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Phosphorylation of Src by phosphoinositide 3-kinase regulates beta-adrenergic receptor mediated EGFR transactivation

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Abstract

β_2 -adrenergic receptors (β_2 AR) transactivate epidermal growth factor receptors (EGFR) through formation of a β_2 AR-EGFR complex that requires activation of Src to mediate signaling. Here, we show that both lipid and protein kinase activities of the bifunctional phosphoinositide 3-kinase (PI3K) enzyme are required for β_2 AR-stimulated EGFR transactivation. Mechanistically, generation of phosphatidylinositol (3,4,5)-tris-phosphate (PIP3) by the lipid kinase function stabilizes β_2 AR-EGFR complexes while the protein kinase activity of PI3K regulates Src activation by direct phosphorylation. The protein kinase activity of PI3K phosphorylates serine residue 70 on Src to enhance its activity and induce EGFR transactivation following β AR stimulation. This newly identified function for PI3K, whereby Src is a substrate for the protein kinase activity of PI3K, is of importance since Src plays a key role in pathological and physiological signaling.

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Conflict of Interests

The authors declare they have no conflicts of interest with this article.

Author Contributions

LJW, KMA, KX, SVNP and HAR conception and design of research; LJW, KMA, MLM, SM, ALB and KX performed experiments; LJW, KMA, MLM, ALB and KX analyzed data; LJW, KMA, SVNP and HAR interpreted results; LJW, KMA, MLM and SM prepared figures; LJW, KMA, MLM drafted manuscript; LJW, KMA, MLM, SVNP, HAR edited and revised manuscript; LJW, KMA, MLM, ALB, KX, SVNP and HAR approved final version of manuscript.

1. INTRODUCTION

Phosphatidylinositol-4,5-bisphosphate 3-kinases (PI3Ks) are a widely distributed family of enzymes uniquely characterized as having both lipid and protein kinase activities[1, 2]. The lipid kinase activity of PI3K catalyzes the phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP₂) to form phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) to activate downstream pathways necessary for cell survival, proliferation, cytoskeletal arrangement, receptor endocytosis and many Akt-dependent pathways[3–5]. Considerably less well appreciated is that PI3K contains serine/threonine protein kinase activity and this activity regulates cellular events such as survival and proliferation[6, 7]. Our recent work has shown that both lipid kinase and protein kinase activities of PI3K are required to regulate β -adrenergic receptor (β AR) endocytosis and signaling[8–10].

β ARs belong to the large family of seven transmembrane spanning receptors (7TMRs) that couple to G-proteins following agonist activation[11, 12]. Agonist stimulation of β ARs leads to a conformation change in the receptor followed by the activation of heterotrimeric G-proteins, resulting in increased adenylyl cyclase activity and cAMP generation. Following activation of the receptor, G protein-coupled receptor kinases (GRKs) phosphorylate the C-terminal tail of the activated receptor promoting the recruitment of β -arrestin to agonist occupied β ARs. β -arrestin desensitizes the receptor by inhibiting G-protein coupling and targeting the receptor for internalization[11]. While β -arrestin desensitizes β ARs, it also simultaneously initiates a diverse suite of signaling events, one of which is β AR-mediated epidermal growth factor receptor (EGFR) transactivation [13–15].

EGFR transactivation is a mechanism by which the EGF receptor is stimulated, internalized and downstream signaling initiated following agonist stimulation by a GPCR[16–18], such as the β AR[19]. Our current understanding of β AR-EGFR transactivation pathway begins with agonist stimulation of the β ARs and the subsequent phosphorylation of the receptor by GRKs. This is followed by the recruitment of β -arrestin and associated Src to the β AR-EGFR complex [13, 20]. Once Src is recruited to the complex following β AR stimulation, it mediates the activation of matrix metalloproteinases (MMPs) that act to cleave heparin-binding epidermal growth factor (HB-EGF) resulting in EGF ligand shedding[13, 18]. The shed HB-EGF ligand is available to bind and activate EGFRs, leading to downstream EGFR signaling [13, 18, 21]. While considerable research has focused on the mechanisms proximal to the activation of the β AR and EGFR[13, 18, 19, 22], less is known about the intermediate steps, particularly how Src is activated during β AR stimulated EGFR transactivation[13]. In this regard, we postulated that PI3Ks may play an important role in this process.

Following agonist stimulation the β AR is phosphorylated by GRKs to promote β -arrestin recruitment. GRK2 has been shown to recruit PI3K to the β AR complex following receptor stimulation[4]. The lipid kinase activity of PI3K (generates PIP₃ to maintain and stabilize a β -arrestin-adaptor protein AP2 interaction necessary for clathrin mediated endocytosis[23, 24]. Moreover, protein kinase activity of PI3K through phosphorylation of tropomyosin has also been shown necessary for β AR internalization and activation of downstream signaling [10]. Given the integral role of PI3K in β AR endocytosis and signaling we postulated that PI3K may play a critical role in β 2AR stimulated EGFR transactivation. We show here that a

mechanism of β AR stimulated Src activation is mediated by the protein kinase activity of PI3K through its phosphorylation of amino acid residue serine-70 leading to increased Src activity. We also show that the lipid kinase activity of PI3K generates phospholipids needed to maintain the stability of a β_2 AR-EGFR complex for efficient β_2 AR-mediated EGFR transactivation.

2. Materials and Methods

2.1 Plasmid Construction

Site-directed mutagenesis for the Src mutants was performed using the QuikChange II Site-Directed Mutagenesis kit (Agilent) according to the manufacturer's instructions. The GST-Src construct was made by digesting with EcoRI and XhoI and subcloning into the pGEX 4T1 bacterial expression vector. All plasmids were sequenced to verify authenticity.

Plasmids encoding the PIK, LK, PK, and LKPK mutants were previously described [10]. Plasmids encoding HA-Src, CFP- β_2 AR, Flag-EGFR, GFP-EGFR, and YFP-EGFR were obtained from R.J. Lefkowitz (Duke University Medical Center, Durham, NC). The Src Biosensor plasmid was a generous gift from Shu Chien and Yingxiao Wang (University of California at San Diego, La Jolla, Ca).

2.2 Generation of purified PI3K γ protein using baculovirus expression Sf9 insect cells

Baculovirus construct was generated by subcloning wild type full length PI3K γ downstream of 6 $\times$ His tag sequence into the pFastBacHTb vector (Invitrogen) as previously described [10]. The cloned bacmid DNA was used to infect Sf9 insect cells to express recombinant proteins. Sf9 cells expressing PI3K γ protein were collected and re-suspended in cell lysis buffer [HEPES pH 7.4, 50 mM KCl, 300 mM NaCl, 10% glycerol, 14 mM β -mercaptoethanol (β -ME), protease inhibitor cocktail]. The suspended cells were sonicated and incubated with Benzonase (5 U/mL) for 30 min and the suspension was centrifuged at 35,000 $\times g$ for 30 min. The PI3K γ protein was immunoprecipitated using Ni-NTA affinity resin (Invitrogen) following manufacturer's protocol. Wash buffer consisted of cell lysis buffer plus 5 mM imidazole (Sigma). The protein was eluted using elution buffer (Cell lysis buffer plus 150 mM imidazole). The immunoprecipitated PI3K γ was used in in vitro protein kinase assays using Src as substrate and in vitro Src functional assays with enolase from baker's yeast (Sigma) as substrate.

