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Gα and Gβγ Require Distinct Src-dependent Pathways to Activate Rap1 and Ras*

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The Src tyrosine kinase is necessary for activation of extracellular signal-regulated kinases (ERKs) by the β-adrenergic receptor agonist, isoproterenol. In this study, we examined the role of Src in the stimulation of two small G proteins, Ras and Rap1, that have been implicated in isoproterenol's signaling to ERKs. We demonstrate that the activation of isoproterenol of both Rap1 and Ras requires Src. In HEK293 cells, isoproterenol activates Rap1, stimulates Rap1 association with B-Raf, and activates ERKs, all via PKA. In contrast, the activation by isoproterenol of Ras requires Gβγ subunits, is independent of PKA, and results in the phosphoinositol 3-kinase-dependent activation of AKT. Interestingly, β-adrenergic stimulation of both Rap1 and ERKs, but not Ras and AKT, can be blocked by a Src mutant (SrcS17A) that is incapable of being phosphorylated and activated by PKA. Furthermore, a Src mutant (SrcS17D), which mimics PKA phosphorylation at serine 17, stimulates Rap1 activation, Rap1/B-Raf association, and ERK activation but does not stimulate Ras or AKT. These data suggest that Rap1 activation, but not that of Ras, is mediated through the direct phosphorylation of Src by PKA. We propose that the β_2-adrenergic receptor activates Src via two independent mechanisms to mediate distinct signaling pathways, one through $G\alpha_s$ to Rap1 and ERKs and the other through Gβγ to Ras and AKT.

Stimulation of G protein-coupled receptors (GPCRs)[1] triggers a wide range of biochemical and physiological effects. GPCR activation of heterotrimeric G proteins signals to distinct effector molecules through both the G protein α and βγ subunits (1–3). $G\alpha_s$ activation stimulates adenylyl cyclases to elevate intracellular cAMP and activation of PKA. PKA can regulate cell growth and differentiation through cross-talk with the mitogen-activated protein kinase or ERK (extracellular signal-regulated kinase) cascade (4–8).

In HEK293 cells, the β2-adrenergic receptor agonist isoproterenol stimulates endogenous receptors to activate ERKs through a PKA-dependent pathway (9, 10). The activation by isoproterenol of PKA has been shown to induce the activation of Ras via a Src-dependent mechanism that is mediated by Gβγ subunits (10–12). However, isoproterenol and PKA can also activate Rap1 and ERKs in these cells (9). In NIH3T3 fibroblast cells, PKA activation of Rap1 has been proposed to result from the direct phosphorylation by PKA on the Src tyrosine kinase (13). In NIH3T3 cells that do not express B-Raf, PKA and Rap1 antagonize Ras-dependent activation of ERKs (4, 7). In addition to antagonizing Ras, Rap1 can activate ERKs in cells that express the mitogen-activated protein kinase kinase kinase B-Raf (14). However, the contribution of Src in this action of Rap1 has not been examined.

Because it has been shown that isoproterenol couples efficiently to both Ras and Rap1 in HEK293 cells, this model system provides an opportunity to examine the requirement of Src in each process. Surprisingly, we found that Src was required for the activation of both Ras and Rap1 by isoproterenol. However, it activated Rap1 and Ras through distinct mechanisms.

MATERIALS AND METHODS

Reagents—Antibodies specific to phosphorylated-ERK (pERK) that recognize phosphorylated ERK1 (pERK1) and ERK2 (pERK2) at residues threonine 183 and tyrosine 185 were purchased from New England Biolabs (Beverly, MA). Antibodies specific to phosphorylated-AKT (pAKT) that recognize phosphorylated AKT at residue threonine 308 were purchased from Cell Signaling (Beverly, MA). Antibodies to Rap1, Raf-1, B-Raf, ERK2, c-Myc (9E10), Cbl, C3G, and agarose-conjugated antibodies to Myc and hemagglutinin (HA) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies to HA (12CA5) were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Anti-Ras antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). FLAG (M2) antibody, isoproterenol, and epidermal growth factor (EGF), were purchased from Sigma. Forskolin, PP2 (AG1879; 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyazolo[3,4-d]pyrimidine), LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-bemzopyran-4-one), and *N*-(2-(*p*-bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide (H89) were purchased from Calbiochem (Riverside, CA). Nickel-nitrilotriacetic acid-agarose was purchased from Qiagen Inc. (Chatswoth, CA).

Cell Culturing Conditions and Treatments—HEK293, SYF, and Src^{2+} cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum, penicillin/streptomycin, and L-glutamine at 37 °C in 5% CO_2. Cells were maintained in serum-free Dulbecco's modified Eagle's medium for 16 h at 37 °C in 5% CO_2 prior to treatment with various reagents for immunoprecipitation assay, and Western blotting. In all experiments, cells were treated with EGF (100 ng/ml), isoproterenol (10 μM), or forskolin (10 μM) for 5 min unless otherwise indicated. PP2 (10 μM), H89 (10 μM), and LY294002 (10 μM), were added to cells 20 min prior to treatment, unless otherwise indicated.

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[1] The abbreviations used are: GPCR, G protein-coupled receptors; PKA, protein kinase A; ERK, extracellular-regulated signal kinase; HA, hemagglutinin; EGF, epidermal growth factor; GST, glutathione *S*-transferase; SFK, Src family kinases; ARK, β-adrenergic receptor kinase; HEK, human embryonic kidney; PI3K, phosphoinositol 3-kinase.

Western Blotting and Immunoprecipitation—Cell lysates and Western blotting were prepared as described (7). Briefly, protein concentrations were quantified using the Bradford protein assay. For detection of Raf-1, ERK2, Myc-ERK2, FLAG, Rap1, Ras, pERK1/2, Cbl, C3G, and pAKT, equivalent amounts of protein per treatment condition were resolved by SDS-PAGE, blotted onto polyvinylidene difluoride (Millipore Corp., Bedford, MA) membranes, and probed with the corresponding antibodies according to the manufacturer's guidelines. For immunoprecipitation of Myc-ERK2, Myc-Cbl, FLAG-Src, and HA-AKT equal amounts of cell lysate per condition were precipitated at 4 °C for 4–6 h in lysis buffer. Proteins were then resolved by SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and probed with the indicated antibodies. In all cases, the results illustrated are from representative experiments, repeated at least three times.

Plasmids and Transfections—The Src, SrcS17A, and SrcS17D plasmids were all generated as previously described (13). The SrcY527F mutants were synthesized using PCR primers containing sequences corresponding to the 5′ end of the Src cDNA and sequences corresponding to the 3′ end of the Src cDNA, with the sequence corresponding to tyrosine 527 replaced with that for phenylalanine (Y527F). SrcWT, SrcS17A, and SrcS17D were amplified with these primers and subcloned into a FLAG-pcDNA3 to create SrcY527F, SrcS17A/Y527F, and SrcS17D/Y527F, respectively. Cbl-ct, encoding the carboxyl-terminal amino acids 541 to 906, was provided by Dr. Brian Druker (OHSU, Portland, OR). Hemagglutinin-tagged AKT (HA-AKT) was provided by Dr. Thomas Soderling (Vollum Institute, Portland, OR). The transducin (cone) cDNA was provided by the Guthrie cDNA Resource Center (www.guthrie.org/AboutGuthrie/Research/cDNA). Seventy to eighty percent confluent HEK293, SYF, or Src^{2+} cells were co-transfected with the indicated cDNAs using a LipofectAMINE 2000 kit (Invitrogen) according to the manufacturer's instructions. The control vector, pcDNA3 (Invitrogen Corp.), was included in each set of transfections to assure that each plate received the same amount of DNA. Following transfection, cells were allowed to recover in serum-containing media for 24 h. Cells were then starved overnight in serum-free Dulbecco's modified Eagle's medium before treatment and lysis.

