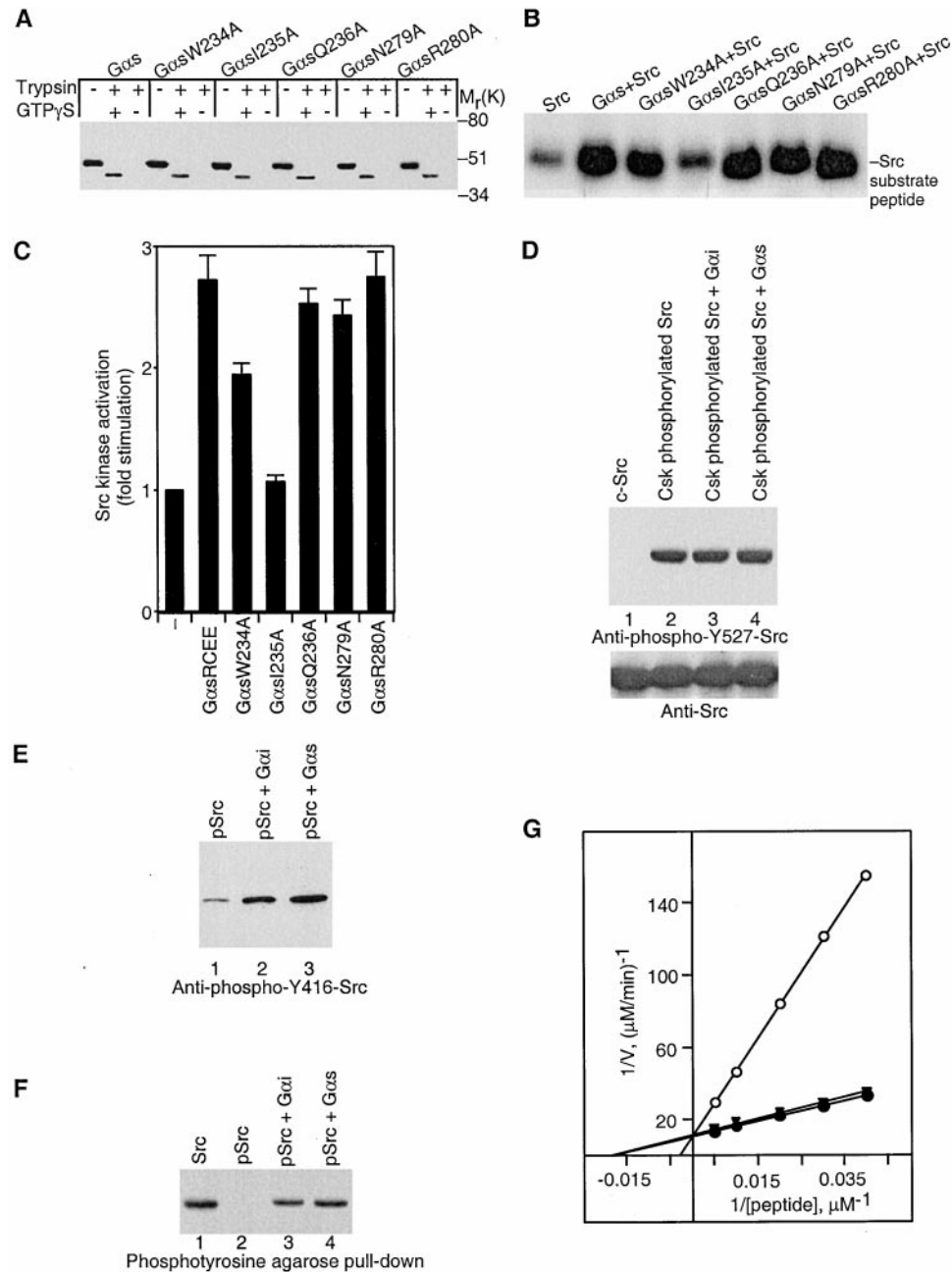


Figure 6. Activation Mechanism of c-Src by Gαs

(A) Trypsin digestion protection assay.

(B) In vitro c-Src kinase assay with purified mutant Gαs proteins.