2.3 Expression and purification GST-Src fusion protein

A glutathione S-transferase (GST)-Src fusion protein was generated by subcloning full length wild type (WT) Src cDNA into pGEX 4T1 between Eco RI and Xho I restriction sites. The recombinant plasmids were sequenced to verify in frame subcloning. Serine/Threonine to Alanine (S35A, T37A, S39A, S43A, S70A and S75A) point mutations of Src were generated by site directed mutagenesis using Quickchange II Site-Directed mutagenesis kit (Agilent) following manufacturer's instructions. All plasmids were sequenced to verify the authenticity. The plasmids harboring WT and mutant Src were transformed into E. Coli strain BL21 and fusion proteins were purified as previously described [10]. Briefly, GST-Src fusion protein expression was induced in cultured BL21 cells using 0.1 mM iso-propyl-1-thio- β -D-galactopyranoside (IPTG). Cells were pelleted

and lysed using lysis buffer (PBS, pH 7.2; 1 mM DTT; 1 mM EDTA; protease inhibitor cocktail; 3 U/mL Benzonase; 1 % Triton X-100; 1 mg/mL Lysozyme). GST-Src fusion protein was isolated from the supernatant using Glutathione Sepharose beads. GST beads were washed in wash buffer (PBS, pH 7.2; 1 mM DTT; 1 mM EDTA). The fusion protein was eluted using elution buffer (50 mM Tris, pH 8.0; 0.4 M NaCl; 50 mM L-Glutathione reduced; 0.1 %, Triton X-100, 1 mM DTT). Protein concentrations were estimated and resolved using SDS-PAGE and Coomassie staining to check expression levels. Purified Src was used as substrate for in vitro phosphorylation assays and Src functional assays.

2.4 Cell Culture and Transient Transfection

HEK-293 cells stably expressing WT- β_2 AR were cultured as described previously [13]. Briefly, cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C. The cells were seeded at a density of 1×10^5 cells for 35-mm confocal dishes, 2×10^5 cells for 6-well dishes, or 1×10^6 cells for 10-cm dishes the day before transfection. Appropriate amounts of plasmids (1–2 μ g/35-mm confocal dish, 2 μ g/6-well dish, or 2–4 μ g/10-cm dish) were used for transient transfection with FuGENE 6 (Roche). Cells were serum starved 48 hours post-transfection and treated with inhibitors and agonists as described.

2.5 Treatment of cells with phospholipids

HEK-293 cells were treated with phospholipids as previously described [23]. Briefly, synthetic DiC16 PtdIns-3,4,5- P_3 (Excelon) dissolved in chloroform was air dried under N_2 . The N_2 air-dried phospholipids were resuspended in a buffer containing 10 mM HEPES and 1 mM EDTA and sonicated to form lipid micelles. HEK-293 cells were treated with saponin (1 mg/mL) and incubated with PtdIns-3,4,5- P_3 at room temperature for 10 min. The cells were allowed to recover for 30 min by washing off saponin and stimulated with 10 μ M ISO and used for immunoprecipitation studies as described below.

2.6 Immunoprecipitation

Immunoprecipitation was performed as described as described previously [13]. Briefly, cell lysates (700–1000 μ g) were incubated at 4°C overnight with anti-FLAG or anti-HA antibody (1:100) and 35 μ l of Protein G Plus/A agarose beads (Calbiochem) and a control (with no antibody) was also included for each of the experimental sets. Following incubation, the beads with immunoprecipitated receptors were washed three times with 1 $\times$ PBS and loading dye was added to the beads. The beads were incubated at 70°C for 5 min before loading on to SDS-PAGE gels. The immunoprecipitated proteins were analyzed by western blotting.

2.7 Western Blotting

Following stimulation, cells were washed once with PBS and solubilized in lysis buffer containing 20 mM Tris-HCl, pH 7.4, 0.8% Triton X-100, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 100 μ M PMSF, 5 μ g/ml aprotinin and leupeptin. The protein concentrations of the cell lysates were determined using a Bio-Rad protein assay kit with bovine serum albumin as a standard. Samples were mixed with loading dye and denatured by heating at 70°C for 5 min, resolved on SDS-PAGE and transferred to PVDF membrane (Bio-Rad). The

membranes were immunoblotted with primary antibody to EGFR (1:1000, Santa Cruz), phospho-EGFR (Tyr845) (1:1000, Cell Signaling), ERK1/2 (1:5000, Millipore), phospho-ERK1/2 (1:1000, Cell Signaling), Src (1:2000, Cell Signaling), β_2 AR (1:2000, Santa Cruz), HA (1:1000, Sigma), or Flag (1:1200, Sigma). Immunoblots were visualized with an HRP-conjugated secondary antibodies using a chemiluminescence detection system (GE Healthcare).

2.8 EGFR- β_2 AR FRET

HEK-293 cells expressing CFP- β_2 AR and YFP-EGFR were plated onto 35-mm confocal dishes. Prior to imaging media was replaced with imaging buffer (125 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose, 0.2% bovine serum albumin, 10 mM HEPES, pH 7.4) were placed on a 37°C heated stage. Cells were stimulated, and the images were acquired on a Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc.) with a Roper Micromax cooled charge-coupled device camera (Photometrics) utilizing a 63 $\times$ oil immersion lens with a 1.4 NA and SlideBook 4.0 (Intelligent Imaging Innovations). Changes in FRET were measured, and all calculations used have been previously described [15, 25]. Briefly, CFP and FRET images were obtained through a 436/20 excitation filter (20 nm band-pass centered at 436 nm), a 455DCLP (dichroic longpass mirror), and separate emission filters (480/30 for CFP and 535/30 for FRET). YFP intensity was imaged through a 500/20 excitation filter, 515LP dichroic mirror, and 535/30 emission filter. All optical filters were obtained from Chroma Technologies. Excitation and emission filters were switched in filter wheels (Lambda 10-2; Sutter). Integration times were varied between 100 and 300 ms to optimize signal and minimize photobleaching. Spectral bleed through was determined by acquiring CFP, FRET, and YFP intensity images of samples expressing CFP only and YFP only and was linear with respect to fluorophore expression. This imaging system exhibits 43% CFP/FRET bleed through and 24% YFP/FRET bleed through. CFP/YFP bleed through was undetectable. FRET intensity corrected for bleed through (FRETc) was defined as $FRETc = FRET - 0.43 \times CFP - 0.24 \times YFP$. FRETc for all FRET images was calculated on a pixel-by-pixel basis for localization of FRET. All graphs display calculations based on intensity from whole cells or sets of cells.

