Src Docks to A-kinase Anchoring Protein Gravin, Regulating β2-Adrenergic Receptor Resensitization and Recycling*

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Gravin (AKAP12) is a membrane-associated scaffold that provides docking for protein kinases, phosphatases, and adaptor molecules obligate for resensitization and recycling of β2-adrenergic receptors. Gravin binds to the cell membrane in a Ca2+-sensitive manner and to receptors through well characterized protein-protein interactions. Although the interaction of serine/threonine, cyclic AMP-dependent protein kinase with protein kinase A-anchoring proteins is well described and involves a kinase regulatory subunit binding domain in the C terminus of these proteins, far less is known about tyrosine kinase docking to members of this family of scaffolds. The non-receptor tyrosine kinase Src regulates resensitization of β2-adrenergic receptors and docks to gravin. Gravin displays nine proline-rich domains distributed throughout the molecule. One class I ligand for Src homology domain 3 docking, found in the N terminus of gravin, is shown to bind Src. Binding of Src to gravin activates the intrinsic tyrosine kinase of Src. Mutagenesis/deletion of the class I ligand (P15A,P16A) on the N terminus of gravin abolishes both the docking of Src to gravin as well as the receptor resensitization and recycling catalyzed by gravin. The Src-binding peptide-(1–51) of gravin behaves as a dominant-negative for AKAP gravin regulation of receptor resensitization/recycling. The tyrosine kinase Src plays an essential role in the AKAP gravin-mediated receptor resensitization and recycling, an essential aspect of receptor biology.

A-kinase anchoring proteins (AKAP)2 are molecular scaffolds critical for proper cell signaling (1). Gravin (aka AKAP250, AKAP12, and SSeCKS) is a 250-kDa AKAP displaying the ability to dock protein kinases A (PKA) and C (PKC), phosphoprotein phosphatases (such as PP2B), adaptor molecules, as well as a prominent member of the superfamily of G protein-coupled receptors (GPCR), the β2-adrenergic receptor (β2AR) (2).

Gravin interacts with the β2AR primarily through a conserved receptor-binding domain (RBD) of the scaffold, whose interaction with the cytoplasmic, C-terminal tail of this GPCR is enhanced by protein phosphorylation catalyzed by the PKA itself docked to the scaffold (3). This receptor-scaffold interaction is obligate for resensitization and recycling of β2AR following classic agonist-induced desensitization and internalization (4). Gravin associates with the inner leaflet of the cell membrane through two types of domains, in addition to the RBD through which it interacts with a heptihelical GPCR (5): i.e. an N-myristoylation site (6); and, three small, positively charged basic domains that bind to negatively charged phospholipids with high affinity (7, 8). Like the RBD-GPCR interaction that is dynamic and regulated by PKA phosphorylation, the cell membrane/positively charged domain-based interactions of gravin are dynamic, in this case readily reversible by increasing the intracellular concentration of Ca2+ in the presence of calmodulin (8).

The role of serine/threonine protein kinases, such as PKA and PKC, in agonist-stimulated desensitization and internalization of members of the GPCR is well known (9). Gravin as well as AKAP79 has been shown to dock protein kinases PKA, PKC, as well as the β2AR, participants in agonist-stimulated receptor desensitization and internalization (10, 11). Far less is known about the docking of members of another major family of protein kinases, the tyrosine kinases, to AKAP scaffolds. Inhibitors of the non-receptor tyrosine kinase Src (e.g. PP2) as well as expression of dominant negative versions of Src have been shown to display complex effects of regulation of GPCRs (12). Src family kinases can act as direct effectors of GPCR signaling, interacting with GPCRs (13–16), G-protein subunits (17, 18), and β-arrestin (19). Src has been shown to participate in macromolecular complexes with AKAPs and β2AR (4, 16). As gravin has been shown to be a scaffold obligate for the resensitization/recycling of β2AR and Src has been shown to effect both the internalization and recycling arms of receptor trafficking, we test the hypothesis that the AKAP gravin acts as a scaffold for Src regulation of receptor trafficking. We show that Src docks to gravin through a class I ligand for the SH3 domain found in the far N terminus of the scaffold and that its docking and activation are essential for the ability of the AKAP to resensitize and recycle to the cell membrane β2AR that have been internalized in response to agonist-induced desensitization.