(C) In vivo immunocomplex c-Src kinase assay from Gαs mutant transfected cells.

(D) G protein stimulation of c-Src does not involve the change of phosphorylation on the negative regulatory tyrosine residue 527 of c-Src. Equal amounts of purified c-Src (lane 1), Csk-phosphorylated c-Src (lane 2), Csk-phosphorylated c-Src after incubation with 180 nM Gαi-GTPγS (lane 3), or Csk-phosphorylated c-Src after incubation with 300 nM Gαs-GTPγS (lane 4) were loaded onto an SDS-PAGE gel. After transfer to membrane filter, an antibody for phospho-Tyr527-containing c-Src (Biosource International) was used for Western blotting to examine the phosphorylation state of Tyr527 in c-Src.

(E) G protein stimulation of c-Src increased the autophosphorylation on Tyr416 of c-Src. Equal amounts of Csk-phosphorylated c-Src (lane 1), Csk-phosphorylated c-Src after incubation with Gαi-GTPγS (lane 2), or Csk-phosphorylated c-Src after incubation with Gαs-GTPγS (lane 3) were loaded onto an SDS-PAGE gel. An antibody for phospho-Tyr416-containing c-Src (Biosource International) was used for Western blotting to examine the phosphorylation state of Tyr416 in c-Src.

(F) Phosphotyrosine agarose binding assay. Similar amounts of purified c-Src (lane 1), Csk-phosphorylated c-Src (lane 2), Csk-phosphorylated c-Src after incubation with Gαi-GTPγS (lane 3), or Csk-phosphorylated c-Src after incubation with Gαs-GTPγS (lane 4) were mixed with phosphotyrosine agarose beads. After extensive washes, phosphotyrosine agarose beads bound to c-Src were loaded onto an SDS-PAGE gel and Western blotted with an anti-c-Src antibody.

(G) Double reciprocal plots for phosphorylation of the Src peptide substrate by c-Src in the absence (open circles) or presence of Gαi-GTPγS (filled circles) or Gαs-GTPγS (filled triangles). Data are representative of three experiments.

Table 2. Effect of $G\alpha i$ and $G\alpha s$ on Kinetic Parameters of Src

	K_m (pep)	K_m (ATP)	V_{max}	K_{cat}	K_{cat}/K_m (pep)	K_{cat}/K_m (ATP)
	μM	μM	$\mu M/min$	min^{-1}	$min^{-1} \mu M^{-1}$	$min^{-1} \mu M^{-1}$
pY527 c-Src	368 ± 60	55 ± 11	0.1 ± 0.01	10 ± 1	0.03 ± 0.00	0.18 ± 0.02
+ $G\alpha i$	59 ± 9	32 ± 5	0.1 ± 0.01	10 ± 1	0.17 ± 0.02	0.31 ± 0.03
+ $G\alpha s$	58 ± 8	26 ± 7	0.1 ± 0.01	10 ± 1	0.17 ± 0.02	0.39 ± 0.04

Src activity derived from data such as Figure 6G yielded the K_m and V_{max} values listed. K_{cat} and K_{cat}/K_m were calculated. $K_{m(pep)}$: K_m for the substrate peptide. Data are representative of at least three independent measurements.

downregulated c-Src can cause a conformational change, release the phospho-Tyr527 binding from the SH2 domain, and make the SH2 domain available for interacting with other proteins.

G Proteins Increase c-Src Enzymatic Activity by Decreasing K_m without Affecting V_{max}

We have further studied the effect of $G\alpha s$ and $G\alpha i$ on the kinetic parameters of c-Src in order to gain insights on possible regulatory mechanism. Regulation of enzymatic activity can be studied in terms of changes in K_m (Michaelis constant), V_{max} (maximum velocity), or K_{cat} (maximum turnover rate). After phosphorylation by Csk, we determined that c-Src has a K_m for the peptide substrate of $\sim 370 \mu M$, and K_m for ATP $\sim 55 \mu M$ (Figure 6G and Table 2). These numbers are similar to previous reports of Csk-phosphorylated c-Src and Hck (Wong and Goldberg, 1984; Boerner et al., 1996; LaFevre-Bernt et al., 1998). We found that the major effect of $G\alpha i$ and $G\alpha s$ was to decrease the K_m for the peptide substrate to $\sim 60 \mu M$ (Figure 6G and Table 2). The change on K_m for ATP was less than 2-fold. V_{max} was not affected (Table 2). These data bolster the idea that G protein binding changes the conformation of c-Src, allowing easier access for the substrate to the active site.

