c-Src Tyrosine Kinase Binds the β2-Adrenergic Receptor via Phospho-Tyr-350, Phosphorylates G-protein-linked Receptor Kinase 2, and Mediates Agonist-induced Receptor Desensitization*

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The nonreceptor tyrosine kinase Src has been implicated in the switching of signaling of β2-adrenergic receptors from adenyllylcyclase coupling to the mitogen-activated protein kinase pathway. In the current work, we demonstrate that Src plays an active role in the agonist-induced desensitization of β2-adrenergic receptors. Both the expression of dominant-negative Src and treatment with the 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) inhibitor of Src kinase activity blocks agonist-induced desensitization. Agonist triggers tyrosine phosphorylation of the β2-adrenergic receptor and recruitment and activation of Src. Because phosphorylation of the Tyr-350 residue of the β2-adrenergic receptor creates a conditional, canonical SH2-binding site on the receptor, we examined the effect of the Y350F mutation on Src phosphorylation, Src recruitment, and desensitization. Mutant β2-adrenergic receptors with a Tyr-to-Phe substitution at Tyr-350 do not display agonist-induced desensitization, Src recruitment, or Src activation. Downstream of binding to the receptor, Src phosphorylates and activates G-protein-linked receptor kinase 2 (GRK2), a response obligate for agonist-induced desensitization. Constitutively active Src increases GRK phosphorylation, whereas either expression of dominant-negative Src or treatment with the PP2 inhibitor abolishes tyrosine phosphorylation of GRK and desensitization. Thus, in addition to its role in signal switching to the mitogen-activated protein kinase pathway, Src recruitment to the β2-adrenergic receptor and activation are obligate for normal agonist-induced desensitization.

The nonreceptor tyrosine kinase family of Src functions in a wide spectrum of cell signaling (1). The recruitment and activation of Src kinases is obligate for the mediation of Ras activation by G-protein-linked receptors (GPLRs)1 (2, 3). GPLRs display four well known responses to the stimulation by agonists: namely, activation, desensitization, internalization, and eventual resensitization (4, 5). The activation of adenyllylcyclase in response to agonist stimulation of β2-adrenergic receptors results in elevation of intracellular cyclic AMP levels, activation of protein kinase A, and later phosphorylation of the receptor by protein kinase A and G-protein-linked receptor kinases (GRKs) (6). Phosphorylation leads to desensitization and receptor sequestration, dependent upon the binding of β-arrestin to the phosphorylated receptor as well as to clathrin (7, 8). Internalization occurs via clathrin-mediated processes (9) and resensitization/dephosphorylation follows by the action of the protein phosphatase 2A (10) or 2B (11). These activities are organized by the protein kinase A-anchoring protein AKAP250 or gravin (12). Gravin acts as a scaffold for interactions among protein kinase A, protein kinase C, PP2B, and the β2-adrenergic receptor (13).

Src has been shown to participate in the formation of β2-adrenergic receptor-Src complexes, in association with β-arrestin, to switch the signaling from adenyllylcyclase to activation of Ras and its downstream effectors, the mitogen-activated protein kinase (3). Src has been reported to phosphorylate and activate GRKs (14), observations confined to systems in which the elements are overexpressed at high levels or examined in reconstituted systems, leaving unanswered the question whether or not Src interacts directly with the β2-adrenergic receptor and functions in desensitization. The proposed model for Src action defines a temporal sequence in which GRK2 phosphorylates the β2-adrenergic receptor, and an Src-arrestin complex then targets to the receptor, facilitating internalization and signaling to Ras (3). In the current work we reveal that Src targets a conditional SH2-binding site of the β2-adrenergic receptor, is recruited to the phospho-Tyr-350 receptor, is activated, and then phosphorylates GRK2. This temporal sequence is shown to be obligate for agonist-induced desensitization.