EXPERIMENTAL PROCEDURES

HA-tagged Fusion Proteins—To isolate and quantify gravin and gravin fragments, fusion proteins with the hemagglutinin
antigen (YPDYVPDHYL, HA) on the N terminus were created. The HA-tagged fusion proteins of full-length gravin, N-terminal-truncated, C-terminal-truncated, and other alanine-substituted gravin mutants were constructed as previously described (3) and inserted into the expression vector pCDNA3. To generate HA-tagged gravin in which amino acids 1–51 are deleted (gravin Δ1–51), the sense primer was engineered to contain a 5' NheI site and nucleotides encoding the HA tag followed by nucleotides corresponding to 154–174. The antisense primers synthesized correspond to the BamHI restriction site in gravin. The PCR products were subcloned into HA-gravin pcDNA3 between NheI and BamHI restriction sites.

The proline to alanine substitution mutants of HA-gravin were engineered according to the standard protocol for PCR-mediated mutagenesis. All of the polymerase chain reactions were performed using Pfu polymerase (Stratagene, La Jolla, CA). The identity of the amplified sequences was confirmed by direct DNA sequencing.

Cell Culture—Human epidermoid carcinoma cells (A431) from the ATCC collection were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin (60 μg/ml), and streptomycin (100 μg/ml) and grown in a humidified atmosphere of 5% CO2 and 95% air at 37 °C.

Immunoprecipitation and Immunoblotting Studies—For most studies, A431 cells were transiently transfected with an expression vector harboring the cDNA or an HA-tagged protein. Cells were harvested and treated with a lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 10 mM dithiothreitol, 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 KH2PO4, 10 mM sodium molybdate, and 20 mM Tris-HCl, pH 7.4) at 4 °C for 20 min to promote cell lysis. After centrifugation of the cell debris at 10,000 × g for 20 min, the supernatant was added to a final 30–μl reaction containing the Src/ATP mixture, 10 μCi of [γ-32P]ATP, and 150 μM of the Src kinase substrate (KVEKIGEGTYGYVVK, amino acids 6–20 of p34<sup>Src</sup>). After a 30-min incubation at 30 °C, the reactions were terminated with 20 μl of 40% trichloroacetic acid. A 25–μl aliquot of each reaction mixture was then spotted onto P81 paper blot. The assay blots were washed 5 times for 5 min each with 0.75% phosphoric acid, followed by a final, 3-min wash with acetone. The assay blots were air-dried and transferred to scintillation vials for counting.

Knockdown of Gravin with Antisense Morpholinos—Antisense morpholino oligonucleotides (morpholinos) were designed, synthesized, and purified to cell culture grade (Gene Tools, LLC). The morpholinos and protocol for knockdown (KD) of cellular gravin expression were optimized and reported earlier (3, 8). The extent of the suppression of gravin expression by antisense morpholinos in these studies was >75 ± 6.3%. The antisense morpholinos were designed to target the 5'-untranslated region of the mRNA for native gravin and do not recognize gravin mRNA transcribed from the expression vector. Prior to their addition to A431 cell cultures, morpholinos were mixed in a ratio of 1:1 (w/w) with EPEI special delivery solution (Gene Tools, LLC). Cells were treated with the anti-gravin morpholinos (5 μg/ml) for 3 days. Whole cell lysates of the morpholino-treated cells were subjected to SDS-PAGE and the resolved proteins were blotted and stained with anti-gravin antibody. Under standard conditions, morpholinos antisense for gravin suppressed the cellular level of the AKAP by more than 90% (8). An additional treatment with morpholinos antisense to gravin was performed prior to transient transfection of the cells with either wild-type (WT) or mutant forms of gravin. The morpholinos are designed to suppress the expression of endogenous gravin, whereas not interfering with the gravin expressed through use of mammalian expression vectors. Following this protocol, cells were analyzed for β-adrenergic agonist-induced (i.e., Iso, 10 μM) desensitization and internalization of β2AR, as well as the recovery (i.e., resensitization and recycling of β2AR) after washout of agonist.
Desensitization and Internalization of β2AR—Two days prior to the analysis of agonist-induced desensitization, the A431 cells were seeded in 96-well microtiter plates at a density of 25,000–50,000 cells/well. Routinely cells were serum-starved overnight, prior to the analysis. Desensitization was accomplished by pretreating the cells with the β-adrenergic agonist Iso (10 μM) for 30 min. Under these conditions, subsequent β-adrenergic stimulation of cyclic AMP accumulation is severely blunted (i.e. desensitized) and cell surface-localized β2AR is reduced precipitously by internalization (9). Further details of the desensitization protocol employed herein as well as the assay of intracellular accumulation of cyclic AMP are described elsewhere (4).