Discussion

G proteins and tyrosine kinases are two major cellular signal transducers. They transmit signals from a wide

variety of cell surface receptors to generate physiological responses. Their malfunction often leads to diseases. Although activation of G protein-coupled receptors can elicit rapid stimulation of cellular protein tyrosine phosphorylation, the mechanism by which G proteins activate protein-tyrosine kinases is not completely understood. Since tyrosine kinases have been shown to form networks with a diverse array of cellular signal transduction pathways, this G protein-tyrosine kinase link provides novel mechanisms for G protein signaling to modulate a broad range of physiological responses. Therefore, it is essential to understand how these two common signaling components communicate with each other.

G Protein Regulation of Src

Src-family tyrosine kinases have been shown to play critical roles in G protein-coupled receptor signaling. However, the link between Src and these G protein-coupled receptors was not clear. Until now, G protein α subunits have not been studied for their ability to increase c-Src kinase activity. There was one report that $G\beta\gamma$ subunits, when overexpressed in COS-7 cells, increased (by ~ 2 -fold) c-Src Tyr416-autophosphorylation (Luttrell et al., 1996). Here, we present evidence that activated $G\alpha s$ and $G\alpha i$, but neither $G\alpha q$, $G\alpha 12$, nor $G\beta\gamma$, directly regulate Src-family tyrosine kinases by activating the downregulated, C-terminal phosphorylated kinase. We have also shown that, in Src-family tyrosine kinase knockout SYF cells, $G\alpha sQ227L$ and $G\alpha iQ205L$

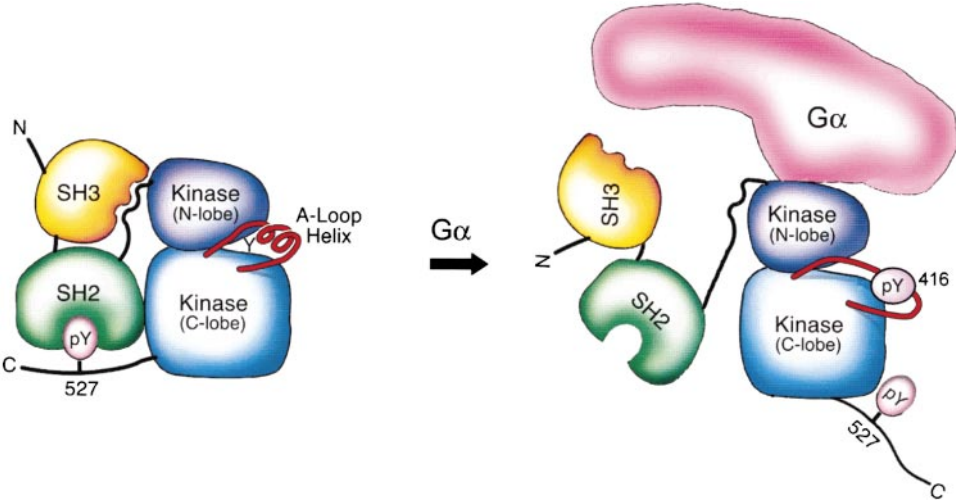


Figure 7. Proposed Model for G Protein Activation of c-Src
The drawing of the downregulated c-Src was adapted from Xu et al. (1999) and Moarefi et al. (1997). The contact sites of $G\alpha$ protein and of c-Src are postulated.

