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Ga and GBy 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-adrenergic receptor activates Src via two independent mechanisms to mediate distinct signaling pathways, one through Gαs to Rap1 and ERKs and the other through Gβγ to Ras and AKT.

Stimulation of G protein-coupled receptors (GPCRs) 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). Gαs 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 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]pyrazol-3,4-dipyrimidinyl), LY294002 (2-(4-morpholinyl)-8-phenyl-4H-benzopyran-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. (Chatsworth, CA).

Cell Culturing Conditions and Treatments—HEK293, SYF, and Src− cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum, penicillin/streptomycin, and 1-glutamine at 37 °C in 5% CO2. Cells were maintained in serum-free Dulbecco's modified Eagle's medium for 16 h at 37 °C in 5% CO2 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.
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 carboxy-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−/− cells were co-transfected with the indicated cDNAs using a LipofectAMINE 2000 kit (Invitrogen) according to the manufacturer’s instructions. The control vector, pcDNAs3 (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 RafGDS (GST-RafGDS) was expressed in Escherichia coli following induction by isopropyl-β-D-thiogalactopyranoside (GST-RafGDS 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 MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 mM NaF, 0.1 μM aprotinin, and 1 mM NaN3). Active Rap1 was isolated as previously described by Franke et al. (15). Equivalent amounts of supernatants (150 μg) were incubated with the GST-RafGDS-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 are shown.

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 MgCl2, 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-nitrioltriacetic 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 Rap1 proteins were detected by Western blotting (7, 9).

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. 1A). Isoproterenol also activated endogenous Ras via SFKs, but this proceeded through a PKA-independent pathway (Fig. 1B).

To determine which SFK was mediating these effects, we utilized a pair of cell lines derived from mouse embryonic 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−/−, 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. 2A), but was retained in Src−/− cells (Fig. 2B). Similarly, the ability of isoproterenol to activate Ras was also absent in SYF cells (Fig. 2C), but was retained in Src−/− cells (Fig. 2D). 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...
the PKA-dependent phosphorylation of Src at serine 17 (Ser\textsuperscript{17}). However, the requirement of Ser\textsuperscript{17} phosphorylation in hormonal signaling to Ras, Rap1, and ERKs has not been examined. To address the requirement of Ser\textsuperscript{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\textsuperscript{17} was replaced with an alanine (SrcS17A), was unable to reconstitute the activation by isoproterenol of Rap1 (Fig. 2A). Moreover, expression of a second Src mutant in which Ser\textsuperscript{17} was replaced with an aspartate (SrcS17D) resulted in constitutive activation of Rap1 (Fig. 2A). 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\textsuperscript{2+} cells inhibited the activation by isoproterenol of Rap1 (Fig. 2B), 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\textsuperscript{2+} cells was additive (Fig. 2B), 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. 2C). Interestingly, in Src\textsuperscript{2+} cells, SrcS17A did not interfere with Ras activation by isoproterenol but appeared to enhance Ras activation in these cells (Fig. 2D). On the other hand, SrcS17D was unable to activate Ras in either cell line (Fig. 2C 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\textsuperscript{2+} cells was selective. Moreover, SrcS17D did not potentiate the stimulation by isoproterenol of Ras in Src\textsuperscript{2+} cells (Fig. 2D), 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. 2C). 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...
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. 4A). 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. 4A).

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. 4B). Taken together, these data demonstrate that phosphorylation of Ser17 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. 4B). Isoproterenol also induced the association of Cbl with SrcWT but not SrcS17A (Fig. 4C). SrcS17D induced this association in the absence of isoproterenol (Fig. 4C). Similarly, isoproterenol induced an association between C3G and transfected Cbl that was mimicked by SrcS17D, but not SrcS17A (Fig. 4D). In addition, we detected an isoproterenol-dependent association between wild type Src and C3G that was also mimicked by SrcS17D (Fig. 4E). This suggests that the association of Cbl/ C3G with Src following isoproterenol stimulation was dependent on phosphorylation of Ser17.