Affinity Assay for Rap1 Activation—A GST fusion protein of the Rap1-binding domain of RalGDS (GST-RalGDS) was expressed in *Escherichia coli* following induction by isopropyl-1-thio-β-D-galactopyranoside (GST-RalGDS was a gift from Dr. Johannes Bos, Utrecht University, The Netherlands). Cells were grown as described and were stimulated at 37 °C for the indicated times and lysed in ice-cold lysis buffer (50 mM Tris-Cl (pH 8.0), 10% glycerol, 1% Nonidet P-40, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 mM NaF, 0.1 μM aprotinin, and 1 mM $NaVO_4$). Active Rap1 was isolated as previously described by Franke *et al.* (15). Equivalent amounts of supernatants (500 μg) were incubated with the GST-RalGDS-Rap1 binding domain coupled to glutathione beads. Following a 1-h incubation at 4 °C, beads were pelleted and rinsed three times with ice-cold lysis buffer, proteins were eluted from the beads using 2× Laemmli buffer and applied to a 12% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membrane, blocked in 5% milk for 1 h, and probed with either α-Rap1/Krev-1 or FLAG antibody overnight at 4 °C, followed by incubation with an horseradish peroxidase-conjugated anti-rabbit IgG antibody (or an anti-mouse IgG for anti-FLAG Western blots). Proteins were detected using enhanced chemiluminescence. All experiments were repeated at least three times and representative gels are shown.

Affinity Assay for Ras Activation—HEK293 cells were grown as described, stimulated, and lysed in ice-cold lysis buffer. Activated Ras was assayed as previously described (7). Briefly, equivalent amounts of lysates from stimulated cells were incubated with GST-Raf1-RBD (Ras-binding domain) as specified by the manufacturer (Upstate Biotechnology, Lake Placid, NY). Proteins were eluted with 2× Laemmli buffer and applied to a 12% SDS-polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride membrane, blocked at room temperature for 1 h in 5% milk, and probed with either Ras or FLAG antibody overnight at 4 °C, followed by horseradish peroxidase-conjugated anti-mouse secondary antibodies. Proteins were detected using enhanced chemiluminescence. All experiments were repeated at least three times and representative gels shown.

Nickel Affinity Chromatography—HEK293 cells were transfected using LipofectAMINE reagent with polyhistidine-tagged Rap1 (His-Rap1) as previously described (7, 9). Briefly, cells were lysed in ice-cold buffer containing 1% Nonidet P-40, 10 mM Tris, pH 8.0, 20 mM NaCl, 30 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mg/ml aprotinin and supernatants were prepared by low speed centrifugation. Transfected His-tagged proteins were precipitated from supernatants containing equal amounts of protein using nickel-nitrilotriacetic acid-agarose and washed with 20 mM imidazole in lysis buffer and eluted with 500 mM imidazole and 5 mM EDTA in phosphate-buffered saline. The eluates containing His-tagged proteins were separated on SDS-PAGE and Raf-1 proteins were detected by Western blotting (7, 9).

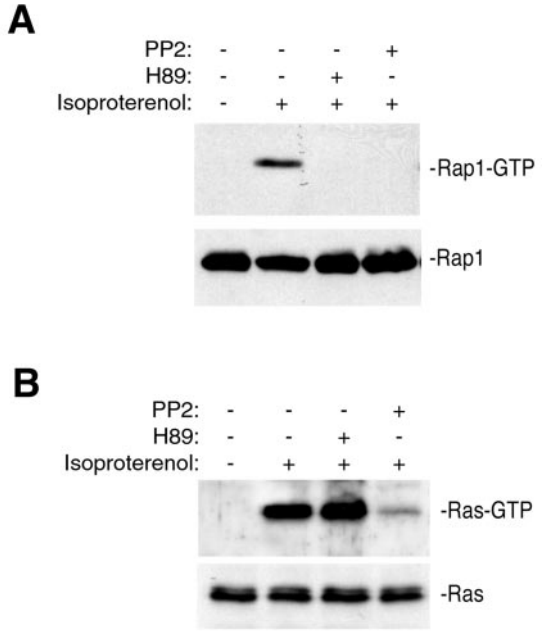

FIG. 1. **Activation by isoproterenol of Rap1 and Ras.** *A*, the activation by isoproterenol of the small G protein Rap1 is PKA- and SFK-dependent. HEK293 cells received no pretreatment or were pretreated with H89 or PP2, and then treated with isoproterenol, as indicated (see "Experimental Procedures"). Cell lysates were prepared as described under "Experimental Procedures" and endogenous GTP-loaded Rap1 was examined by Western blot (*upper panel*). The *lower panel* is a Western blot demonstrating equivalent loading of total Rap1 in whole cell lysates used for the Rap1 assay, using the Rap1 antibody. *B*, the activation by isoproterenol of Ras is SFK-dependent and PKA-independent. HEK293 cells were treated as in *A*. Cell lysates were examined for endogenous GTP-loaded Ras as described under "Experimental Procedures" and probed for Ras-GTP (*upper panel*). The *lower panel* demonstrates that equivalent amounts of total Ras in whole cell lysates were loaded, as evidenced by anti-Ras Western blot.

RESULTS

In HEK293 cells, isoproterenol activated endogenous Rap1 in a PKA-dependent manner, as previously shown (9). Interestingly, this activation required Src family kinases (SFKs) as it was blocked by the inhibitor PP2 (16) (Fig. 1*A*). Isoproterenol also activated endogenous Ras via SFKs, but this proceeded through a PKA-independent pathway (Fig. 1*B*).

To determine which SFK was mediating these effects, we utilized a pair of cell lines derived from mouse embryo fibroblasts that lack SFKs. One cell line, SYF, was developed from mice deficient in the genes encoding Yes, Fyn, and Src, and has been shown to lack SFK activity (17). The second cell line, Src^{2+}, originated from mice deficient in only Yes and Fyn and maintained the wild type Src gene and normal Src protein levels (17). The ability of isoproterenol to activate Rap1 was absent in SYF cells (Fig. 2*A*), but was retained in Src^{2+} cells (Fig. 2B). Similarly, the ability of isoproterenol to activate Ras was also absent in SYF cells (Fig. 2*C*), but was retained in Src^{2+} cells (Fig. 2*D*). Additionally, both actions of isoproterenol could be reconstituted by transfecting wild type Src (SrcWT) into SYF cells (Fig. 2, *A* and *C*). Taken together, these data demonstrate that Src is required for activation by isoproterenol of both Ras and Rap1 in these cells.