EXPERIMENTAL PROCEDURES

Cell Culture—Human epidermoid carcinoma cells (A431) and Chinese hamster ovary (CHO) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin (100 μg/ml), and streptomycin (100 μg/ml) and grown in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Cells were stably transfected with mammalian expression vectors harboring the constitutively active form (Y327F) or the dominant-inhibitory form (K295R/Y327F) of c-Src, the hamster wild-type β2-adrenergic receptor, the hamster Y350F mutant form of the β2-adrenergic receptor, or a green fluorescent protein-tagged β2-adrenergic receptor fusion protein (8), as previously described (13). Suppression via Antisense Oligodeoxynucleotides—Antisense, sense, and control missense oligodeoxynucleotides with the same base composition, but in scrambled order, were synthesized and purified to cell

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1 The abbreviations used are: GPLR, G-protein-linked receptor; GRK, G-protein-linked receptor kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; CHO, Chinese hamster ovary; ODN, oligodeoxynucleotide; GFP, green fluorescent protein; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.
culture-grade (Oneron, Alameda, CA), as described (15). Before addition to cells, oligodeoxynucleotides were mixed at a ratio of 1:3 (w/w) with N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (Roche Molecular Biochemicals), a cationic diacylglycylcer with liposomal form that serves as a delivery vehicle. A431 cells were treated with oligodeoxynucleotides (ODNs, 5 μg/ml) for at least 48–72 h prior to the analysis of the expression of the target molecule. The sequences employed for the antisense and sense ODNs for Src were 24 nucleotides in length, terminating with the initiator codon for Src. Missense was created using a random sequence with the same base composition as the antisense ODN for Src.

Immunoprecipitation and Immunoblotting by Antibodies against β2-Adrenergic Receptor, c-Src, or GRK2—For most studies, A431 cells were either untreated or stimulated with 10 μM isoproterenol for periods up to 30 min. Cells were harvested and lysed in a lysis buffer (1% Triton X-100, 0.5% Nonidet-P40, 10 mM diethiothreitol, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 100 μg/ml bacitracin, 100 μg/ml benzamidine, 2 mM sodium orthovanadate, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM sodium pyrophosphate, 50 mM K2HPO4, 10 mM sodium molybdate, and 20 mM Tris-HCl, pH 7.4) at 4 °C for 20 min. After centrifugation of the cell debris at 10,000 × g for 15 min, the lysates were precleared with protein A/G-agarose for 90 min at 4 °C. The mixture was then subjected to immunoprecipitation for 2 h with antibodies specific for the β-adrenergic receptor (C6404, c-Src, or GRK2). The primary antibodies were linked covalently to a protein A/G-agarose matrix. Immune complexes were separated three times with RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM diethiothreitol, 1% Triton X-100, pH 8.0) and separated on 4 to 12% linear gradient SDS-acrylamide Laemmli gels. Immunoblotting and detection of Src, of GRK2, and of the β2-adrenergic receptor by immunostaining were performed as previously described (13). The anti-phospho-Src (Y416) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY).

Epifluorescence Imaging—Microscopy of live cells stably transfected to express a green fluorescent protein-tagged β2-adrenergic receptor fusion protein was performed on the Eclipse TE300 (Nikon) inverted microscope equipped with a × 40 objective and a set of filters (13). Images were acquired using MicroMax Imaging System (Princeton Instruments Inc.) and WinView32 software. Fluorescent dyes were imaged sequentially in frame-interface mode to eliminate spectral overlapping between the channels (12).

Radioligand Binding Assay—The number of β2-adrenergic receptors was determined by radioligand binding. Approximately 106 stably transfected CHO cells were incubated with 30 μM [125I]iodocyanopindolol (PerkinElmer Life Sciences) in the absence or presence of 10 μM propranolol (for defining the amount of nonspecific binding) at 23 °C for 60 min. Cells were washed three times with RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM diethiothreitol, 1% Triton X-100, pH 8.0) and freed from any cellular debris. Immune complexes were formed on separate occasions.