Analysis of β2AR Localization and Recycling to the Cell Membrane—The internalization of β2AR correlates well with the extent of agonist-induced desensitization observed by assay of intracellular accumulation of cyclic AMP. Using radioligand equilibrium binding assays with A431 cells and a cell-impermeant, tritiated antagonist ([3H]CGP-12177), the complement of cell surface-localized β2ARs as well as internalized β2AR (by subtraction) was determined. Cultures of A431 cells were treated with Iso for 30 min (i.e. “desensitized and internalized”) or treated with Iso for 30 min then washed free of agonist for 60 min (i.e. “resensitized and recycle”). The cells were then washed with ice-cold phosphate-buffered saline and resuspended in Dulbecco’s modified Eagle’s medium containing 20 mM HEPES, pH 7.4, and the hydrophilic, membrane-impermeant β2-adrenergic antagonist [3H]CGP-12177 (70 nM). Binding was performed at 4 °C for 6 h. The cells then were diluted with cold buffer, collected on GF/C membranes at reduced pressure, and washed twice in rapid succession. The amount of radioligand bound to the washed cell mass collected on the filter provides a direct assay of the cell surface complement of receptors. The amount of bound ligand was quantified by liquid scintillation spectrometry (4, 22).

Statistical Analysis—The experiments were performed at least in triplicate. All data are expressed as mean ± S.E. for at least three separate experiments. Statistical significance (p value of <0.05) is denoted with an asterisk and is derived from comparison of experimental data with the respective controls by two-way analysis of variance for repeated measures. The InStat statistics program (GraphPad, San Diego, CA) was used for statistical computations.

RESULTS

Src Docks to the N-terminal Region (1–200) of AKAP Gravin—Human A431 epidermoid carcinoma cells were employed in this investigation as these cells display 35,000–65,000 β2AR/cell and provide a well studied model of agonist-stimulated receptor desensitization and trafficking (23), a hallmark of members of the superfamily of GPCRs (24). Earlier, it had been shown that inhibition of Src activity alters agonist-induced desensitization (15), whereas, little is known about a role of Src, if any, in resensitization and trafficking of GPCRs. We investigated first the effects of the Src family inhibitor 4-amine-5-(4-chlorophenyl)-7-(t-buty1)pyrazolo[3,4-d]pyrimidine (PP2) on resensitization of desensitized, internalized β2AR, a process catalyzed by the AKAP gravin (Fig. 1). Cells were treated with β-adrenergic agonist for 30 min to stimulate β2AR internalization. The cells were washed free of agonist and allowed to recycle the internalized β2AR back to the cell membrane, a process that could be followed through equilibrium binding experiments with intact A431 cells and use of a radioligand [3H]CGP-12177, a cell-impermeant ligand able only to bind to those receptors accessible to the bulk solution, i.e. on the exterior of the cell membrane (Fig. 1). The amount of β2AR internalized within 30 min of Iso (10 μM) treatment was ~50% (+ Isoproterenol) and the amount of β2AR that remains internalized 60 min following the washout of agonist (W60) was determined. Washout of agonist for 60 min provokes complete recovery of the internalized β2AR trafficking to the cell membrane. When cells are treated with the Src family inhibitor PP2 (50 nM), in contrast, the recycling of internalized β2AR is markedly attenuated, i.e. sequestered β2AR remains internalized. These data identify a critical role of Src in the recycling of agonist-internalized β2AR, a process known to be gravin-dependent.