2.9 Confocal Microscopy

Visualization of EGFR internalization was performed by confocal microscopy as described previously [13, 14, 26]. Briefly, to monitor internalization of EGFR following stimulation, HEK-293 cells stably expressing β_2 ARs transfected with GFP-EGFR were plated on 35-mm glass bottom culture dishes. Following stimulation, cells were washed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde and Fluoromount-G was added for storage. EGFR internalization following ISO or EGF stimulation was visualized with a LSM-780 laser scanning microscope. Using a 63 $\times$ oil immersion objective with a 1.4 NA at RT camera with a GaAsP high QE 32 channel spectral array utilizing Zen acquisition software. Quantification of EGFR-GFP internalization was performed using on a Marianas inverted microscope imaging system based on a Zeiss Axio Observer Z1 fitted with a CSU-X1 spinning disk confocal. Images were acquired at 40 $\times$ (1.3 NA) on a Photometrics Evolve 16-bit EMCCD camera. Slidebook 6 acquisition software was used. All acquired images were analyzed using ImageJ.

2.10 Src Biosensor Activity Assay

Src activity was determined using a FRET based Src biosensor as previously described [27, 28] with minor modifications. The Src biosensor consists of a CFP-YPet FRET pair at either end of a flexible linker domain containing a Src kinase peptide (WMEDYDYVHLQG, from the known Src target p130cas[29, 30]). NIH 3T3 cells were transiently transfected with 3 μ g β_2 AR, 2 μ g Src biosensor, and 2 μ g of mCherry-tagged Src mutants. Fibroblasts were plated onto poly-d-lysine coated dishes and starved for 36 hours in MEM containing 0.5% FBS. Prior to imaging, media was changed to CO₂-independent media (Invitrogen) and the cells were imaged on a Deltavision Elite microscope using a 60 $\times$ oil immersion objective with a 1.42 NA and an Evolve back-thinned EM-CCD camera. Images were acquired every 2 min using CFP-YFP-mCherry filter sets. Excitation of the samples was performed by an InsightSSI 7 channel solid-state illumination source. Wortmannin (50 nM) or PP2 (10 μ M) were added 30 min prior to stimulation with ISO (10 μ M) or PDGF (50 nM). Vehicle or agonist was added after the third time point. Acquired images were analyzed using ImageJ. Normalized emission ratios (CFP/FRET signal) are reported.

2.11 In vitro Src phosphorylation assay

Protein kinase assays were performed using Sf9 purified PI3K γ as a kinase and bacterial purified WT and mutant Src proteins as substrates. Four pmoles of PI3K γ and 250 pmoles of Src proteins were resuspended in 45 μ l reaction buffer (20 mM HEPES pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 5 μ M ATP, 1 mM DTT, 0.2 mM EDTA) containing 10 μ Ci of [γ -³²P] ATP. Phosphorylation was performed at 30 $^{\circ}$ C for 40 min and the reactions were stopped by adding 15 μ l of 4 $\times$ Laemmli Sample Buffer. Samples were then resolved on SDS-PAGE gels, stained with Coomassie blue to confirm equal loading of the substrate, and then gels were dried. Src phosphorylation was visualized by autoradiography.

2.12 In vitro Src functional assay

Src protein was in vitro phosphorylated using 4 pmoles of PI3K γ and 250 pmoles of Src substrate in 30 μ l reaction buffer (20 mM HEPES pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 5 μ M ATP, 1 mM DTT, 0.2 mM EDTA) containing 5 μ Ci of [γ -³²P] ATP for 40 min at 30 $^{\circ}$ C. The reactions were continued for another 30 min after supplementing with 3 nmoles of enolase from baker's yeast and another 5 μ Ci of [γ -³²P] ATP and increasing the reaction volume to 45 μ l. Reactions were stopped by adding 15 μ l of 4 $\times$ Laemmli Sample Buffer, resolved on SDS-PAGE, stained with Coomassie to check for equal loading of the Src and enolase proteins. The gels were dried and enolase phosphorylation was visualized by autoradiography.

2.13 Mass spectrometry

HEK-293 cell lines stably expressing Src were established by transfecting cells with a neomycin-resistant HA-Src expression plasmid and selecting colonies grown in medium containing G418 (600 μ g/mL). Stable isotope labeling of amino acids for SILAC experiments was performed by culturing the cells separately in "light" or "heavy" medium containing G418, 10% fetal bovine serum, and 1% penicillin/streptomycin for at least for 6 passages (approximately two and a half weeks)[31]. The cells were grown in regular MEM

containing 10% FBS, 1% penicillin/streptomycin, and G418 for non-SILAC experiments. The cells were transfected with 3 μ g of β_2 AR encoding cDNA, serum starved for two hours 48 hours post-transfection and pretreated with DMSO or LY-294002 (20 μ M) for 20 min prior to stimulation with ISO (10 μ M) for 5 min. The cells were washed, lysed, and immunoprecipitated as described above. In SILAC experiments, the “light” and “heavy” lysates were mixed in a 1:1 ratio prior to immunoprecipitation[31]. The immunoprecipitates were resolved by SDS-PAGE and then stained with Coomassie. The appropriate bands were excised, destained with 25 mM ammonium bicarbonate in 50% acetonitrile and the cysteine residues were reduced using 10 mM DTT and alkylated with 55 mM iodoacetamide. The samples were digested with trypsin (20 μ g/ μ l) overnight at 37°C. The phosphopeptides were phospho-enriched using immobilized metal ion affinity chromatography (IMAC) and desalted. The phosphopeptides were then reconstituted in 10 μ l Water’s phosphopeptide buffer (50 mM citric acid, 2% acetonitrile, and 0.1% TFA) and analyzed by LC/MS/MS using a Thermo Scientific LTQ Orbitrap XL as previously described [31].

2.14 Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean and analyzed by either one-way ANOVA or two-way, repeated measures ANOVA followed by post-hoc tests as described. A *P* value of less than 0.05 was considered significant.

3. RESULTS

3.1 PI3K is required for isoproterenol (ISO)-induced EGFR transactivation

Since PI3K is recruited to the β_2 AR complex following ISO, we tested if PI3K is required for β_2 AR-mediated EGFR transactivation by transfecting HEK-293 cells stably expressing β_2 ARs with EGFR-GFP, pre-treating with vehicle or PI3K inhibitors and stimulating with ISO or EGF. We performed confocal microscopy to monitor EGFR-GFP internalization following agonist stimulation and showed pharmacologic inhibition of PI3K blocked ISO-stimulated EGFR internalization but did not affect EGF-mediated EGFR internalization (Figure 1a).

Because PI3K is a bi-functional enzyme with both lipid kinase and protein kinase activity [32] we tested whether either or both lipid and protein kinase activity of PI3K is required for β_2 AR-mediated EGFR transactivation. We used our previously characterized PI3K γ mutants that have no activity (PIK), retain only lipid kinase activity (LK), retain only protein kinase activity (PK), or maintain both lipid and protein kinase activity (LKPK) (Figure 1b) [10]. These mutants function as dominant negatives only at the level of the receptor complex due to their recruitment by GRK2[10] and have previously shown not to alter cytosolic activity of PI3K [10]. HEK-293 cells stably expressing β_2 ARs were transfected with EGFR-GFP, the various PI3K γ mutants (PIK, LK, PK, LKPK), and then stimulated with ISO. Confocal microscopy was again utilized to monitor EGFR-GFP internalization. We found that ISO-stimulated EGFR internalization was prevented in the presence of LK, PK, or PIK mutants but was maintained with LKPK expression (Figure 1c). These data demonstrate that both the lipid and protein kinase activities of PI3K γ are required for β_2 AR-mediated EGFR transactivation.