Cyclic AMP Assay—One day prior to the analysis, A431 cells and CHO clones stably transfected with β2-adrenergic receptor and β2-adrenergic receptor-GFP-pcDNA3 were seeded in 96-well plates at the density of 20,000 cells/well. Cells were washed and challenged with or without 10 μM isoproterenol in 50 μl of HEM buffer (20 mM Hepes, pH 7.4, 135 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM NaHCO3, containing Ro-20–1724 (0.1 μM; Calbiochem)) and adenosine deaminase (0.5 unit/ml) for the times indicated in the figure legends. The first challenge with agonist was for 0–60 min. For some studies, the cells first challenged with agonist were washed free of agonist (three times) at the end of the first incubation and then challenged again for 5 min with agonist. The washes were performed by addition of 100 μl of ice-cold ethanol. The agonist-stimulated cyclic AMP production was determined as described elsewhere (15).

RESULTS

The nonreceptor tyrosine kinase Src has been shown to play a prominent role in linking G-protein-linked receptors to Ras and downstream signaling to the mitogen-activated protein kinase network (2, 3). For the β2-adrenergic receptor, Src has been shown to act as a switch from receptor regulation of Gs and adenylcyclase to activation of Ras following the normal progression of receptor activation, desensitization, and sequestration. The role of Src in desensitization is far less clear. Src has been proposed to target to GPLRs via its interaction with β-arrestin, suggesting in a temporal sense that Src would be functioning in post-receptor desensitization. To test the hypothesis that Src may be participating in events prior to receptor sequestration and Ras activation, we focused on an analysis of β2-adrenergic receptor-mediated activation and desensitization, using the accumulation of intracellular cyclic AMP as the readout.

Human epidermoid A431 carcinoma cells are a widely used model of β2-adrenergic receptor action (18, 19). Challenging A431 cells with the β2-adrenergic agonist isoproterenol (10 μM) leads to peak accumulation of cyclic AMP at 5 min, which declines to approximately half-maximal levels within 30–60 min, reflecting ongoing agonist-induced desensitization (Fig. 1). The cyclic AMP response of A431 cells to agonist, following a 30 or 60 min first challenge with isoproterenol, a washout of agonist, and a second challenge with isoproterenol for 5 min, is rectified to the level of naive cells that have been challenged with agonist for 5 min. To explore what role, if any, Src may have in agonist-induced desensitization of β2-adrenergic receptors, we performed the same experiments in A431 cells pretreated for 30 min with the Src family tyrosine kinase inhibitor PP2 (Fig. 1). In the presence of the Src inhibitor, agonist-induced desensitization was abolished, and intracellular cyclic AMP levels continued to increase over the period of 60 min of challenge with agonist. These data suggest that Src activity is important not only for subsequent downstream signaling to Ras but also for agonist-induced desensitization itself. To test the observations by an independent approach that targets Src only, A431 cells were treated for 4 days with ODNs antisense to Src to suppress Src levels. Treatment with ODNs antisense to Src also abolished agonist-induced desensitization (Fig. 1). Suppression of Src levels by antisense ODNs yields increased cyclic AMP accumulation in A431 cells challenged with isoproterenol for up to 60 min. Treatment of cells with ODNs sense or missense to Src, in contrast, was without effect (data not shown).

To further test this newly discovered role for Src in agonist-induced desensitization of β2-adrenergic receptors, we tested the effects of stable transfection of A431 cells with an expression vector harboring either the constitutively active form (V527F) or the dominant-negative form (K295R/T527F) of c-Src (Fig. 2). Stable expression of the dominant-inhibitory form of Src abolishes agonist-induced desensitization of β2-adrenergic
proper targeting to instances, the introduction of constitutively active Src without energetic receptors first have been activated. Under these circumstances.