We searched the protein sequence of human gravin scanning for proline-rich sequences (PRS) to which a Src homology 3 (SH3) domain might bind. The putative ligands for SH3 domains are displayed along with several well characterized landmarks in gravin (Fig. 2A). Nine PRSs predicted to bind SH3 were distributed throughout the molecule, the first found in the N terminus of 1PXXP 15 and the ninth found at 1PXXP 147, in close proximity to the highly conserved PKA RII subunit binding site (1540–1553) in the C terminus (residue 1783) of this AKAP. Any one or more of these nine PRSs may bind Src and play a role...
in Src-dependent recycling of internalized β2AR. To facilitate analysis of these putative SH3 binding domains, we expressed gravin and targeted fragments of gravin as fusion proteins tagged with the HA antigen. The HA tag enabled isolation and quantification of expression of full-length and mutant gravin fragments (Fig. 2B).

Full-length HA-gravin and HA-gravin fragments were expressed in A431 cells, harvested from whole cell lysates of the clones, subjected to immune precipitation with anti-HA antibody followed by SDS-PAGE, transferred electrophoretically to nitrocellulose blots, and stained with anti-HA antibodies (Fig. 2C). Staining of the blots with anti-HA antibody allows comparison of the relative levels of expression of HA-tagged gravin versus that of the gravin fragments. To test for Src binding to gravin-based complexes, we probed the immune complexes of gravin pulldowns (or pulldowns of gravin fragments) by anti-HA antibody, subjected to SDS-PAGE, blotting, and staining with anti-Src antibodies (Fig. 2C). Immune complexes of full-length HA-tagged gravin-(1–1783) stained positive for Src (Fig. 2C). Immune complexes obtained of each expressed gravin fragment that harbored the N terminus of the AKAP (e.g. 1–200, 1–365, 1–652, and 1–938) also stained positive for Src. Immune complexes of gravin fragments lacking the N terminus (e.g. 840–1782 and 554–938), in sharp contrast, displayed no positive staining for Src. We further refined the analysis of the N-terminal region, making use of additional deletions. This deletion analysis revealed that the Src binding domain of gravin occurs in the first 51 residues of the scaffold (Fig. 2C). Immune complexes of N-terminal truncates HA-(52–652) gravin and HA-(52–1782) gravin do not stain positive for Src, whereas those of HA-tagged gravin stain prominently for Src binding (Fig. 2C), focusing attention on the N-terminal PRSs of gravin-(1–51) as the site(s) of Src binding.

Inspection of the protein sequence of the N-terminal 1–51 region of gravin reveals two PRS, both potential sites for Src binding (Fig. 3). The more N-terminal of the two sites with the sequence 104RXPPXP15 is a classic type 1 ligand for SH3 domains (25). Addition of a C-terminal Pro residue (Pro146) may influence the interaction with an SH3 domain (25). The second of the two N-terminal sites with the sequence 224PXXPXP27 may also dock an SH3 domain. The next PRS to be encountered is at 104RXPPXP15 and six more are found C-terminal in the 51–1783 primary sequence. The deletion of the gravin-(52–1783) region that harbors seven PRS did not alter the ability of the gravin to dock Src. If the results from the Src binding studies to gravin and its fragments are correct, either the loss (truncations) or mutagenesis of the N-terminal region harboring the two PRS should influence gravin biology. This premise was tested functionally using two independent assays of Src binding function.