The ability of cAMP to activate Rap1 in selected cell types has recently been shown to require Src and PKA (13), through

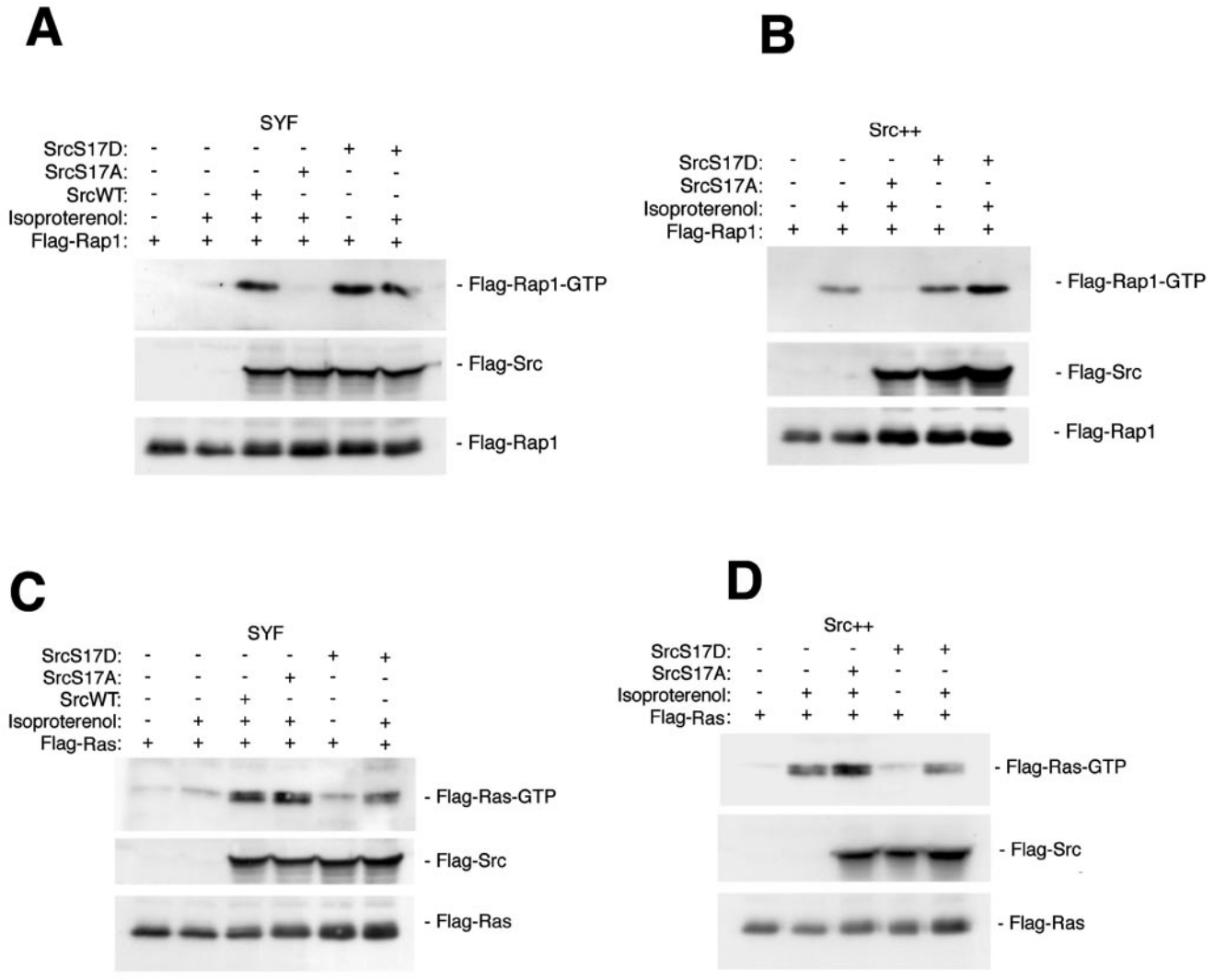

FIG. 2. **Rap1 and Ras activation by isoproterenol require Src kinase.** *A* and *B*, PKA phosphorylation of serine 17 on Src is necessary and sufficient for Rap1 activation by isoproterenol. SYF cells (*A*) or Src^{2+} cells (*B*) were transfected with FLAG-Rap1 and the indicated Src cDNAs, or pcDNA3 vector alone. Cells were stimulated with isoproterenol or left untreated, as indicated. Cell lysates were examined for active GTP-loaded Rap1 (Flag-Rap1-GTP). *C* and *D*, phosphorylation of Src by PKA on serine 17 is not required for Ras activation by isoproterenol. SYF cells (*C*) or Src^{2+} cells (*D*) were transfected with FLAG-Ras and the indicated Src cDNAs or vector alone. Cells were stimulated with isoproterenol or left unstimulated, and cell lysates were examined for active GTP-loaded Ras (*Flag-Ras-GTP*). In all experiments, the level of FLAG-Src and FLAG-Rap1 or FLAG-Ras are shown in the *middle* and *lower panels*.

the PKA-dependent phosphorylation of Src at serine 17 (Ser^{17}). However, the requirement of Ser^{17} phosphorylation in hormonal signaling to Ras, Rap1, and ERKs has not been examined. To address the requirement of Ser^{17} phosphorylation in Ras and Rap1 signaling, we examined their activation by isoproterenol in cells expressing one of two SrcS17 mutants. Unlike SrcWT, the expression of a mutant Src, where Ser^{17} was replaced with an alanine (SrcS17A), was unable to reconstitute the activation by isoproterenol of Rap1 (Fig. 2*A*). Moreover, expression of a second Src mutant in which Ser^{17} was replaced with an aspartate (SrcS17D) resulted in constitutive activation of Rap1 (Fig. 2*A*). This is similar to previous results from cells treated with forskolin, an activator of adenylyl cyclases (13). Importantly, this activation of Rap1 by SrcS17D was not further stimulated by isoproterenol, suggesting that SrcS17D was maximally activating Rap1. The expression of SrcS17A in Src^{2+} cells inhibited the activation by isoproterenol of Rap1 (Fig. 2*B*), suggesting that SrcS17A was interfering with signals through endogenous Src in these cells. In contrast to the response in SYF cells, the activation of Rap1 by SrcS17D and isoproterenol in Src^{2+} cells was additive (Fig. 2*B*), presumably reflecting the contribution of the activation by isoproterenol of endogenous Src.

Conversely, the activation by isoproterenol of Ras was not blocked by these mutants. In SYF cells, expression of SrcS17A reconstituted Ras activation by isoproterenol to a level similar to that seen using SrcWT (Fig. 2*C*). Interestingly, in Src^{2+} cells, SrcS17A did not interfere with Ras activation by isoproterenol but appeared to enhance Ras activation in these cells (Fig. 2*D*). On the other hand, SrcS17D was unable to activate Ras in either cell line (Fig. 2*C* and *D*). These data suggest that SrcS17A was capable of mediating a Src-dependent pathway to Ras, demonstrating that the interference of Rap1 activation by SrcS17A in Src^{2+} cells was selective. Moreover, SrcS17D did not potentiate the stimulation by isoproterenol of Ras in Src^{2+} cells (Fig. 2*D*), suggesting that, unlike SrcS17A, SrcS17D alone could not participate efficiently in pathways to activate Ras.