The constitutively active, mutant form of Src abolishes agonist-induced or the dominant-negative mutant of Src (CA-Src (Y527F)) were used to study cyclic AMP accumulation in response to isoproterenol over time. The cells were challenged with 10 μM isoproterenol (ISO) for 0, 5, 30, and 60 min, and intracellular cyclic AMP accumulation was determined. Some cells were first treated with isoproterenol for 30 or 60 min and then washed free of agonist and rechallenged with isoproterenol for 5 min. Assays of cyclic AMP accumulation were performed in triplicate. The data shown are mean values ± S.E. from three to five separate experiments performed on separate occasions.

The increase in the amount of Src associated with the β2-adrenergic receptor following the challenge with isoproterenol was phosphorylated at Tyr-416 and therefore activated. Thus, not only were Src expression and activity necessary for agonist-induced desensitization of β2-adrenergic receptors but association and activation by this G-protein-linked receptor were also increased sharply in response to agonist.

We tested the hypothesis that agonist treatment of A431 cells activates endogenous Src activity in A431 cells (Fig. 3). Cells were challenged with isoproterenol (10 μM) for periods up to 60 min. At the conclusion of the activation, whole-cell extracts were prepared, and the extracts were subjected to an immune precipitation “pull down” assay using antibodies against the β2-adrenergic receptor (antibody CM04) and the insulin catalyze the phosphorylation of the β2-adrenergic receptor on residue Tyr-416. The precipitates were subjected to SDS-PAGE, and the resultant immunoblots were stained with anti-phosphotyrosine antibody (PY99). These data show conclusively and un-

![Fig. 2. Expression of a dominant-negative, but not the constitutively active, mutant form of Src abolishes agonist-induced desensitization of the cyclic AMP response to stimulation by a β2-adrenergic agonist. A431 cells (control) and clones stably transfected to express either the Y527F constitutively active mutant of Src (CA-Src (Y527F)) or the dominant-negative mutant of Src (DN-Src (K255R/Y527F)) were used to study cyclic AMP accumulation in response to isoproterenol over time. The cells were challenged with 10 μM isoproterenol (ISO) for 0, 5, 30, and 60 min, and intracellular cyclic AMP accumulation was determined. Some cells were first treated with isoproterenol for 30 or 60 min and then washed free of agonist and rechallenged with isoproterenol for 5 min. Assays of cyclic AMP accumulation were performed in triplicate. The data shown are mean values ± S.E. from three to five separate experiments performed on separate occasions.](http://www.jbc.org/)

![Fig. 3. Stimulation of cells with isoproterenol provokes recruitment of Src to the β2-adrenergic receptor and tyrosine phosphorylation (activation) of Src. A431 cells were challenged with 10 μM isoproterenol (ISO) for 0, 5, 30, and 60 min and then used to harvest whole-cell extracts. The extracts were subjected to immune precipitation (IP) pull down assays with antibodies against the β2-adrenergic receptor (CM04), and the immune complexes were subjected to SDS-PAGE. The resultant immunoblots (IB) were stained with antibodies specific for the phospho-Tyr-417 activated version of Src, and the immune precipitates were subjected to SDS-PAGE. The resultant immunoblots (IB) were stained with antibodies specific for the phospho-Tyr-417 activated version of Src, and the immune precipitate on SDS-PAGE, and immunoblotting. The immunoblots (IB) were stained with anti-phosphotyrosine antibody (PY99) and used to establish equivalent loading for each lane. Shown is a blot representative of three such experiments with essentially identical results. β2AR, β2-adrenergic receptor.](http://www.jbc.org/)