3.2 PI3K γ phosphorylates specific amino acid residues of Src

It is known that GPCRs can activate Src[33] and that Src is required for β AR-mediated EGFR transactivation[13, 14]. To test whether the protein kinase activity of PI3K directly phosphorylates Src following β_2 AR stimulation, we used stable isotope labeling (SILAC) in β_2 AR expressing cells (Figure 2a). Two separate sets of HEK-293 cells stably expressing HA-Src and β_2 AR were grown in either “light” medium containing arginine and lysine residues with normal ^{12}C or “heavy” medium labeled with ^{13}C . Cells maintained in “heavy” medium were stimulated with ISO, while cells in the “light” medium were pre-treated with LY-294002 prior to ISO. Following stimulation, lysates of cells grown in “heavy” and “light” medium were mixed at 1:1 ratio and HA-Src immunoprecipitated from the mixed lysates. The HA-Src band was excised and subjected to trypsin digestion. The trypsin digested Src band was extracted, purified and analyzed for phosphopeptides by liquid chromatography-mass spectrometry (LC-MS). Because the “heavy” peptides have a greater mass due to ^{13}C incorporation than the “light” peptides, two distinct peaks could be detected for specific peptides allowing for detection of differentially phosphorylated peptides in response to ISO. LC-MS analysis revealed several PI3K-dependent Src phosphorylation sites in the N-terminal region of Src (Figure 2b and Table 1). Treatment with ISO revealed a peptide containing the phosphorylation sites S35, T37, S39, and S43 that was increased 9.6 fold, while a second peptide containing S70 and S75 was increased 3.5-fold compared to the ISO+LY294002 treated group. These two peptides showed the greatest increase in phosphorylation with ISO stimulation with A-scores more than 20 indicating high levels of confidence and accuracy in identifying the specific phosphorylated residues (Table 1)[34].

To test which of the specific amino acid residues of Src identified by mass spectrometry were potential direct PI3K phosphorylation sites we performed in vitro kinase assays with purified WT and mutated Src proteins. We generated Src mutants containing single amino acid mutations to alanine as informed by the SILAC experiment. Non-phosphorylatable Src mutants (S35A, T37A, 39A, S43A, S70A or S75A) and wild-type Src were purified from bacteria and used as substrates in an in vitro kinase assay using Sf9 baculo-virus purified PI3K γ . The in vitro kinase assay was performed in the presence of PP2, a Src inhibitor, to prevent the intrinsic autophosphorylation of Src [33]. WT Src phosphorylation was significantly increased in presence of PI3K γ , which was markedly reduced following addition of LY-294002 (Figure 2c). Importantly, reduced phosphorylation was observed for the S39A and S70A Src mutants suggesting that PI3K γ may phosphorylate Src at residues S39 and S70 in vitro (Figure 2c).

3.3 Phosphorylation of Src on serine residue 70 (S70) by PI3K γ modulates Src kinase activity

To directly examine which amino acid residue of Src is phosphorylated by PI3K to modulate its activity we performed Src kinase activity assays using enolase as a substrate. Purified WT and Src mutants were first subjected to in vitro phosphorylation by PI3K γ . After addition of enolase, PI3K γ mediated WT Src phosphorylation markedly increased enolase phosphorylation (Figure 2d). In contrast, enolase phosphorylation by Src was abolished when PI3K γ -dependent Src phosphorylation was carried out in presence of LY-294002 (Figure 2d). While initial in vitro kinase experiments using purified proteins suggested that

either S39A or S70A were PI3K dependent sites, only the S70A Src mutant showed reduced Src kinase activity in the presence of PI3K γ . These data suggest that S70 phosphorylation is the likely amino acid residue critical for PI3K γ -mediated Src activation (Figure 2d).

3.4 Phosphorylation of Src by PI3K at S70 increases cellular Src activity

To demonstrate that serine residue S70 of Src is a critical site for PI3K mediated regulation of Src activity during β_2 AR-EGFR transactivation, we used a FRET-based Src biosensor assay [27, 28]. The Src biosensor consists of a CFP-YPet FRET pair at either end of a flexible linker domain containing a Src kinase peptide (WMEDYDYVHLQG, from the known Src target p130cas [29, 30]). In the presence of minimal Src activity, the FRET pair remains in close proximity and a CFP-YPet FRET signal is detectable. Upon Src activation, phosphorylation of the p130cas Src substrate occurs, which induces a conformational change resulting in the loss of CFP-YPet FRET signal. NIH 3T3 cells were transiently co-transfected with the β_2 AR, the Src biosensor and individual Src plasmids. Minimal Src activity was observed in the absence of stimulation as measured by the CFP/FRET ratio. Following platelet derived growth factor (PDGF) or ISO stimulation, Src activity increased to similar levels over a 15 min time period (Figure 3a). In contrast, ISO mediated Src activation was completely abolished with pre-treatment with either wortmannin (PI3K inhibitor) or PP2 (Src inhibitor) (Figure 3a). Importantly, expression of the Src mutant S70A abolished the ISO mediated Src activation whereas PDGF mediated Src activation was unaffected (Figure 3b). All of the Src mutants responded normally to both PDGF and ISO stimulation (Table 2) except S70A. These findings further support our in vitro data demonstrating that phosphorylation of serine residue S70 by PI3K is a key regulatory step for Src activation.

3.5 PI3K-dependent phosphorylation of serine residue 70 (S70) of Src is necessary for β_2 AR mediated EGFR transactivation

We next generated mCherry tagged Src mutants (WT, S35A, T37A, S39A, S45A, S70A and S75A) and individually co-transfected these mutants with EGFR-GFP into HEK 293 cells stably expressing β_2 ARs. ISO-mediated EGFR transactivation was assessed by EGFR-GFP internalization using confocal microscopy. Following ISO stimulation robust EGFR internalization was observed with WT Src, but was abolished by overexpression of the S70A Src mutant (Figure 4a, second column). As expected, pre-treatment of cells with Src inhibitor PP2 abolished ISO-induced EGFR internalization (Figure 4a, third column). Expression of tagged WT Src or the S70A Src mutant did not affect EGF dependent EGFR internalization (Figure 5a, fourth column). Over expression of the other S to A mutants did not affect either EGF or ISO dependent EGFR internalization (Figure 4b). In separate experiments, EGFR internalization was quantified by counting the number of cells showing internalized EGFRs. 125 to 150 cells were counted per treatment and data are presented as percent cells with internalized EGFRs (Figure 5). Only mutation of S70 showed impaired ISO induced EGFR internalization. These data, combined with the Src biosensor data, strongly suggest that phosphorylation of serine residue 70 (S70) is critical for Src activity during β_2 AR-mediated EGFR transactivation.