Surprisingly, SrcS17D could activate Ras to a moderate degree in SYF cells, but only in conjunction with isoproterenol (Fig. 2*C*). This appears inconsistent with the selectivity of SrcS17D toward Rap1. One mechanism by which SrcS17D might function as an activator of Rap1 is to bind endogenous

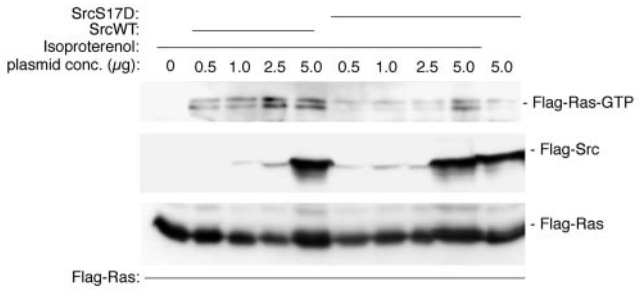

FIG. 3. **Concentration curve for the activation by Src of Ras in isoproterenol-treated SYF cells.** The activation by Src of Ras is dose-dependent. SYF cells were transfected with FLAG-Ras (5 μg) and FLAG-SrcWT or FLAG-SrcS17D at the indicated plasmid concentrations. Cells were left unstimulated or treated with isoproterenol, as indicated, and cell lysates were examined for active GTP-loaded Ras (*Flag-Ras-GTP*). The levels of FLAG-Src and FLAG-Ras are shown in the *middle* and *lower panels*, respectively.

proteins that target Src toward Rap1. In this model, it might be expected that the selectivity of SrcS17D toward Rap1 would not be apparent if it was overexpressed. To examine further the finding that SrcS17D could participate in the activation by isoproterenol of Ras in SYF cells, we compared the effect of increasing concentrations of both transfected SrcWT and SrcS17D in these cells (Fig. 3). Increasing amounts of transfected SrcWT potentiated the activation by isoproterenol of Ras at all concentrations examined, especially at low to moderate doses (Fig. 3). In contrast, the ability of SrcS17D to carry a signal from isoproterenol to Ras was only apparent at the highest level of expression examined. Therefore, whereas activation by SrcS17D of Rap1 was not further enhanced by isoproterenol, isoproterenol-dependent activation of Ras by SrcS17D was contingent on overexpression. These data are consistent with a model that SrcS17D interacts with an endogenous protein that channels Src toward a Rap1 pathway, but that when overexpressed, SrcS17D can act independently of this pathway.

Next, we examined the mechanism of ERK activation by isoproterenol in HEK293 cells (9). The ability of isoproterenol to activate Rap1 was modestly enhanced following transfection of SrcWT, but was completely blocked following transfection of SrcS17A (Fig. 4*A*). These data suggest that SrcS17A can interfere with the ability of endogenous Src to mediate the activation by isoproterenol of Rap1 in HEK293 cells. Expression of SrcS17D activated Rap1 constitutively in these cells, and this was not significantly enhanced by isoproterenol (Fig. 4*A*).

Upon its activation, Rap1 binds the effector B-Raf to activate ERKs (5, 9). This recruitment of B-Raf has been used as an index of Rap1 activation in a variety of cell types (9, 18–20). The ability of isoproterenol to stimulate the association of B-Raf with Rap1 was blocked by SrcS17A. SrcS17D stimulated the association of B-Raf with Rap1, and this was modestly enhanced by isoproterenol (Fig. 4*B*). Taken together, these data demonstrate that phosphorylation of Ser^{17} is required for both Rap1 activation and function.

It has previously been suggested that the activation by cAMP of Rap1 by forskolin requires Cbl and the Rap1 exchanger C3G, in NIH3T3 cells (9, 13). To examine whether a similar mechanism might underlie signaling from GPCRs, we examined isoproterenol signaling in HEK293 cells expressing two interfering mutants: Cbl-ct, a carboxyl-terminal fragment that blocks Cbl function (13), and CBR, a truncated protein containing the Crk-binding region of C3G that blocks C3G binding to Crk (9, 21). The ability of isoproterenol to induce the recruitment by Rap1 of B-Raf was blocked by both Cbl-ct and CBR (Fig. 4*B*). Isoproterenol also induced the association of Cbl with SrcWT but not SrcS17A (Fig. 4*C*). SrcS17D induced this association in the absence of isoproterenol (Fig. 4*C*). Similarly, isoproterenol induced an association between C3G and transfected Cbl that was mimicked by SrcS17D, but not SrcS17A (Fig. 4*D*). In addition, we detected an isoproterenol-dependent association between wild type Src and C3G that was also mimicked by SrcS17D (Fig. 4*E*). This suggests that the association of Cbl/C3G with Src following isoproterenol stimulation was dependent on phosphorylation of Ser^{17}.

Mutation of tyrosine 527 of Src to phenylalanine (Y527F) produces a constitutively active (oncogenic) Src by eliminating the inhibitory phosphorylation at Tyr^{527} (22, 23). This mutant constitutively activated both Ras and Rap1 in HEK293 cells (Fig. 5, *A* and *B*). However, introduction of S17A in SrcY527F created a new mutant (SrcS17A/Y527F) that was unable to activate Rap1, whereas SrcS17D/Y527F activated Rap1 constitutively (Fig. 5*A*). Both SrcS17A/Y527F and SrcS17D/Y527F could activate Ras, suggesting that S17A selectively interfered with oncogenic activation by Src of Rap1. In addition, both Cbl-ct and CBR interfered with Rap1 activation, but had no effect on Ras activation, suggesting that the action of endogenous Cbl and C3G were specific for Rap1.

The inability of SrcS17A to interfere with Ras signaling was seen by examining hormonally activated Src in HEK293 cells. Both SrcWT and SrcS17A, but not by SrcS17D, modestly enhanced the activation by isoproterenol of Ras (Fig. 6*A*). Ras activation by isoproterenol is thought to be mediated via $G\beta\gamma$ (10, 24). This was confirmed by experiments in HEK293 cells that showed that the activation by isoproterenol of Ras was blocked by expression of a truncated β-adrenergic receptor kinase (βARK-ct) (Fig. 6*A*), and by expression of transducin, a retinal-specific $G\alpha_s$ subunit (Fig. 6*B*). Both βARK-ct and transducin block signals generated from $\beta\gamma$ subunits by binding to endogenous $\beta\gamma$ (2, 25). As a control, we show that EGF activation of Ras was not blocked by expression of transducin (Fig. 6*B*). In contrast, transducin did not block Rap1 activation by isoproterenol (Fig. 6*C*). Taken together, these data suggest that while Ras and Rap1 are both activated by Src-dependent mechanisms downstream of the β-adrenergic receptor, only Ras activation involves $G\beta\gamma$.

To examine the downstream consequences of Src-dependent signaling in HEK293 cells, we measured ERK activation, using pERK antibodies (pERK1/2). Activation by isoproterenol of ERKs required both PKA and SFKs, as phosphorylation of ERK was prevented by both H89 and PP2 (Fig. 7*A*). Similar results were seen using forskolin (Fig. 7*B*). In contrast, the phosphoinositol 3-kinase (PI3K) inhibitor, LY294002, did not block ERK phosphorylation (Fig. 7*A*). Expression of SrcS17A blocked the activation by isoproterenol of ERKs (Fig. 8*A*), and SrcS17D constitutively activated ERKs (Fig. 8*B*), consistent with a model that the activation by Src of Rap1 was necessary and sufficient for the activation by isoproterenol of ERKs in these cells. Moreover, isoproterenol was unable to further activate ERKs in SrcS17D-expressing cells, suggesting that the phosphorylation of SrcS17 was the predominant mode of ERK activation by isoproterenol.