![Fig. 4. Stimulation of cells with β2-adrenergic agonist leads to increased phosphoryrosine content of the β2-adrenergic receptor. A431 cells were challenged with isoproterenol (ISO) (10 μM) for 0, 5, 30, and 60 min and then used to prepare whole-cell extracts. The cell extracts were subjected to immune precipitation (IP) pull down assays with antibody (CM04) against the β2-adrenergic receptor, separation of the immune precipitate on SDS-PAGE, and immunoblotting. The immunoblots (IB) were stained with anti-phosphotyrosine antibody (PY99) and used to establish equivalent loading for each lane. Shown is a blot representative of three such experiments with essentially identical results. β2AR, β2-adrenergic receptor.](http://www.jbc.org/)
expectedly that the phosphotyrosine content of the β2-adrenergic receptor increased upon challenge of the cells with isoproterenol. Within 5 min of challenge with isoproterenol, the content of phosphotyrosine more than doubled, increasing to 2.5- and then 3.0-fold by 30 and 60 min, respectively. We confirmed these data using metabolic labeling of an independent hamster smooth muscle cell line (DDT-MF2 vas deferens) with [32P]Pi, overnight and stimulation with 10 μM isoproterenol for 5 or 15 min (data not shown).

Earlier studies demonstrated both in vivo and in vitro that phosphorylation of the β2-adrenergic receptor at Tyr-350 generates an SH2-binding site that Grb2, 1-phosphatidylinositol 3-kinase, or dynamin can bind via SH2 domains (23, 24). We tested whether Tyr-to-Phε substitution of the Tyr-350 residue of the β2-adrenergic receptor would alter association of the receptor with Src (Fig. 5). These studies were performed in CHO-K1 cells that express very low levels of endogenous β2-adrenergic receptors. Stably transfected CHO clones were created that express either the wild-type β2-adrenergic receptor or the Y350F mutant form of the receptor. Clones were selected that express comparable levels of expression of β2-adrenergic receptors, 0.25 pmol of receptor/mg of membrane protein, as measured using the high affinity, radiolabeled β2-adrenergic antagonist ligand iodocyanopindolol. CHO clones were challenged with isoproterenol (10 μM) for periods up to 60 min. Whole-cell extracts were prepared from both clones and then employed for pull-down assays for the β2-adrenergic receptor using the CM04 anti-receptor antibody that recognizes an exofacial epitope of the receptor not influenced by the Tyr-350 site localized to the cytoplasmic, C-terminal tail of the β2-adrenergic receptor. The immune precipitates were subjected to SDS-PAGE and immunoblotting and stained with either anti-phosphotyrosine 4G10 antibody or anti-receptor CM02 antibody to establish equivalent loading of samples. For the clones expressing wild-type receptor, the phosphotyrosine content increased (Fig. 6), as observed above. In the cells expressing the Y350F mutant form of the β2-adrenergic receptor, however, phosphotyrosine content at 30 and 60 min post-challenge with isoproterenol was reduced in comparison with that obtained for wild-type receptors. An increase in phosphotyrosine content was observed at 5 min in the clones expressing the Y350F mutant form of the receptor, which presumably reflects increased phosphorylation of either Tyr-132 and Tyr-141 in the second intracellular loop or Tyr-354 and/or Tyr-364 in the cytoplasmic, C-terminal tail of the β2-adrenergic receptor. Each of these tyrosine residues has been characterized earlier with respect to phosphorylation, but only the Tyr-350 site creates an SH2-binding site upon phosphorylation (20).

Using the cyclic AMP response to isoproterenol stimulation as a readout, we examined in parallel the effects of the Y350F mutation of the β2-adrenergic receptor on agonist-induced desensitization in these CHO clones (Fig. 7). Clones stably expressing either the wild-type or the Y350F mutant forms of the receptor were challenged with isoproterenol for up to 60 min. In some cases (30 and 60 min) the clones were then washed free of the agonist and then challenged a second time for 5 min with isoproterenol, and intracellular cyclic AMP accumulation was
CHO clones expressing either wild-type (WT) β2-adrenergic receptors or the Y350F mutant form of the β2-adrenergic receptor were challenged with 10 mM isoproterenol (ISO) for 0, 5, 30, or 60 min, and intracellular cyclic AMP accumulation was determined. Some cells were first treated with isoproterenol for 30 or 60 min and then washed free of agonist and rechallenged with isoproterenol for 5 min. Assays of cyclic AMP accumulation were performed in triplicate. The data shown are mean values ± S.E. from three to five separate experiments performed on separate occasions.