induced tyrosine phosphorylation of many of cellular proteins was severely reduced, suggesting that Src-family tyrosine kinases are the major mediator of $G_{\alpha s}$ - and $G_{\alpha i}$ -induced protein tyrosine phosphorylation in cells. Furthermore, we have demonstrated that the switch II region of $G_{\alpha s}$ is involved in interacting with Src. In general, G proteins, including heterotrimeric and Ras-family G proteins, use their effector domains to contact the downstream effectors. For Ras, the switch I region is a core effector domain essential for all effector interactions. The switch II region of $G_{\alpha s}$ and $G_{\alpha i}$ has been shown to be important in interacting with their common effector adenylyl cyclase. Our data suggest that $G_{\alpha s}$ uses some residues such as I235 in the switch II region, and likely residues in other regions, to interact with the catalytic domain of Src. These studies significantly advance our understanding of an important aspect of the cross-talk between G protein-coupled receptors and tyrosine kinases. Furthermore, these experiments provide a possible mechanism of action by which the *gip2* mutant of $G_{\alpha i}$ and the *gsp* mutant of $G_{\alpha s}$ induce oncogenicity (Landis et al., 1989; Lyons et al., 1990).

cAMP-PKA Independent Signaling by $G_{\alpha s}$ and $G_{\alpha i}$

There are previous reports indicating that some biological effects of $G_{\alpha s}$ and $G_{\alpha i}$ could not be explained by their opposing effects on adenylyl cyclases (Morris and Malbon, 1999). Both activated mutants (GTPase deficient) of $G_{\alpha s}$ and $G_{\alpha i}$ are oncogenes, found in certain human tumors (Landis et al., 1989; Lyons et al., 1990). Both $G_{\alpha s}$ and $G_{\alpha i}$ can transform cells and activate the mitogen-activated protein kinase (MAPK) pathways (Pace et al., 1991; Gupta et al., 1992a, 1992b; Pace et al., 1995). Activated $G_{\alpha i}$ subunits induced a transformed phenotype in fibroblasts independent of inhibition of adenylyl cyclase (Gupta et al., 1992b). Also, $G_{\alpha s}$ and $G_{\alpha i}$ regulate adipogenesis in mouse 3T3-L1 cells and stem cell differentiation of F9 teratocarcinoma cells into primitive endoderm, which could not be explained by changes in intracellular cAMP (Wang et al., 1992; Watkins et al., 1992). Furthermore, inhibition of magnesium uptake in S49 cells by isoproterenol or prostaglandin E_1 had previously been shown to be $G_{\alpha s}$ -dependent, but cAMP and PKA-independent (Maguire and Erdos, 1980). Similarly, in differentiating wing epithelial cells of *Drosophila*, activation of $G_{\alpha s}$ leads to formation of wing blisters (Wolfgang et al., 1996). This pathway was genetically demonstrated to be independent of PKA (Wolfgang et al., 1996). Recently, we found that engagement of β -adrenergic receptors initiated a $G_{\alpha s}$ -dependent, PKA-independent pathway leading to apoptosis in S49 cells (Gu et al., 2000). These reports, together with our observations, clearly indicate that $G_{\alpha s}$ and $G_{\alpha i}$ can signal through novel transduction pathways, in addition to the classically defined cAMP second messenger system. The relative contribution of these different effector systems to the physiology of G proteins in organisms remains to be addressed.

Proposed Activation Mechanism

c-Src kinase activity can be modulated by either tyrosine phosphorylation or conformational changes. Our data indicated that neither $G_{\alpha s}$ nor $G_{\alpha i}$ changed the Tyr527 phosphorylation state, suggesting that autodephosphorylation of Tyr527 is not the regulatory mechanism