3.6 Akt activity is not required for β_2 AR mediated EGFR transactivation

Since PI3K generates the D-3 phospholipid PIP₃ to activate the serine/threonine kinase Akt, we wanted to exclude the possibility that Akt rather than PI3K was phosphorylating Src to promote EGFR transactivation. β_2 AR overexpressing cells were transfected with FLAG-EGFR and stimulated with ISO or EGF in presence of wortmannin (PI3K inhibitor, 50 nM, 30 min pretreatment), tricarbine (Akt inhibitor, 10 μ M, 30 min pretreatment) or PP2 (Src inhibitor 10 μ M, 30 min pretreatment). Significant EGFR phosphorylation was observed following ISO, which was markedly reduced in the presence of either wortmannin or PP2 (Figure 6). Despite Akt inhibition with tricarbine, significant EGFR phosphorylation was observed with ISO (Figure 7). Similarly, ISO mediated ERK activation was observed in the presence of tricarbine (Figure 7). Importantly, EGF dependent EGFR phosphorylation and ERK activation was not inhibited in the presence of either wortmannin or tricarbine, but markedly reduced only in the presence of the Src inhibitor, PP2 (Figure 6). These data suggest that Akt is not mechanistically involved in ISO stimulated β_2 AR-EGFR transactivation.

3.7 PI3K is necessary for β_2 AR-EGFR complex formation

The formation of a β_2 AR-EGFR complex is thought to be integral to β_2 AR-mediated EGFR transactivation[15]. To investigate whether PI3K activity is necessary for this complex formation, β_2 ARs were immunoprecipitated from cells expressing FLAG- β_2 AR, EGFR, and kinase-dead PI3K mutants (PIK or PI3K γ) and probed for co-immunoprecipitating EGFR. ISO stimulation resulted in a significant increase in the association between β_2 AR and EGFR compared to non-treated samples (Figure 7a). In contrast, co-expression of PIK or PI3K γ abolished the β_2 AR-EGFR association (Figure 7a). Similarly, pre-treatment of the cells with the PI3K inhibitor LY-294002 also prevented co-immunoprecipitation of β_2 AR and EGFR (Figure 7b).

To further demonstrate the requirement of PI3K activity for the β_2 AR-EGFR association, fluorescence resonance energy transfer (FRET) experiments were performed using CFP-tagged β_2 ARs and YFP-tagged EGFRs. Following ISO stimulation there was a time dependent decrease in the FRET signal as the β_2 AR-EGFR complex was internalized and individual receptors were sorted to different intercellular compartments. Pretreatment with LY-294002 markedly increased this loss of FRET signal, suggesting a decrease in the β_2 AR-EGFR complex formation (Figure 7c). Taken together, these data show that PI3K activity is necessary for the formation and maintenance of the β_2 AR-EGFR complex.

3.8 Lipid kinase activity of PI3K is responsible for β_2 AR-EGFR complex formation

To determine which of the kinase activities of PI3K is responsible for β_2 AR-EGFR complex formation we transiently transfected FLAG- β_2 AR stable cells with EGFR and either the PI3K mutants LK or PK. FLAG- β_2 ARs were immunoprecipitated and probed for EGFR. ISO stimulation resulted in significant β_2 AR-EGFR association in control cells, and cells expressing the lipid kinase only (LK) mutant of PI3K γ (Figure 7d). Expression of the PK mutant of PI3K, which only has protein kinase activity, prevented the association of β_2 ARs and EGFRs into a complex following ISO stimulation (Figure 7d). However, in rescue experiments the in vitro addition of PIP₃ (0.5 μ M), the product of the lipid kinase activity of

PI3K to saponin treated PK expressing cells restored the β_2 AR-EGFR association following ISO (Figure 7d). Taken together, these data support the concept that PIP3 generated by lipid kinase activity of PI3K is necessary for β_2 AR-EGFR complex formation, to promote efficient ISO-stimulated β_2 AR mediated EGFR transactivation.

4. DISCUSSION

Our current understanding of the EGFR transactivation mechanism is that agonist stimulation of β ARs lead to β -arrestin recruitment to the receptor complex along with Src, whose activation stimulates MMP mediated cleavage of HB-EGF. HB-EGF then stimulates EGFRs in an autocrine/paracrine manner to induce EGF dependent signaling [13–15, 18, 19, 33]. While many components of this pathway have been previously explored, it was still unknown how Src is activated following GPCR stimulated EGFR transactivation. Our study provides insights into the mechanistic underpinnings of the regulatory signaling pathway that connects the activation of β_2 AR to EGFR during transactivation, specifically at the level of Src activation.

We show that the bi-functional enzyme PI3K, which is recruited to the β_2 AR complex following ISO stimulation[4, 23] plays a critical role in β_2 AR-mediated EGFR transactivation. Our data show that both the protein and lipid kinase activities of PI3K are necessary for β_2 AR-EGFR transactivation to occur. First, we show that the protein kinase activity of PI3K regulates Src activity, a known activator of MMPs[35], to promote cleavage of the EGFR ligand HB-EGF mediating EGFR transactivation[13, 18]. Utilizing SILAC based mass spectroscopy and generation of single point Src mutants, we show that PI3K phosphorylation of serine residue 70 on Src increases its activity. Since the S70A Src mutant inhibited β_2 AR-mediated EGFR transactivation but not EGFR activation by EGF, S70 Src phosphorylation by PI3K appears to be a required step in the process of EGFR transactivation. We further show that the lipid kinase activity of PI3K is required for maintaining the β_2 AR-EGFR complex following ISO stimulation, which appears to be required for the efficient cross-activation between the β AR and EGFR to stimulate downstream signaling molecules ERK and Akt/PKB in a Src dependent manner (Figure 8). Activation of ERK and Akt/PKB have been used as measures of downstream signaling following ISO-mediated EGFR transactivation. However, it is important to note that ERK and Akt/PKB can be activated by multiple pathways following receptor activation that include β -arrestin dependent mechanisms[36]. Although, ERK and Akt/PKB can be activated by multiple pathways, when EGFR is overexpressed, ISO-induced EGFR transactivation appears to be the dominant mechanism by which ERK and Akt/PKB are activated. Inhibition of PI3K by wortmannin or Src by PP2 results in significant inhibition in ERK and Akt/PKB activation following ISO stimulation consistent with the idea that EGFR transactivation is the dominant pathway regulating these downstream signals.