Expression of the Y350F mutant form of the β2-adrenergic receptor does not alter the ability of the receptor to activate adenylyl cyclase, because the cyclic AMP response of the mutant receptor is essentially equal to that observed for the clones expressing the wild-type receptor (Fig. 7). What is striking, however, is that the clones expressing the Y350F mutant form of the β2-adrenergic receptor display a desensitization response that is substantially reduced in magnitude from that observed in clones expressing the wild-type receptor. At both 30 and 60 min post-challenge with isoproterenol, clones expressing the wild-type receptor display complete desensitization, whereas the CHO clones expressing the Y350F mutant retain ~50% of the cyclic AMP response observed following a single 5-min challenge with agonist. Although the fundamental observations obtained from the studies of the A431 cells and CHO clones stably expressing wild-type receptors are the same, the cellular context does play a role in the magnitude of the biological readout of cyclic AMP accumulation.

Because phosphorylation of the β2-adrenergic receptor by protein kinase A and GRK2 precedes association of the phospho-receptor with β-arrestin and because β-arrestin acts as an adapter for clathrin-mediated internalization of the β2-adrenergic receptor, we explored the phosphorylation state of GRK2 in A431 cells in the basal and isoproterenol-stimulated states (Fig. 8). Src has been shown to be capable of phosphorylating Tyr-350 and the SH2-binding site of the β2-adrenergic receptor created upon phosphorylation, but rather via β-arrestin, following the phosphorylation of the β2-adrenergic receptor by GRK2. As an alternative, we hypothesize that Src associates directly with, and is activated by, the phospho-Tyr-350 β2-adrenergic receptor. Src then is activated through association with the β2-adrenergic receptor and phosphorylates GRK2, driving the desensitization response. The β2-adrenergic receptor phosphorylated by Src-activated GRK2 then favors association of β-arrestin and clathrin with the receptor complex. This hypothesis was tested by measuring the amount of phosphotyrosine content (phosphorylation) in GRK2 under the same conditions that were employed to test agonist-induced desensitization and sequestration.

A431 wild-type and stably transfected clones were either untreated or challenged with isoproterenol (10 μM) for periods of up to 60 min, and the whole-cell extracts were prepared for immune precipitation pull-down assays using antibodies specific for GRK2 (Fig. 8). The immune precipitates were subjected to SDS-PAGE, and the resultant immunoblots were stained with either anti-phosphotyrosine antibodies (PY99) or antibodies against GRK2 to establish equivalent loading for each lane. Shown is a blot representative of three such experiments with essentially identical results.

![Figure 8](https://example.com/figure8.png) **Fig. 8.** Inhibition of Src kinase activity or expression of dominant-negative Src blocks the stimulation of increased phosphotyrosine content (activation) of GRK2 in response to β-adrenergic agonist. A431 cells with or without pretreatment for 30 min with the Src kinase inhibitor PP2 or clones stably transfected to express the constitutively active (CA-Src) or the dominant-negative (DN-Src) form of Src were challenged with isoproterenol (ISO) (10 mM) for 0, 5, 30, and 60 min and then used to prepare whole-cell extracts. The cell extracts were subjected to immune precipitation (IP) pull-down assays with antibody against GRK2, separation of the immune precipitate on SDS-PAGE, and immunoblotting. The immunoblots (IB) were stained with anti-phosphotyrosine antibody (PY99) as well as with anti-GRK2 antibody to establish equivalent loading for each lane. Shown is a blot representative of three such experiments with essentially identical results.
The clones stably expressing the GFP-tagged receptor could be monitored in live cells by epifluorescence microscopy. To study receptor localization in the unstimulated, control situation, the GFP-tagged receptor was stimulated for 30 min with isoproterenol (10^{-6} M) to induce desensitization of the adrenergic receptor. Agonist-induced desensitization and GRK2 phosphorylation are known cyclic AMP response was accompanied by a second wave of responses involving a new cast of G-protein partners and signaling to the mitogen-activated protein kinase pathway via Ras (3, 4). The latter response to mitogen-activated protein kinase protein has been shown to involve internalization in some cell lines (26) and a scaffold role of the β2-adrenergic receptor for mitogen-activated protein kinase signaling elements in other cell lines (25). Thus, much of what we know about the role of Src in GPLR generally and β2-adrenergic receptors specifically involves agonist-induced responses that occur temporally after desensitization.