used by G proteins. $G_{\alpha s}$ and $G_{\alpha i}$ did increase the autophosphorylation of Tyr416. Our binding data showing that $G_{\alpha s}$ and $G_{\alpha i}$ interact with the catalytic domain of Src suggest that a conformational change model is likely. Indeed, our phosphotyrosine binding experiment showed that G protein stimulation caused the release of phospho-Tyr527 from the SH2 domain, making it available for interacting with other proteins. In a similar manner, activation of Hck kinase activity by the Nef protein of human immunodeficiency virus-1 (HIV-1), comes about through a conformational change, which occurs in the absence of dephosphorylation of the tail Tyr527 (Briggs et al., 1997; Moarefi et al., 1997). Our enzymatic kinetic measurement revealed that the major effect of $G_{\alpha s}$ and $G_{\alpha i}$ was to decrease the K_m for the peptide substrate. These data suggest that G protein binding changes the conformation of c-Src and allows the peptide substrate easier access to the active site. This model is consistent with the structural data of Src-family tyrosine kinases. In the downregulated state, two intramolecular interactions stabilize the restrained conformation of the kinase domain (Sicheri et al., 1997; Williams et al., 1997; Xu et al., 1997). The activation loop forms an α helix that packs between the upper and lower lobes of the catalytic domain, thus blocking the peptide substrate binding site (Schindler et al., 1999; Xu et al., 1999). In the active state, the activation loop swings away from the entrance of the catalytic cleft, allowing access of the substrate to the active site (Yamaguchi and Hendrickson, 1996). This activation mode has also been observed in the activation of cyclin-dependent kinase (cdk). Cyclin binding to the catalytic domain repositions cdk's activation loop and permits access of substrates to the active site (Jeffery et al., 1995). We propose that G protein binding to the catalytic domain modulates the position and conformation of the activation loop, as well as other elements in the catalytic domain. This could lead to relief of steric hindrance at the entrance to the catalytic cleft, increased accessibility of the active site to substrates, exposure of the side chain of Tyr416 making it a better substrate for autophosphorylation (the Tyr416 hydroxyl group is buried in the catalytic cleft in the downregulated form), and thus increased kinase activity (Figure 7). Regardless of the detailed chemical mechanism, our data demonstrate that c-Src and Hck are novel direct effectors of $G_{\alpha s}$ and $G_{\alpha i}$ proteins.

Experimental Procedures

Protein Purification

$G_{\alpha i1}$ and $G_{\alpha s}$ were purified from *E. Coli* as described (Lee et al., 1994). The pQE60- $G_{\alpha i1}$ and pQE60- $G_{\alpha s}$ plasmids were transformed into bacteria strain BL21(DE3)pLysS. Myristoylated $G_{\alpha i1}$ was synthesized with coexpression of yeast protein N-myristoyltransferase (Linder et al., 1991). One liter of bacterial culture was grown at room temperature until the absorbance at 600 nm was ~ 1.4 . G protein expression was induced with 0.2 mM IPTG (Research Products International) for 18 hr at room temperature. The bacterial pellet was resuspended into lysis buffer (50 mM HEPES [pH 8.0], 3 mM $MgCl_2$, 20 mM β -mercaptoethanol, 0.7% CHAPS, and protease inhibitor cocktail tablet [Boehringer Mannheim]) on ice. Lysozyme (0.1 mg/ml) was added and the sample was incubated on ice for 30 min. After cell lysis by sonication, the lysate was spun down at $10,000 \times g$ for 30 min at 4°C. Ni-NTA agarose resin (5 ml, from Qiagen) was added into the supernatant after preequilibration of the resin with lysis buffer. The mixture was gently agitated overnight at 4°C and packed into a C16/20 column (Pharmacia), washed with lysis buffer plus 100 mM NaCl, and eluted with lysis buffer plus a linear gradient

of imidazole (10 mM to 500 mM). $G_{\alpha i1}$ was most abundant in the 20 mM imidazole elution fraction, whereas $G_{\alpha s}$ was abundant in the 200 mM imidazole fraction. Elutions were concentrated and changed to Mono-Q buffer (50 mM Tris [pH 8.0], 2 mM DTT, and 1 mM EDTA) by using Millipore Ultrafree filters and further purified on a Mono Q column (Pharmacia) with a linear gradient of NaCl (100 mM to 800 mM). $G_{\alpha s}$ elute fractions were changed to HPHT buffer (10 mM Tris [pH 8.0], 1 mM DTT, and 10 mM K_2HPO_4) and purified by using a Bio-Rad ChT-II cartridge with a linear gradient of phosphate (20 mM to 500 mM). Neither $G_{\alpha s}$ nor $G_{\alpha i}$ was palmitoylated. Protein concentration, purity, and identity were analyzed by silver stain and Western blot. $G_{\alpha i1}$ and $G_{\alpha s}$ were activated in the presence of 10 mM $MgSO_4$ and 100-fold molar excess of GTP γ S at 30°C for 1 hr. Free GTP γ S was removed by gel filtration.