Previously, we and others have suggested that Ras was not required for ERK activation by isoproterenol in HEK293 cells (9, 20). This is despite the fact that Ras is activated by isoproterenol (Fig. 6, *A* and *B*). To examine the physiological role of Ras signaling in these cells, we examined a well known Ras effector, PI3K and its target, AKT (26). Isoproterenol activated AKT, as measured by phosphorylation-specific antibodies to phosphothreonine 308 (pAKT) (Fig. 9*A*). This phosphorylation required PI3K as LY294002 blocked isoproterenol-induced phosphorylation at this site (Fig. 9*A*). Moreover, this phospho-

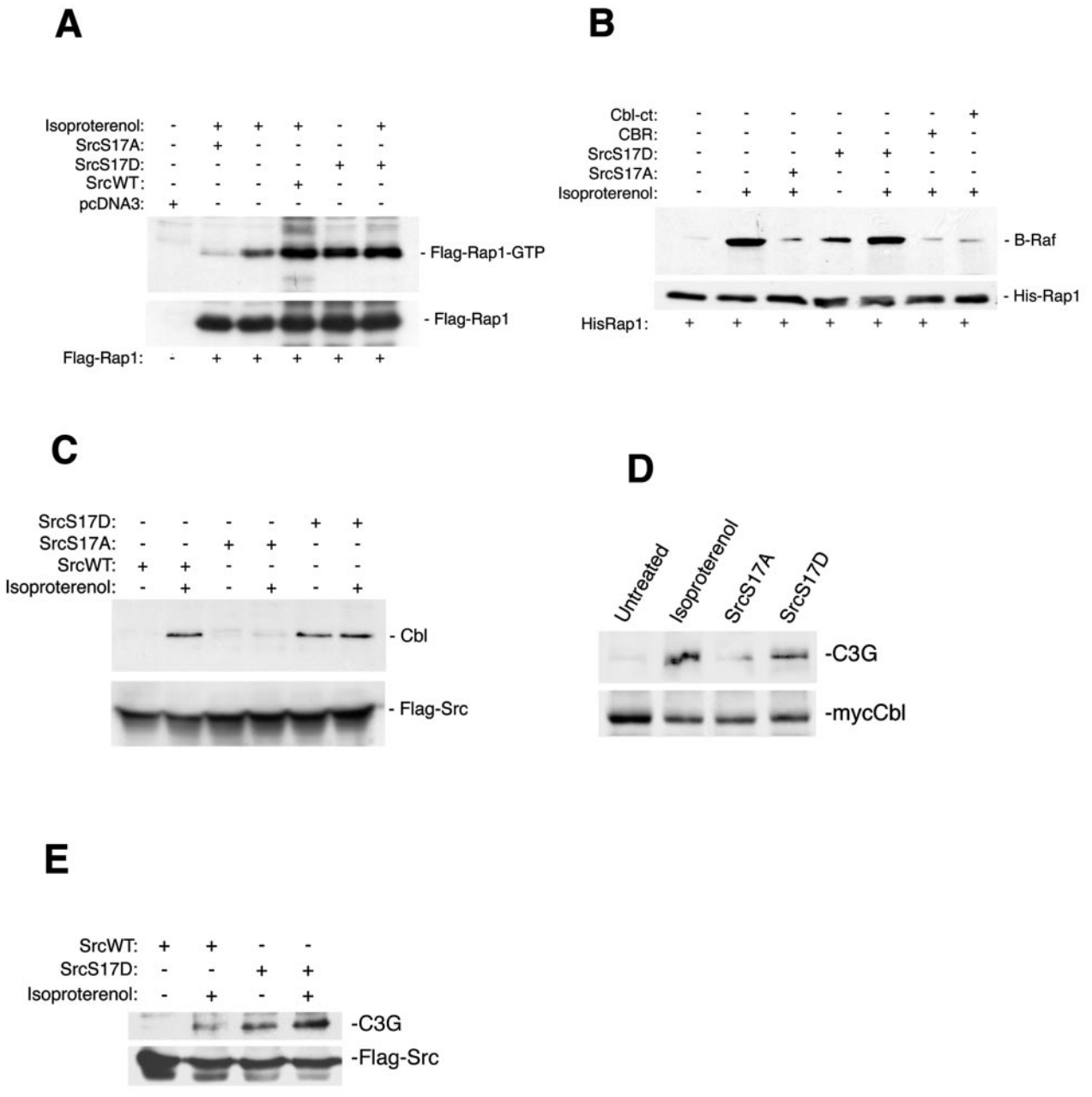

FIG. 4. **PKA phosphorylation of Src mediates Rap1 activation and its association with B-Raf following isoproterenol stimulation.** *A*, serine 17 of Src mediates the activation by isoproterenol of Rap1. HEK293 cells were transfected with pcDNA3, FLAG-Rap1, or the indicated Src plasmids. Cells were treated with isoproterenol as indicated, and cell lysates were examined for active Rap1 (*Flag-Rap1-GTP*, *upper panel*). The *lower panel* demonstrates similar expression levels of FLAG-Rap1 protein. *B*, B-Raf association with Rap1 following isoproterenol stimulation requires Src, Cbl, and C3G. HEK293 cells were transfected with His-tagged Rap1 (*His-Rap1*), in the presence or absence of SrcS17A, SrcS17D, CBR, and Cbl-ct as indicated. Cells were treated with isoproterenol, and cell lysates were passed over a nickel column to isolate His-Rap1 and associated proteins, and eluates were probed by Western blotting for endogenous B-Raf, as described under "Experimental Procedures" (*upper panel*). The *lower panel* shows a Western blot indicating that similar protein amounts of transfected His-Rap1 protein were assayed in each treatment condition. *C*, isoproterenol induces the association of Src with Cbl in HEK293 cells. HEK293 cells were transfected FLAG-tagged SrcWT, SrcS17A, and SrcS17D, as indicated. Cells were treated with isoproterenol and equivalent amounts of lysates immunoprecipitated with FLAG antibody and the presence of endogenous Cbl within the eluates was examined by Western blot (*upper panel*). The *lower panel* shows a Western blot indicating that similar protein amounts of FLAG-Src proteins were assayed in each treatment condition. *D*, isoproterenol induces the association of C3G with Cbl in HEK293 cells. HEK293 cells were transfected with Myc-Cbl and either SrcS17A or SrcS17D, as indicated. Cells were treated with isoproterenol or left untreated and equal amounts of lysates immunoprecipitated with Myc antibody and the presence of endogenous C3G within the eluates were examined by Western blot (*upper panel*). The *lower panel* shows a Western blot indicating that similar protein amounts of Myc-Cbl proteins were assayed in each treatment condition. *E*, isoproterenol induces the association of Src with C3G in HEK293 cells. HEK293 cells were transfected FLAG-tagged SrcWT or SrcS17D and treated with isoproterenol as indicated. Equivalent amounts of lysates were immunoprecipitated with FLAG antibody and the presence of endogenous C3G within the eluates was examined by Western blot (*upper panel*). The *lower panel* shows a Western blot indicating that similar protein amounts of FLAG-Src proteins were assayed in each treatment condition.

rylation was blocked by PP2, but not H89, suggesting the requirement of SFKs, but not PKA (Fig. 9*A*).