PI3Ks are a unique family of enzymes that are characterized by both lipid and protein kinase activities[2, 10]. Although PI3K is well recognized for its lipid kinase activity, accumulating evidence shows that the protein kinase activity of the PI3K may be as important in mediating downstream signals that could contribute to physiology as well as pathology[8, 10, 37]. Correspondingly, our studies show that both activities of PI3K are necessary for β_2 AR-

stimulated transactivation of EGFR. In this regard, we used previously characterized PI3K γ mutants that selectively contain lipid kinase (LK) or protein kinase (PK) or lipid and protein kinase activities (LKPK) (Fig. 1B)[10] based on structure of PI3K γ [32]. All the PI3K γ mutants contain the phosphoinositide (PIK) domain of PI3K γ as we our previous studies had shown that G-protein coupled receptor kinase (GRK2) interacts with PI3K γ via the PIK domain[23] and in part, recruits PI3K γ to the β_2 AR complex following ISO stimulation. Therefore, overexpression of PIK domain leads to displacement of endogenous PI3K γ from GRK2 impairing PI3K γ activity at the β_2 AR complex. Therefore, PIK domain containing PI3K γ mutants (LK, PK or LKPK) displace endogenous PI3K γ upon overexpression selectively targeting lipid kinase (LK) or protein kinase (PK) or lipid and protein kinase (LKPK) activity of PI3K γ to the β_2 AR complex upon ISO stimulation. By using PI3K γ mutants that harbor only a single enzymatic function, we found that each activity selectively regulates a specific function in the process of β_2 AR mediated EGFR transactivation. Using a similar rationale, but utilizing a different set of PI3K mutants, Bondeva et al. showed that that lipid kinase activity of PI3K γ classically regulates protein kinase B (PKB)/(Akt), while the protein kinase activity of PI3K γ activates mitogen-activated protein kinase (MAPK) pathways[37]. Similarly, our previous studies have shown that both protein and lipid kinase activity of PI3K γ are required for agonist-mediated β_2 AR internalization[10]. Consistent with this concept of dual activity for PI3K, it was shown that PI3K α can phosphorylate the regulatory p85 α subunit to inhibit PI3K α recruitment to the receptor complex[38]. These studies support the concept that specific kinase activities of PI3K confer diverse regulatory functions for PI3K in start of cellular signaling.

Our data provides new evidence that the protein kinase activity of PI3K is also required for transactivation, likely by regulating Src activity. Src is a non-receptor tyrosine kinase known to play a critical role in the activation of MMPs[18, 39], thereby mediating shedding of the EGFR ligand HB-EGF [35]. Although a key role of Src activity in transactivation is well established, its regulation and activation in EGFR transactivation is not well understood. Previous studies have shown that following ISO stimulation Src is recruited to the β_2 AR complex by β -arrestin[20], and the generation of PIP3 by PI3K stabilizes β -arrestin complexes at the plasma membrane. In this context, we show that PI3K, in addition to stabilizing the β_2 AR-EGFR complex, via its lipid kinase activity, directly phosphorylates Src to enhance its activity and lead to EGFR activation and internalization and ERK signaling. These observations show that Src activation is critical for EGFR transactivation as measured by EGFR internalization. Consistent with our previous work, we show that inhibition of Src by PP2 abolishes ISO-mediated EGFR transactivation as assessed by phospho-EGFR, phospho-Akt and phospho-ERK activation (Fig. 6), while inhibition of Akt does not impair EGFR transactivation. These findings support the concept that Src plays a key role in ISO-mediated EGFR transactivation.

We used SILAC labeling to identify that Src is phosphorylated by the protein kinase activity of PI3K, and demonstrate its role as a key regulatory step in Src activation. Although our SILAC labeling studies identified multiple putative sites on Src that could be phosphorylated by PI3K, our biochemical and cellular studies show that PI3K mediated Src phosphorylation of Serine 70 is the critical amino acid residue in β_2 AR-mediated EGFR transactivation. Indeed, our data suggest that phosphorylation of Src on serine 70 by PI3K is a required

process for transactivation activity since overexpression of a serine to alanine Src point mutant abolished ISO-mediated Src activation and EGFR transactivation, but had no effect on PDGFR activation of Src.

The current concept for Src activation is that Src is maintained in an auto-inhibitory state by phosphorylation of tyrosine 527 that intra-molecularly binds with the SH2 domain to stabilize an inactive conformation[40]. In addition to the SH2, SH3, and tyrosine kinase domains, Src possesses a unique domain consisting of the first 83 N-terminal amino acids, which confers most of the sequence diversity among non-receptor tyrosine kinases [41]. Phosphorylation of tyrosine 213 by the PDGFR is thought to result in de-stabilization of the SH2 domain leading to Src activation[42]. Similarly, cyclin dependent kinase 1 (CDK1) is known to mediate phosphorylation of N-terminal serine (S72) and threonine (Thr34 & 46) residues of Src. This phosphorylation activates Src and leads to transition through the cell cycle[40, 43]. It also known that PKC and PKA promote Src phosphorylation; however, their role in Src activation is not well understood[44, 45]. Thus, it is considered that the phosphorylation of Src at the N-terminus leads to a conformational change that releases the inhibitory intra-molecular interactions. This phenomenon is also observed in other non-receptor tyrosine kinases [44, 46]. Consistent with this mechanism of Src activation, we show that phosphorylation of serine 70 by PI3K enhances Src activity and is needed for β_2 AR-mediated EGFR transactivation.

Classically, PI3K lipid kinase activity generates PIP₃ phospholipids that recruit pleckstrin homology (PH) domain containing proteins and the phosphoinositide dependent kinase (PDK) mediated activation of PKB/Akt[47]. It is not precisely known how phospholipid engagement with the PH domain proteins regulate their activity, but activation is thought to be associated with translocation from cytosol to the plasma membrane[48], mediating a conformational change by either relieving intra-molecular inhibition or permitting enhanced proximity with their substrates or binding partners[49]. Our data shows that lipid kinase activity of PI3K appears to be selectively required for stabilization of β_2 AR-EGFR complex following ISO stimulation. Indeed, stabilization of β_2 AR-EGFR complex is restored following exogenous addition of PIP₃ (the product of the lipid kinase activity) in presence of the protein kinase mutant of PI3K γ that does not have lipid kinase activity. The lipid kinase activity of PI3K also appears to be important for the association of β_2 AR with the multi-functional scaffold protein, β -arrestin. β -arrestin is known to differentially interact with D-3 phosphoinositides with highest affinity towards PIP₃ followed by PIP₂ and PIP [50]. PIP₃ binding to β -arrestin stabilizes activated β_2 ARs to clathrin-coated pits, as mutants of β -arrestin deficient in phosphoinositide binding are not effectively recruited to the coated pits [24]. Moreover β_2 AR mediated ERK activation requires formation of a complex between β_2 AR and β -arrestin wherein the activation of ERK is determined by the stability of the β -arrestin- β_2 AR complex [15]. Thus, PIP₃ generated by PI3K allows for a stabilized interaction between β -arrestin- β_2 AR-EGFR perhaps providing the platform for the formation of a multi-receptor complex that is required for effective ISO-mediated β_2 AR transactivation of EGFR.

Since Src is a key nodal signal transducer[51], such precise regulation may provide a mechanism for specificity in cellular signaling. For example, we show that PI3K γ

phosphorylates Src, which mediates EGFR transactivation. Transactivation also leads to the activation of PI3K α /Akt signaling in a Src dependent manner as Src inhibition by PP2 leads to loss in Akt activation. Such a signaling mechanism could provide a positive feedback loop whereby activated PI3K α phosphorylates Src to initiate further downstream activation of Akt. Although this is an intriguing concept for sustained signaling post-transactivation, our current data do not definitely prove this notion and will need to be the subject of future studies. Our identification of Src regulation by PI3K is significant given the expansive role of Src in mediating cell differentiation, proliferation, motility, and survival. PI3K is a key signaling molecule downstream of G-protein coupled receptors (7TMRs) and regulation of Src by PI3K demonstrates that presence of a hitherto unknown mechanism that could activate Src. These observations are significant given that 7TMRs are involved in regulating a wide-range of responses including cardiac contraction, vision, taste and pain[52].