In the current hypothesis for agonist-induced desensitization, it has been shown that Src both phosphorylates and activates GRK2 itself (demonstrated in vivo in the current work), arguing that the proper sequence of Src association with the

**DISCUSSION**

The Src family of nonreceptor tyrosine kinases have been shown to play important roles in the biology of G-protein-linked receptors. In response to agonist binding, GPLRs typically demonstrate several discrete, temporally linked responses, the first of these being activation of the receptor, transducing activation of the cognate heterotrimeric G-protein(s) to which the receptor is coupled. Following activation, virtually all GPLRs display agonist-induced desensitization, sequestration/internalization, and a resensitization phase. Recently, the β2-adrenergic receptor was shown to display signaling "switching" in which the desensitization of its well known cyclic AMP response was accompanied by a second wave of responses involving a new cast of G-protein partners and signaling to the mitogen-activated protein kinase pathway via Ras (3, 4). The latter response to mitogen-activated protein kinase protein has been shown to involve internalization in some cell lines (26) and a scaffold role of the β2-adrenergic receptor for mitogen-activated protein kinase signaling elements in other cell lines (25). Thus, much of what we know about the role of Src in GPLR generally and β2-adrenergic receptors specifically involves agonist-induced responses that occur temporally after desensitization.

Herein we explored the possible role of Src in events that precede rather than follow desensitization. The results demonstrate an important role of Src in the ability of the β2-adrenergic receptor to undergo agonist-induced desensitization. The primary results on which this claim is made are as follows. Treating cells with the cell-permeable PP2 inhibitor of Src kinases impairs desensitization; suppression of the expression of Src specifically leads to a loss in agonist-induced desensitization for the β2-adrenergic receptor; and expression of a dominant-negative mutant (K295R/Y527F) of Src nullifies agonist-induced desensitization and enables an impressive cyclic AMP response for periods up to 60 min post-challenge with agonist. These data implicate Src itself, rather than other Src family members, in the desensitization of β2-adrenergic receptors in response to agonist challenge. Other Src kinase family members, however, may play analogous roles for GPLRs other than β2-adrenergic receptors.

How Src is recruited to the receptor complex is not understood. Based upon pull-down assays, it has been suggested that Src associates with at least one well known member of the desensitization complex, β-arrestin. The interaction between β-arrestin and Src appears to be either a weak SH3 motif found in the N terminus of β-arrestin and/or a binding site that interacts with the catalytic domain of Src. Because the current hypothesis for agonist-induced desensitization suggests that GRKs first are recruited to the phosphorylated GPLR, followed by Src/Thr phosphorylation of the receptor and eventual recruitment of β-arrestin to the phosphorylated GPLR, the proposed association of Src with β-arrestin as a targeting mechanism seems untenable. In addition, it has been shown that Src both phosphorylates and activates GRK2 itself (demonstrated in vivo in the current work), arguing that the proper sequence of Src association with the
or bAR agonist-induced desensitization and commencement clathrin-mediated internalization. Inhibition of Src kinase with PP2, by expression of a dominant-negative receptor. The Ser/Thr-directed phosphorylation of the receptor SH2-binding site activates Src and catalyzes its phosphorylation/activation of GRK2, a Ser/Thr kinase that phosphorylates the agonist-activated receptor. The Ser/Thr-directed phosphorylation of the β2-adrenergic receptor recruits β-arrestin to the complex to complete agonist-induced desensitization and commence clathrin-mediated internalization. Inhibition of Src kinase with PP2, by expression of a dominant-negative mutant of Src or the Y350F mutation of the β2-adrenergic receptor, abolishes the ability of Src to be recruited/activated in response to β-adrenergic agonist and thereby precludes both desensitization and eventually full internalization of the receptor-Src complex. Not shown for the sake of simplicity is the scaffold molecule gravin that nucleates the complex of the receptor with several additional kinases and phosphatases. βAR or bAR, β-adrenergic receptor; TK, tyrosine kinase.