The requirement for Ras in the activation by isoproterenol of AKT in HEK293 cells was demonstrated by the ability of the interfering mutant of Ras, RasN17, to block this effect (Fig. 9*B*) (7). To examine the requirement of Src in greater detail, we

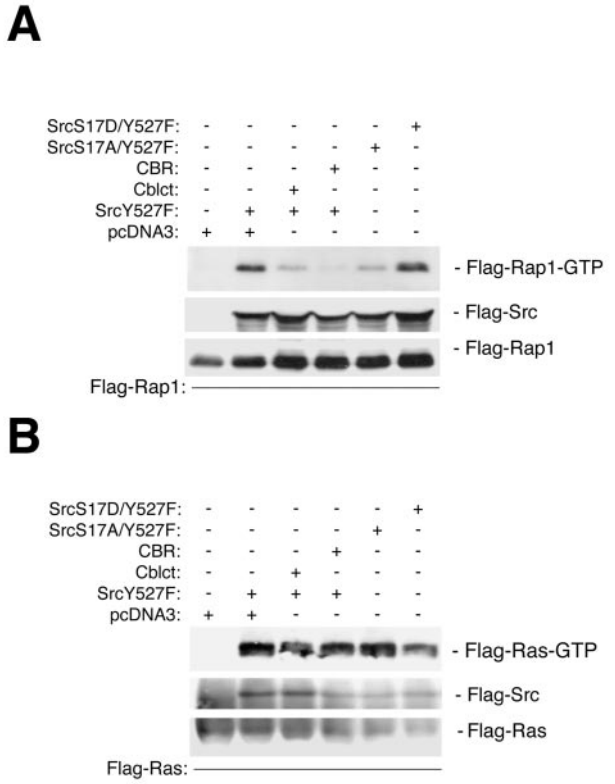

FIG. 5. **Constitutively active Src (SrcY527F) activates both Ras and Rap1.** *A*, SrcY527F activates Rap1. HEK293 cells were transfected with FLAG-tagged Rap1 (*Flag-Rap1*), in the presence or absence of pcDNA3, SrcY527F, SrcS17A/Y527F, SrcS17D/Y527F, CBR, and Cbl-ct as indicated. Cell lysates were assayed for FLAG-Rap1 activation (*Flag-Rap1-GTP*), as described above. The *middle* and *lower panels* show Western blots indicating the expression of FLAG-Src and FLAG-Rap1, respectively, in each treatment condition. *B*, SrcY527F activates Ras. HEK293 cells were transfected with FLAG-tagged Ras (*Flag-Ras*), in the presence or absence of pcDNA3, SrcY527F, SrcS17A/Y527F, SrcS17D/Y527F, CBR, and Cbl-ct as indicated. Cell lysates were assayed for FLAG-Ras activation (*Flag-Ras-GTP*), as described above. The *middle* and *lower panels* show Western blots indicating the expression of FLAG-Src and FLAG-Ras, respectively, in each treatment condition.

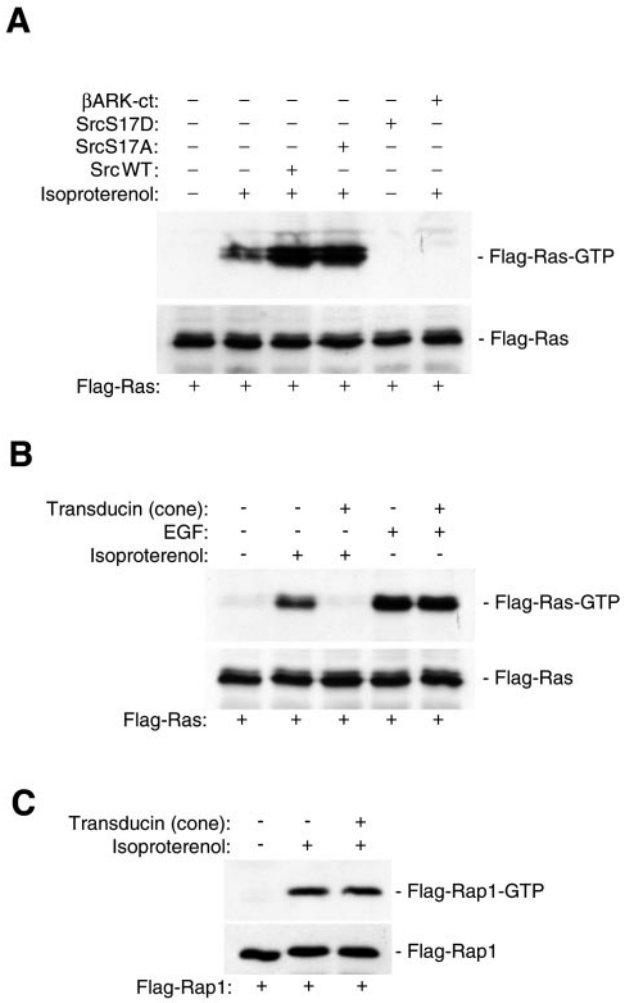

FIG. 6. **Isoproterenol activation of Rap1 and Ras occurs through distinct heterotrimeric G protein subunits.** *A*, Ras activation by isoproterenol is independent of PKA phosphorylation of Src. HEK293 cells were transfected with FLAG-Ras in the presence or absence of SrcWT, SrcS17A, SrcS17D, or βARK-ct, as indicated, and stimulated with isoproterenol. Cell lysates were examined for active Ras (*Flag-Ras-GTP*) as indicated in the *upper panel*. The *lower panel* demonstrates similar expression levels of total FLAG-Ras protein. *B*, isoproterenol activation of Ras occurs via the βγ subunits of the β-adrenergic receptor. HEK293 cells were transfected with FLAG-Ras in the presence or absence of the transducin cDNA and stimulated with either isoproterenol or EGF (as a control), as indicated. The activation of FLAG-Ras (*Flag-Ras-GTP*) is shown in the *upper panel*. Expression of total FLAG-Ras is indicated in the *lower panel*. *C*, Rap1 activation by isoproterenol does not require Gβγ subunits. HEK293 cells were transfected with FLAG-Rap1, with and without transducin, and stimulated with isoproterenol, as indicated. Cell lysates were examined for the activation of FLAG-Rap1 (*Flag-Rap1-GTP, upper panel*) as well as for equivalent FLAG-Rap1 expression within whole cell lysates (*lower panel*).

expressed the Src mutants SrcS17A and SrcS17D in these cells. SrcS17A did not block the activation of AKT by isoproterenol nor did SrcS17D result in constitutive activation of AKT. Like their actions on Ras, SrcS17A and SrcWT enhanced phosphorylation of AKT to similar levels, suggesting that, although overexpression of SrcS17A selectively interfered with effectors of Rap1, it mimicked the action of wild type Src on effectors downstream of Ras.