Our current findings shed new light on the underlying mechanism by which β AR-EGFR transactivation occurs. Our new understanding of the β AR-EGFR transactivation pathway begins with stimulation of the β AR, phosphorylation by GRK, recruitment of β -arrestin associated Src and PI3K γ to the receptor[13, 20, 23]. Once at the receptor, the lipid kinase domain of PI3K produces PIP₃, which stabilizes the β AR-EGFR complex, while the protein kinase domain activates Src by phosphorylating serine residue 70. Once activated, Src activates MMP's that cleave HB-EGF [13, 18] and allow for EGFR stimulation, internalization and activation of downstream signaling (Figure 8).

5. Conclusion

In conclusion, our study identifies PI3K as a critical regulator for β_2 AR-mediated EGFR transactivation by promoting Src function through its protein kinase activity and facilitating β_2 AR-EGFR complex formation through the generation of phosphatidylinositol by its lipid kinase activity. This newly discovered function for PI3K shows how this dual specificity kinase regulates a fundamental biological signaling pathway.

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HIGHLIGHTS

1. PI3K is required for β_2 AR-EGFR Transactivation
2. Protein kinase activity of PI3K phosphorylates Src to induce its activation
3. Lipid kinase activity of PI3K stabilizes β_2 AR-EGFR interaction during transactivation.

Figure 1. PI3K is required for β_2 AR-stimulated EGFR transactivation

HEK-293 cells stably expressing β_2 AR were transfected with EGFR-GFP and pretreated with the PI3K inhibitors, wortmannin (50 nM, 30 mins) or LY-294002 (20 μ M, 30 mins).

These cells were stimulated with either the β AR agonist isoproterenol (ISO, 10 μ M) or the EGFR agonist epidermal growth factor (EGF, 10 ng/ml) for 5 min. **(a)** Confocal microscopy revealed that wortmannin and LY-294002 blocked ISO-stimulated internalization of GFP-EGFR but not EGF-mediated internalization. Scale bar, 20 μ m. Images representative of 3 independent experiments. **(b)** β_2 AR stable cells were also transfected with EGFR-GFP and one of the PI3K mutants (PIK, LK, PK, or LKPK; inset shows the structure of each mutant with amino acids noted). These cells were stimulated with ISO or EGF for 5 min. Confocal microscopy revealed that the LK, PK and PIK mutants blocked ISO-stimulated internalization of GFP-EGFR, whereas LKPK restored internalization. Scale bar, 20 μ m. Images are representative of 3 independent experiments.

Figure 2. PI3K γ mediated phosphorylation of Src enhances Src activity

(a) Schematic diagram illustrating the stable isotope labeling SILAC methodology. HEK-293 cells stably expressing both β_2 AR and HA-Src were grown in the presence (heavy) or absence (light) of ^{13}C labeled lysine and arginine for several passages prior to experimentation. Cells grown in the light medium were pre-treated with the PI3K inhibitor LY-294002 (20 μM , 20 min) then stimulated with ISO (10 μM , 5 min). Cells grown in the heavy medium were treated with only ISO (10 μM , 5 min). Cell lysates were mixed 1:1, HA-Src was immunoprecipitated, trypsin digested and enriched for phosphopeptides, and

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were analyzed using LC-MS. **(b)** Diagram illustrating the PI3K-dependent Src phosphorylation sites determined SILAC-based mass spectrometry experiments. LC-MS identified two distinct peptides with increased phosphorylation in the ISO treated group compared to the LY-294002 + ISO group. These two peptides contained 7 phosphorylatable residues (S35, T37, S39, S43, S70 and S75). **(c)** Sf9 purified recombinant PI3K γ protein was used for in vitro phosphorylation assay using purified bacterial purified WT and Serine to Alanine mutants of Src as substrates. Total Src was stained by Coomassie. Phosphorylation levels were measured by densitometric analysis using ImageJ. Data analyzed with a one-way ANOVA and pairwise multiple comparisons made by Holm-Sidak's test, * $p < 0.02$ vs. WT-Src without PI3K γ , † $p < 0.01$ vs. WT-Src with PI3K γ (n=5). **(d)** Sf9 purified recombinant PI3K γ protein was subjected to in vitro phosphorylation assay using purified bacterial purified WT and Serine to Alanine mutants of Src as substrates. After in vitro phosphorylation, enolase was added as a Src substrate. Phosphorylation of enolase was detected by autoradiography. Total enolase and Src were stained by Coomassie, while total PI3K γ was immunoblotted. Phosphorylation of enolase represented as intensity of the bands measured by ImageJ. Data were analyzed by one way ANOVA and pairwise multiple comparisons made by a Holms-Sidak test, * $p < 0.001$ vs. WT-Src without PI3K γ , † $p < 0.002$ vs. WT-Src with PI3K γ (n=5).

Figure 3. S70A Src mutant inhibits PI3K-dependent Src activity

NIH 3T3 cells transiently transfected with the β_2 receptor, Src biosensor and either WT or S70A Src plasmids were assayed for Src activity by using a FRET based biosensor. (a) Cells transfected with WT Src were responsive to both platelet-derived growth factor (PDGF, 50 nM) and ISO (10 μ M). The response to ISO was blocked by pretreatment with both Src (PP2, 10 μ M, 30 min) and PI3K (wortmannin, 50 nM, 30 min) inhibitors. Data were analyzed using a two-way, repeated measure ANOVA followed by Holms-Sidak multiple comparisons test. $p < 0.001$ for the interaction between WT NS and WT ISO or WT PDGF,

$p < 0.001$ for the interaction between WT ISO vs. WT ISO + PP2 or wortmannin. * $p < 0.01$ WT NS vs. WT ISO or PDGF, † $p < 0.01$ WT ISO vs. WT ISO + PP2 or wortmannin. Data are means $\pm$ SEM. $n = 4$ for each group with 2–4 replicates per n . **(b)** The S70A Src mutation did not affect PDGF stimulated Src activity, but abolished the ISO stimulated increase in Src activity. Data were analyzed using a two-way, repeated measure ANOVA followed by Holms-Sidak multiple comparisons test. $p < 0.001$ for the interaction between S70A NS vs. S70A PDGF. * $p < 0.01$ S70A NS vs. S70A PDGF. Data are means $\pm$ SEM. $n = 4$ for each group with 2–4 replicates per n .