c-Src (human c-Src(Δ N85), without the first 85 amino acid residues) was purified from Sf9 cells as described in Xu et al. (1997). Insect cells from 6L culture bearing c-Src(Δ N85) were lysed in 50 ml sonication buffer (25 mM HEPES [pH 7.6], 150 mM NaCl, 5 mM DTT, 5 mM Benzamidine, 1 mM PMSF, 2.5 μ g/ml leupeptin, and 2.5 μ g/ml pepstatin A) and then diluted into 300 ml dilution buffer (25 mM HEPES [pH 7.6], 25 mM NaCl, 5 mM DTT, 5 mM Benzamidine, 1 mM PMSF, 2.5 μ g/ml leupeptin, and 2.5 μ g/ml pepstatin A). The lysate was cleared by centrifugation at $35,000 \times g$ for 4 hr with an SS-34 rotor (Sorvall). The supernatant was then loaded onto a 200 ml DEAE-Sepharose FF column (XK-50, Pharmacia) that had been preequilibrated with 25 mM HEPES [pH 7.6], 25 mM NaCl, and 5 mM DTT. After the column was washed until the OD_{280} went back to the base line, a 1L linear gradient was run up to 25 mM HEPES [pH 7.6], 5 mM DTT, and 0.6 M NaCl. Fractions containing Src were identified by Western blot and combined. Proteins were precipitated by 60% $(NH_4)_2SO_4$ at 4°C, and the flocculent white precipitate was collected by centrifugation for 45 min at $40,000 \times g$ at 4°C. The pellet was resuspended into 25 mM HEPES (pH 7.6) and 5 mM DTT and dialyzed against 2L 25 mM HEPES (pH 7.6) and 5 mM DTT at 4°C. The dialyzed solution was further passed through a 10 ml γ -aminophenyl ATP agarose column (Upstate Biotechnology) and eluted with a linear gradient of NaCl to 1 M. Src with different phosphorylation states was separated by phosphotyrosine chromatography. More specifically, purified c-Src(Δ N85) was concentrated to more than 1 mg/ml in 25 mM HEPES (pH 7.6), 25 mM NaCl, and 5 mM DTT and loaded onto a 20 ml phosphotyrosine column (Sigma). Protein was eluted with 25 mM HEPES (pH 7.6), 1 M NaCl, and 5 mM DTT. Purified protein was maintained in a storage buffer containing 25 mM HEPES (pH 7.6), 0.1 M NaCl, and 5 mM DTT. The yield of Tyr527-phosphorylated Src was increased by incubating nonphosphorylated protein (1 mg/ml) in storage buffer with purified recombinant Csk (1 mg/ml), in the presence of 10 mM $MnCl_2$ and 0.1 M ATP. Hck (human Hck(Δ N77), without the first 77 amino acid residues) was purified from Sf9 cells as described in Sicheri et al. (1997). Csk (chicken Csk) was purified from *E. coli* as described in Sondhi et al. (1998). Phosphorylation of Src and Hck by Csk, and removal of Csk and unphosphorylated c-Src by chromatography was done as described in Xu et al. (1997).

Glutathione S-transferase fusion proteins: DNA sequences corresponding to the indicated regions of c-Src cDNA were amplified by PCR and subcloned into pGEX-2T (Pharmacia). Each construct was confirmed by DNA sequencing. GST fusion proteins were expressed in BL21 cells harboring pREP4groESL and purified on glutathione-agarose beads (Sigma) (Bence et al., 1997; Ma and Huang, 1998). Fusion proteins bound to the beads were used for binding assays.

Kinase Assays

Kinase assay was performed as previously described (Bence et al., 1997; Jiang et al., 1998). Purified c-Src or Hck kinase (10 ng) (final concentration 10 nM) in Src kinase buffer (30 mM HEPES [pH 7.4], 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 10 μ M ATP) was combined with 2 μ g (70 μ M) Src substrate peptide (KVRKIGEGTYGVVK). Csk could not phosphorylate this substrate peptide (Sondhi et al., 1998). The appropriate amount of purified G protein subunit was added and kinase buffer was used to bring the total reaction volume to 20 μ l. Ten μ Ci [γ - 32 P]ATP (3000 Ci/mmol) was added and the mixture incubated at 30°C for 15 min. The reaction was stopped by adding

Laemmli sample buffer. After incubation at 90°C for 5 min, the substrate peptide was separated on 20% SDS-PAGE gel, dried, autoradiographed, and quantified with a phosphorimager. Bands were cut out of the gel and counted in a scintillation counter.