DISCUSSION

The small G proteins Ras and Rap1 have both been proposed to mediate the ability of cAMP to activate ERKs (5, 8, 10, 27–29). In many cell types, PKA inhibits Ras activation of Raf-1 and ERKs (6, 30, 31). The studies shown here, and previous studies (9), suggest that PKA can activate signals to B-Raf and ERKs while inhibiting Ras-dependent activation of Raf-1. The results from this study differ from other results performed in HEK293 cells that suggest that the activation by isoproterenol of ERKs requires both PKA and Ras-dependent activation of Raf-1 (10). It is possible that these distinct results reflect differences among clonal isolates of HEK293 cells in their expression of B-Raf and/or the protein 14-3-3, whose levels may determine the ability of cAMP to activate B-Raf (28).

The studies shown here describe a novel mechanism of the activation by Src of ERKs, via Rap1 and B-Raf. This activation was mediated by PKA and Rap1 and was blocked by phosphorylation site mutants of Src (13). We propose that Src activation of ERK via Rap1 requires the phosphorylation of Src by PKA. Hormonal activation of ERKs via PKA has previously been shown to use Rap1/B-Raf in a number of systems, including those involving thyroid stimulating hormone (32) and isoproterenol (29), although the role of Src was not examined in those studies. In one system examining adenosine activation of ERKs in Chinese hamster ovary cells (33), both cAMP and agonists activated Rap1/B-Raf and ERKs. ERK activation in this system required both PKA (33) and Src (20). In addition, a few examples have been reported of an SFK dependence of

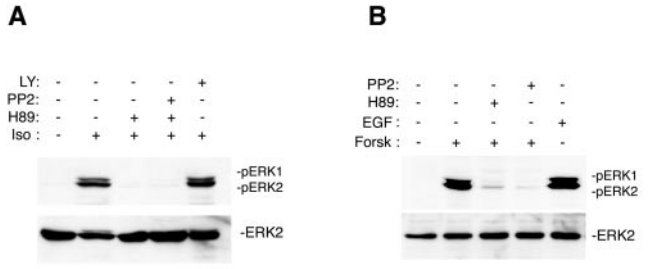

FIG. 7. **cAMP activation of ERKs is both PKA- and Src-dependent.** *A*, activation by isoproterenol of endogenous ERKs requires both PKA and Src. HEK293 cells received no pretreatment or were pretreated with H89, PP2, or LY294002, and stimulated with isoproterenol, as indicated. Cell lysates were analyzed by Western blotting for pERK1/2 (pERK1/pERK2, *upper panel*) or total ERK2 as a control for protein loading (*lower panel*). *B*, forskolin activation of endogenous ERKs requires both PKA and Src. HEK293 cells received no pretreatment or were pretreated with H89 or PP2 and stimulated with either forskolin or EGF (positive control), as indicated. Cell lysates were examined by Western blotting for pERK1/2 (pERK1/pERK2, *upper panel*) or total ERK2 as a control for protein loading (*lower panel*).

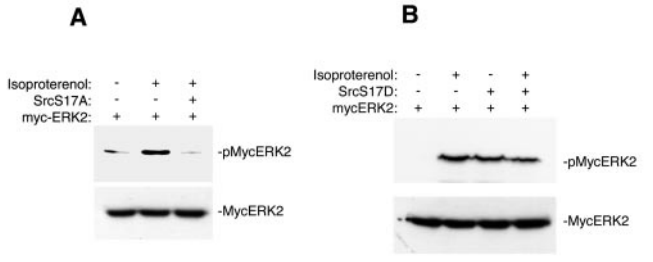

FIG. 8. **Activation by isoproterenol of ERKs requires serine 17 phosphorylation of Src.** *A*, PKA phosphorylation of Src on serine 17 is necessary for ERK activation by cAMP. HEK293 cells were transfected with Myc-ERK2 in the presence or absence of SrcS17A, and stimulated with isoproterenol, as indicated. Myc-ERK2 was immunoprecipitated from cell lysates using an agarose-coupled Myc antibody followed by Western blotting for phospho-ERK (*pMycERK2*, *upper panel*) or total Myc-ERK2 with a Myc antibody, as a control for protein loading (*lower panel*). *B*, expression of SrcS17D mimics the activation by isoproterenol of ERKs. HEK293 cells were transfected with Myc-ERK2 in the presence or absence of SrcS17D, and stimulated with isoproterenol, as indicated. Cell lysates were examined as in *A* for phosphorylated pMyc-ERK2 and expression of pMyc-ERK2.

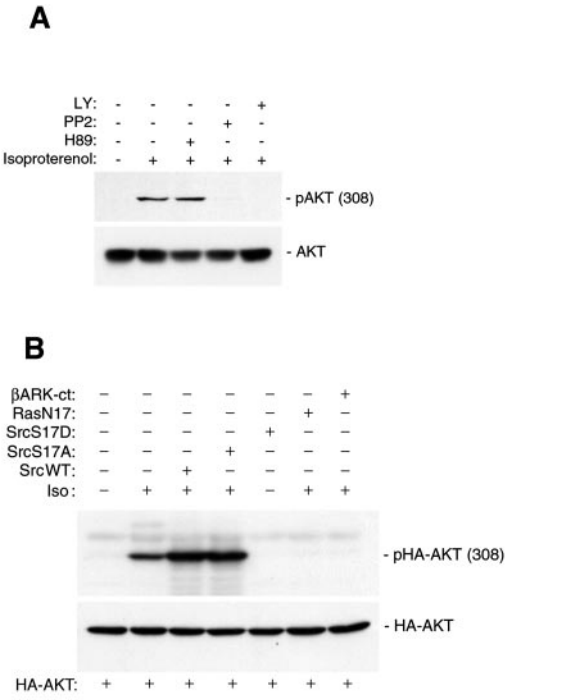

FIG. 9. **AKT activation by isoproterenol is PKA-independent but requires both Ras and G$\beta\gamma$.** *A*, activation by isoproterenol of AKT proceeds through Src and PI3K but not PKA. HEK293 cells received no pretreatment or were pretreated with H89, PP2, or LY294002, and stimulated with isoproterenol, as indicated. Cell lysates were analyzed by Western blotting for phospho-AKT (pAKT 308, *upper panel*) or total AKT as a control (*lower panel*). *B*, AKT activation by isoproterenol is Ras- and $\beta\gamma$-dependent. HEK293 cells were transfected with HA-AKT in the presence or absence of SrcWT, SrcS17A, SrcS17D, RasN17, or βARK-ct, and stimulated with isoproterenol, as indicated. Similar amounts of cell lysate (as determined by protein concentrations) were immunoprecipitated using an agarose-coupled HA antibody followed by Western blotting for phospho-AKT (*pHA-AKT (308)*, *upper panel*) or total HA-AKT with an HA antibody (*lower panel*).

activation by PKA of ERKs, in astrocytic cells (34), neuronal cells (35), and adipocytes (36, 37). It will be important to determine whether PKA phosphorylation of Src participates in Rap1/B-Raf signaling to ERKs in these systems as well.