**Mutation of tyrosine 527 of Src to phenylalanine (Y527F) produces a constitutively active (oncogenic) Src by eliminating the inhibitory phosphorylation at Tyr527** (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. 5A). 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 activation 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. 6A). Ras activation by isoproterenol is thought to be mediated via Gβγ (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. 6A), and by expression of transducin, a retinal-specific Gαs subunit (Fig. 6B). Both βARK-ct and transducin block signals generated from Gαs subunits by binding to endogenous βγ (2, 25). As a control, we show that EGF activation of Ras was not blocked by expression of transducin (Fig. 6B). In contrast, transducin did not block Rap1 activation by isoproterenol (Fig. 6C). 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 Gβγ.

To examine the downstream consequences of Src-dependent signaling in HEK293 cells, we measured ERK activation, using pERK antibodies (pERK1/2). Activation of isoproterenol of ERKs required both PKA and SFKs, as phosphorylation of ERK was prevented by both H89 and PP2 (Fig. 7A). Similar results were seen using forskolin (Fig. 7B). In contrast, the phosphoinositide 3-kinase (PI3K) inhibitor, LY294002, did not block ERK phosphorylation (Fig. 7A). Expression of SrcS17A blocked the activation by isoproterenol of ERKs (Fig. 8A), and SrcS17D constitutively activated ERKs (Fig. 8B), 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. 9A). This phosphorylation required PI3K as LY294002 blocked isoproterenol-induced phosphorylation at this site (Fig. 9A). Moreover, this phospho-
rylation was blocked by PP2, but not H89, suggesting the requirement of SFKs, but not PKA (Fig. 9A).

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. 9B) (7). To examine the requirement of Src in greater detail, we...
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 phospho-
rylation 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 PKA phosphorylation of Src. PKA 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, 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. Expression of total FLAG-Ras is indicated in the lower panel. C, Rap1 activation by isoproterenol does not require Gβy 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).
Specifically, SrcS17A interfered with the activation by isoproterenol of endogenous ERKs required 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). 

The studies presented here using HEK293 cells suggest that 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 Ser17 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 Gαs to activate Rap1 and Gβγ, to activate Ras, respectively. Whereas Src is required for both Rap1 and Ras activation, only Rap1 activation required PKA (Fig. 1). Sequestration of Gβγ subunits blocked only Ras activation, not Rap1. Taken together, these data suggest that Gαs and PKA were responsible for Rap1 activation, whereas Gβγ, 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 specificSrc 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

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**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 (pMyc-ERK2, 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 Gβγ. 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, upper panel) or total AKT as a control (lower panel). B, AKT activation by isoproterenol is Ras- and βγ-dependent. HEK293 cells were transfected with HA-AKT in the presence or absence of SrcWT, SrcS17A, SrcS17D, RasN17, or βARK-c, 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, upper panel) or total HA-AKT with an HA antibody (lower panel).
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 iso-protenol nor SrcS17D could activate Ras in SYF cells, iso-protenol could activate Ras modestly when SrcS17D was expressed to high levels. The requirement of iso-protenol 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 Ser17 site to the NH2-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 saturable. 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, 56–62).

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 related protein Sin (63, 70) to activate a Crk/C3G/Rap1 pathway in certain cells, Src can activate the adaptor Cas (64), or the related protein Sin (63, 70) to activate a Crk/C3G/Rap1 pathway. 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 iso-protenol and the βγ subunits of heterotrimeric G proteins through both Ras and PI3K, but not by constitutively active Goa (75, 76). A more recent study has confirmed that β-adrenergic receptor stimulation activates AKT independently of PKA, and through a βγ- and PI3K-dependent mechanism (7). In our study, interfering with Src function using SrcS17A had no effect on the ability of Src to couple Gβγ 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 βγ, Src, and PI3K.

In summary, we have shown that stimulation of HEK293 cells with iso-protenol activated two independent pathways mediated by Goa to PKA/Rap1/ERK and Gβγ to Ras/AKT, respectively. Both pathways required Src, but only signaling from Goa/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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