In Vitro Binding Assay

In vitro affinity chromatographic binding assay was done as previously described (Ma and Huang, 1998). GST pull-down assay was similarly performed using GST-fusion proteins attached to glutathione agarose beads and purified $G_{\alpha s}$ -GTP γ S and $G_{\alpha i}$ -GTP γ S.

Coimmunoprecipitation Assay

Immunoprecipitation and Western blot were done as previously described (Langhans-Rajasekaran et al., 1995; Wan et al., 1997). In some cases, cells were incubated with cholera toxin (30 ng/ml) or cell extracts were incubated with GTP γ S (100 μ M) for 90 min.

Immunocomplex Kinase Assay

Immunocomplex kinase assay was done as previously described (Wan et al., 1997). Src immunoprecipitation was done with a monoclonal antibody to Src (Oncogene Science). Src kinase assay was performed with 5 μ g of the purified glutathione-S-transferase and the cytoplasmic domain of human erythrocyte band 3 fusion protein (GST-CDB3) as substrate (Wan et al., 1997).

Immunoblot Analysis

Western blotting was done as previously described (Wan et al., 1997). Anti-pTyr527-c-Src and anti-pTyr416-c-Src rabbit polyclonal antibodies were from BioSource International. Anti-G protein antibodies and antibodies for c-Src and Hck were from Oncogene Sciences or Santa Cruz Biotechnology. Anti-phosphotyrosine antibody pY102 was from New England BioLab.

SYF cells

SYF cells, a murine embryonic fibroblast cell line derived from mutant embryos deficient for the three ubiquitously expressed members of Src-family tyrosine kinases c-Src, Yes, and Fyn were from American Type Culture Collection and maintained in DMEM supplemented with 10% fetal calf serum (Klinghoffer et al., 1999). Transfection of SYF cells was done with Effectene Transfection Reagent (Qiagen) following the manufacturer's protocol. SYF/c-Src cells were obtained by expressing c-Src in SYF cells via the retroviral vector pLHCX and were selected with 400 μ g/ml hygromycin B (Calbiochem). Expression of c-Src was checked by Western blot with anti-c-Src antibody. NIH3T3 murine embryonic fibroblast cells (ATCC) were transiently transfected with Cytofectene Transfection Reagent (Bio-Rad).

Mutagenesis

Site-directed mutagenesis of $G_{\alpha s}$ mutants was performed with the GeneEditor in vitro site-directed mutagenesis system (Promega). The DNA template was pQE60- $G_{\alpha s}$ (Lee et al., 1994). These $G_{\alpha s}$ mutants were also subcloned into pcDNA1.1/Amp mammalian expression vector with $G_{\alpha s}$ RCEE background (Grishina and Berlot, 1997), in which the arginine at position 201 of $G_{\alpha s}$ was mutated to cysteine to inhibit GTPase activity, and an EE epitope was generated. Mutant $G_{\alpha s}$ proteins were purified as described in the Protein Purification section.

Phosphotyrosine-Agarose Binding Assay

The phosphotyrosine agarose binding assay to examine the accessibility of the SH2 domain was performed as described (Sun et al., 1998).

Enzyme Kinetics Analysis

When K_m [$_{app}$] was determined, [ATP] was fixed at 100 μ M. When K_m [ATP] was determined, the concentration of the peptide substrate was fixed at 1 mM. Data were analyzed with Lineweaver-Burk reciprocal plot: $1/v = K_m/V_{max} \cdot 1/[S] + 1/V_{max}$. In this $1/v$ versus $1/[S]$ plot, the slope equals to K_m/V_{max} and the intercept on the $1/v$ axis equals to $1/V_{max}$. K_{cat} was calculated as $V_{max}/[E]$.

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