Src can activate ERKs via Ras in other cell types (3). For example, Src can trigger Shc phosphorylation and subsequent activation of the Ras exchanger SOS (38, 39). Src can also activate Ras through the transactivation of the EGF receptor and related receptors following stimulation of GPCRs coupled to either G_i or G_q (12, 40–42). In one report, transactivation of the EGF receptor occurred following stimulation of the β2-adrenergic receptor (43) via $\beta\gamma$ subunits and the assembly of multiprotein complexes at clathrin-dependent sites of endocytosis (44). Direct association of Src with the adaptor β-arrestin has been shown to be involved in the clathrin-mediated endocytic event (45, 46). The role of PKA in this model of Src activation is to phosphorylate the β2 receptor itself, inducing a switch from Gα to G_i and a subsequent activation of Src complex via G$\beta\gamma_i$ (10), although recent studies disagree as to the importance of PKA phosphorylation of the β2 receptor in the activation by isoproterenol of ERK (47, 48).

The studies presented here using HEK293 cells suggest that isoproterenol activation of ERKs required PKA, Src, and Rap1. Specifically, SrcS17A interfered with the activation by isoproterenol of Rap1 but not that of Ras. More importantly, SrcS17D maximally activated ERKs, with no additional activation was provided by isoproterenol. This observation strongly suggests that the major signaling pathway used by isoproterenol to activate ERKs in these cells is via the phosphorylation of Src at Ser^{17} by PKA.

The Src tyrosine kinase has been shown to regulate a diverse number of cellular effects including stimulating (49–51) and inhibiting cell growth (13), regulating cell adhesion (52, 53), and regulating apoptosis (54, 55). Because of the many distinct cellular functions of Src, it is likely that the ability of Src to activate specific pathways is tightly regulated. The studies presented here provide some insight into the signaling specificity of Src. We propose that activation of the β-adrenergic receptor triggers two concurrent Src-dependent pathways, involving Gα_s to activate Rap1 and G$\beta\gamma_s$ to activate Ras, respectively. Whereas Src is required for both Rap1 and Ras activation, only Rap1 activation required PKA (Fig. 1). Sequestration of $\beta\gamma$ subunits blocked only Ras activation, not Rap1. Taken together, these data suggest that Gα_s and PKA were responsible for Rap1 activation, whereas $\beta\gamma$, but not PKA, was responsible for Ras activation. Other studies have suggested a PKA dependence of the activation by isoproterenol of Ras in these (10) and other cell types (47).

We have identified specific Src mutants that can selectively block (SrcS17A) or enhance (SrcS17D) the activation of Rap1, without effect on the simultaneous activation of Ras. These studies support a model whereby the mechanism of activating

Src dictates the choice of effector pathways, and identify PKA phosphorylation of Src as a potential mechanism to direct Src signals toward Rap1. This is consistent with a recent report that suggested that SrcS17A selectively interfered with ERK activation by cAMP (20). This study, however, did not examine the action of this mutant on either Ras or Rap1 activation.

Although neither isoproterenol nor SrcS17D could activate Ras in SYF cells, isoproterenol could activate Ras modestly when SrcS17D was expressed to high levels. The requirement of isoproterenol for SrcS17D activation of Ras suggests that SrcS17D was not acting constitutively, but was dependent on additional signals generated downstream of the β-adrenergic receptor. Moreover, this action was only seen at high levels of SrcS17D expression, suggesting that some endogenous protein(s) may bind SrcS17D and insulate it from participating in Ras activation when expressed at lower levels.

What mechanism allows SrcS17D to direct Src kinase activity toward selective substrates? It is possible that the proximity of the Ser^{17} site to the NH_2-terminal myristoylation of Src may allow this phosphorylation (or aspartate residue) to influence proper membrane targeting. Indeed, one previous study suggested that the phosphorylation by PKA of Src may be sufficient to redirect membrane localization of Src (56). However, the ability of Src mutants to interfere with Rap1 activation suggests that the cellular interactions that are disrupted by this mutant are saturatable. In addition, that SrcS17D is constitutively active suggest that it is not just being relocalized, but is activated as well. It is also possible that this phosphorylation may influence the binding of additional docking/adaptor proteins that influence both the activity of Src and choice of substrates (54, 57–62).

The ability of Src and SYFs to activate Rap1 via C3G/Crk has been demonstrated in multiple systems (13, 63, 64). In some cells, this is mediated via a Cbl adaptor protein (13, 65–67). Studies shown here identify Cbl as a potential target of activation by Src of Rap1. Moreover, the ability of Cbl to associate with either Src or C3G was mimicked by SrcS17D but not SrcS17A. Although Cbl has been proposed to mediate Src-dependent signals downstream of receptors (68, 69), this is the first report of such a pathway downstream of GPCRs. Future studies will examine whether these or additional proteins regulate Src function following phosphorylation by PKA.

The action of Cbl proposed here is similar to that seen for the Cbl-associated protein, Cas and the related protein Sin. In certain cells, Src can activate the adaptor Cas (64), or the related protein Sin (63, 70) to activate a Crk/C3G/Rap1 pathway. Interestingly, in HEK293 cells, activation of Src by Sin activates Crk/C3G/Rap1, but not Ras. However, expression of the oncogenic Src mutant, SrcY527F, activates Ras signaling pathways as well as Rap1 (63). Here, we confirm that SrcY527F can activate both Ras and Rap1 and demonstrate that the activation of Rap1, but not Ras, required Ser^{17} and C3G. Therefore, Src-dependent signals can be directed down specific pathways in a stimulus and cell type-specific manner. The molecular mechanisms by which Src is channeled toward selective signaling pathways are largely unknown.

β-Adrenergic receptor activation of Ras also required Src, but unlike Rap1, Ras activation required $G\beta\gamma_s$. This activation of Ras was capable of coupling positively to AKT. AKT is a serine/threonine kinase known to participate in a variety of cellular effects including cell survival, adhesion, and cell growth (26, 71–73). Multiple GPCRs have been shown to regulate AKT activation via PI3K (74–76). Data presented here identify a requirement for Ras because the activation by isoproterenol of AKT was independent of PKA and unaffected by SrcS17A. This is consistent with a recent report suggesting that cAMP/PKA/Rap1 does not activate AKT (77).

Studies in both COS-7 cells and HEK293 cells have also shown that AKT can be activated by isoproterenol and the $\beta\gamma$ subunits of heterotrimeric G proteins through both Ras and PI3K, but not by constitutively active $G\alpha_s$ (75, 76). A more recent study has confirmed that β-adrenergic receptor stimulation activates AKT independently of PKA, and through a $\beta\gamma$- and PI3K-dependent mechanism (7). In our study, interfering with Src function using SrcS17A had no effect on the ability of Src to couple $G\beta\gamma$ to Ras and AKT. Rather, overexpression of SrcS17A, like wild type Src, potentiated Ras signaling to AKT. Therefore, the ability of SrcS17A to act as an interfering mutant was selective for Rap1. Taken together, this data would suggest that in HEK293 cells, the β-adrenergic receptor is capable of activating AKT through a pathway involving $\beta\gamma$, Src, Ras, and PI3K.

In summary, we have shown that stimulation of HEK293 cells with isoproterenol activated two independent pathways mediated by $G\alpha_s$ to PKA/Rap1/ERK and $G\beta\gamma$ to Ras/AKT, respectively. Both pathways required Src, but only signaling from $G\alpha_s$/PKA to Rap1 and ERKs required the phosphorylation of Src at serine 17. These results imply a model where Src can specifically couple to downstream effectors depending on its mode of activation, and suggest that Src itself simultaneously regulates multiple β-adrenergic pathways via distinct pathways.

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