Figure 4. Phosphorylation of serine 70 of Src is required for EGFR transactivation

HEK293 cells stably expressing the β_2 AR were transiently transfected with EGFR-GFP and m-Cherry-tagged Src constructs. Following stimulation with vehicle, EGF (10 ng/ml, 30 mins) ISO (10 μ M, 30 mins) with or without PP2 pretreatment (10 μ M, 30 min pretreatment), the samples were fixed with 4% paraformaldehyde, and confocal images were acquired. (a) Cells transfected with WT Src showed EGFR internalization in response to either EGF or ISO. The Src inhibitor PP2 abolished ISO-induced internalization. Mutation of serine 70 of Src inhibited EGFR internalization upon ISO stimulation without affecting

EGF dependent internalization. **(b)** All other Src mutants had no effect on either ISO-dependent or EGF-dependent EGFR internalization. Images are representative of at least 5 captures from 5 independent experiments. Scale bar 20 μm

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Figure 5. Quantification of EGFR Internalization

HEK293 cells stably expressing the β_2 AR were again transiently transfected with EGFR-GFP and m-Cherry-tagged Src constructs. Following stimulation with vehicle, EGF (10 ng/ml, 30 mins) ISO (10 μ M, 30 mins) with or without PP2 pretreatment (10 μ M, 30 min pretreatment), the samples were fixed with 4% paraformaldehyde, and confocal images were acquired. (a) Cells transfected with WT-Src showed a significant increase in number of internalized cells following ISO or EGF treatment, the ISO induced-internalization was blocked by addition of the Src inhibitor PP2. (b) Mutation of serine 70 of Src inhibited

EGFR internalization upon ISO stimulation without affecting EGF dependent internalization. All other Src mutants had no effect on either ISO-dependent or EGF-dependent EGFR internalization. N values are reported above each column. Data are presented as means $\pm$ SEM and were analyzed by a one-way ANOVA and multiple comparisons made by the Holms-Sidak method. * $p < 0.0008$ vs. NS.

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HEK- β 2AR cells transfected with Flag-EGFR

Figure 6. β AR mediated transactivation of EGFR is Akt independent

β 2AR overexpressing HEK293 cells were transfected with Flag-EGFR. 48 h post transfection cells were serum starved overnight and pretreated with DMSO, wortmannin (PI3K inhibitor 50 nM, 30 min), Tricarbaine (Akt inhibitor 10 μ M, 30 min), or PP2 (Src inhibitor 10 μ M, 30 min). Unstimulated cells, cells stimulated with 10 μ M ISO, or 10 ng/ml EGF for 5 min were lysed, immunoblotted for pEGFR, pAkt and pERK. Blots were stripped and re-probed for EGFR, Akt and ERK. Densitometric analysis of blots was done using ImageJ and represented as bar graphs (n=5–6). The data were analyzed by one-way ANOVA

and pairwise comparisons were made using the Student-Newman-Keuls method. * $p < 0.01$ vs. similarly pre-treated, non-stimulated cells (lanes 1–4).

Figure 7. Lipid kinase activity of PI3K is necessary for maintaining the β₂AR-EGFR complex

In co-immunoprecipitation experiments, HEK-293 cells stably expressing β₂AR were transfected with EGFR and were either transfected with PIK, PI3Kγ, LK, or PK or pretreated with LY-294002 (20 μM, 30 min). These cells were stimulated with ISO (10 μM, 5 min). (a) Both PIK and PI3Kγ markedly blocked ISO-stimulated β₂AR-EGFR complex (n = 4). (b) Similarly, LY-294002 pretreatment significantly reduced β₂AR-EGFR association (n = 4). (c) FRET experiments using HEK-293 cells expressing CFP-β₂AR and YFP-EGFR were stimulated with ISO (10 μM) in the presence or absence of LY-294002

pretreatment (20 μ M, 30min). The cells pretreated with LY-294002 had a markedly reduced FRET signal upon ISO stimulation compared to the cells without pretreatment (n = 3). **(d)** PK blocked β_2 AR-EGFR association, while LK phenocopied the β_2 AR-EGFR association seen in the empty vector positive control. Moreover, adding PIP₃ (0.5 μ M) to saponin treated cells transfected with PK rescued β_2 AR-EGFR association (n = 4). Data are presented as means $\pm$ SEM and were analyzed by a two-way ANOVA and multiple comparisons were made using the Holm-Sidak method. * p<0.05 vs. (–) ISO.

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Figure 8. Schematic depicting the role of PI3K in β_2 AR stimulated EGFR transactivation

Agonist-dependent receptor stimulation leads to GRK phosphorylation of the C-terminal tail and β -arrestin recruitment. PI3K generates PIP₃ to recruit AP-2 and clathrin and stabilize the β_2 AR-EGFR complex. PI3K also phosphorylates Src to increase its activity. Src activates MMP-mediated HB-EGF shedding, resulting in EGFR activation and downstream signaling.

Table 1

Complete list of Src phosphorylation sites identified in the SILAC-based mass spectrometry experiments.

Phosphorylation Site	Ascore	Relative abundances ISO/ISO + LY-294002	
		Fold Increase	% Decrease
S17/S35, T37, S39, or S43	89.2/3.5	↑ 9.60-fold	
S70/S75	21.3/26.4	↑ 3.50-fold	
T182 or T183	11.7	↑ 2.15-fold	
S75	26.5	↑ 1.95-fold	
T74	28.9	↑ 1.93-fold	
S212	55.1	↑ 1.86-fold	
Y93	64.8	↑ 1.80-fold	
T218	24.4	↑ 1.74-fold	
Y232	38.4	↑ 1.59-fold	
S104	73.3	↑ 1.57-fold	
T88	20.4	↑ 1.54-fold	
S225	31	↑ 1.42-fold	
Y187	88.3	↑ 1.32-fold	
Y216	51.6	↑ 1.29-fold	
T420	28.9	↑ 1.18-fold	
S17	72.3		↓ 1%
Y439	81.6		↓ 14%
Y338	44.1		↓ 18%
T72	13.3		↓ 19%
S69	16.2		↓ 19%
S70	0		↓ 26%
S51			↓ 52%

Table 2

Src biosensor results for all identified PI3K dependent Src phosphorylation sites. N = 3–4/group.

Treatment		NS		PDGF		ISO	
Src Plasmid		Pre-Treat	End	Pre-Treat	End	Pre-Treat	End
WT n=4		1.01	1.02	1.02	1.14 *	1.02	1.14 *
SEM		0.001	0.014	0.005	0.016	0.006	0.028
S35 n=3		0.99	0.99	1.00	1.16 *	1.01	1.16 *
SEM		0.008	0.019	0.011	0.045	0.006	0.032
T37 n=3		1.00	1.00	1.01	1.15 *	0.99	1.15 *
SEM		0.015	0.004	0.019	0.024	0.011	0.010
S39 n=3		0.99	1.01	1.01	1.17 *	1.02	1.15 *
SEM		0.005	0.003	0.019	0.011	0.015	0.017
S43 n=3		1.01	0.97	1.00	1.22 *	1.02	1.15 *
SEM		0.003	0.032	0.015	0.067	0.014	0.029
S70 n=4		1.00	1.00	1.01	1.12 *	1.00	1.03
SEM		0.011	0.005	0.013	0.032	0.002	0.020
S75 n=3		0.99	0.97	1.00	1.14 *	1.01	1.13 *
SEM		0.006	0.022	0.010	0.012	0.011	0.013

Data were analyzed by two-way repeated measures ANOVA followed by Sidak's multiple comparisons test.

* denotes $p < 0.01$ vs. pre-treatment value.