Cells expressing the Y350F mutant form of the β2-adrenergic receptor fail to display normal agonist-induced desensitization (Fig. 7). The phosphotyrosine content of the β2-adrenergic receptor in response to stimulation by agonist is sharply attenuated when the Y350F mutant receptor is expressed (Fig. 6). Furthermore, the ability of the β2-adrenergic receptor to recruit Src in response to agonist stimulation is abolished by the Tyr-to-Phe substitution of the Tyr-350 residue of the receptor (Fig. 5). These data provide a compelling case for the role of the Tyr-350 residue as a docking site for Src binding and for Src activation upon phosphorylation. A working model that captures these features of Src association with the β2-adrenergic receptor in the process of agonist-induced desensitization is displayed (Fig. 10). According to this model, it is the phosphorylation of Tyr-350 by some tyrosine kinase in response to agonist that initiates the process of desensitization. Upon phosphorylation, the Tyr-350 residue recruits Src to the plasma membrane, both docking and activating Src. Activated Src, in turn, phosphorylates and activates GRK2. GRK2 then catalyzes the further phosphorylation of the β2-adrenergic receptor on Ser/Thr residues, which in turn creates the docking site for β-arrestin, largely completing the desensitization phase of the receptor biology. Acting as an adaptor between the phosphorylated receptor and clathrin, β-arrestin then initiates the sequestration/internalization of the β2-adrenergic receptor, a process essential for desensitization and relocalization to the plasma membrane to occur.

Based upon the model (Fig. 10), one can ask, if the Tyr-350 phosphorylated receptor is necessary to recruit Src in advance of GRK2, what the outcome is of inhibiting Src on the subsequent activation of GRK. The answers to this question were quite clear. Inhibition of Src with PP2, expression of a dominant-negative version of Src, or suppression of Src levels by treatment with antisense oligodeoxynucleotides all blocked agonist-induced desensitization as well as the agonist-induced increase in the phosphotyrosine content (i.e., activation) of
antibodies against GRK2. Similarly, inhibiting Src activity with PP2 was shown to block agonist-induced changes in receptor biology, including desensitization and receptor trafficking. Using autofluorescent protein-tagged β2-adrenergic receptors and epifluorescence, we demonstrated a marked thickening of the band of receptor localized in close proximity to the plasma membrane in response to agonist in the PP2-treated cells. This observation made in live cells is consistent with the notion that Src activity is obligate both for desensitization and for proper trafficking of the β2-adrenergic receptor in response to agonist.

Taken together, these results reveal a novel, important role for Src in the regulation of β2-adrenergic receptors that may well extend to many other members of the GPCR superfamily. Src is critical for agonist-induced desensitization. For example, inhibiting Src activity with PP2 was shown to inhibit agonist-induced desensitization and receptor trafficking. Using autofluorescent microscopy, we observed when both signaling molecules are overexpressed that receptor internalization is delayed, and that the interaction of Src with β-arrestin and ultimately clathrin. The interaction of Src with β-arrestin, observed when both signaling molecules are overexpressed (3), may reflect a later role of Src in receptor internalization or signaling to Ras. These studies are the first to illuminate the role of Src in agonist-induced desensitization.

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c-Src Tyrosine Kinase Binds the β2-Adrenergic Receptor via Phospho-Tyr-350, Phosphorylates G-protein-linked Receptor Kinase 2, and Mediates Agonist-induced Receptor Desensitization

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