The N-terminal Region (1–51) of AKAP Gravin Is Obligate for Gravin Function—Gravin has been shown to function primarily in the resensitization and recycling of desensitized, internalized β2AR (26, 27). To probe the functional role of the 1–51 N terminus of gravin, we measured in a time course the ability of a washout of agonist (Iso, 10 μM) to reverse the desensitization of the cyclic AMP response (Fig. 4A). In the control, Iso stimulates a maximal desensitization within 5 min, desensitization persists for 30 min. Following the washout of agonist, the amplitude of the desensitization declines by more than 70% within 30 min (W30, not shown) and declines by more than 90% within 60 min (W60, Fig. 4A).

Next we sought to examine the resensitization response of A431 cells made deficient in gravin. Cells treated with morpholinos antisense to human gravin (targeting the 5′-untranslated region of gravin mRNA) for 3 days displayed a knockdown of more than 75% of the cellular complement of gravin (gravin KD, Fig. 4A). In the gravin-deficient cells, desensitization in response to Iso proceeded normally, whereas the resensitization of the cyclic AMP response to Iso was abolished, even following a 60-min washout (W60) of agonist, when recovery is normally complete. Transient transfection of the gravin KD cells with an expression vector harboring WT gravin (not susceptible to suppression by the antisense morpholinos) effectively rescues the desensitization response absent in gravin-deficient cells (Fig. 4A). In sharp contrast, if the gravin-deficient cells were transfected with the same expression vector harboring the Δ1–51 truncate of gravin, no such rescue was observed. These data suggest that unlike the case for the gravin, expression of the Δ1–51 gravin truncate is not able to rescue the resensitization of the β2AR in cells made deficient of gravin.

**FIGURE 2. Docking of Src to AKAP gravin occurs in the N-terminal region of the scaffold.** A, topological schematic of PXXP sites identified in the sequence of full-length human gravin. Landmarks for N-myristoylation (N-myrist site), positively charged domains that participate in calcium-sensitive docking of gravin to the inner leaflet of the cell membrane (positively charged domains), dynamic association of the scaffold with the GPCR β2AR through the RBD, and PKA binding through the RII-binding domain, are shown for context of PXXP sites. B, schematic of gravin fragments employed to detect Src docking sites. HA-tagged full-length gravin (A), C-terminal truncates, as well as N-terminal truncates of gravin were engineered and then expressed in A431 cells to ascertain which of the 9 PXXP sites actually bind Src. C, to investigate Src-binding sites of gravin, A431 cells were transfected with expression vectors harboring HA-gravin or truncated gravin fragments. After 2 days, the cells were harvested and whole cell lysates prepared and then incubated with antibodies against HA tag that were covalently coupled to protein A/G-agarose beads. The immune complexes were collected and subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with an antibody specific for either Src or HA tag. The data presented are representative of at least three separate determinations performed with separate cell lysates. The position of the position of molecular mass standards (kDa) are displayed at the immediate left and the conditions (IP, immune precipitation; IB, immunoblotting) on the far left column.
Thus, KD of gravin by antisense morpholinos, like treatment with the Src family inhibitor PP2 (Fig. 1), blocks the resensitization of the desensitized β2AR (Fig. 4A).

We tested further, using an independent assay, the conclusions derived from the analysis of β2AR resensitization, i.e. that the loss of the 1–51 N-terminal region of gravin abolishes the ability of the AKAP to support the resensitization response. To assess function as recovery (i.e. “recycling”) of agonist–internalized β2AR, we first treated cells with Iso (10 μM for 30 min; +Iso) to promote agonist-induced desensitization and internalization of β2AR (Fig. 4B). Cells made deficient of gravin (gravin KD) fail to display recycling of agonist-stimulated, internalized β2AR, as measured by radioligand binding experiments with the cell-impermeant β-adrenergic antagonist [3H]CGP-12177, a radioligand that can access only cell-surface-localized β2AR. β2AR internalized in response to β-adrenergic agonist is recycled back to the cell membrane within 60 min of a washout of the agonist (W60). At 60 min post-washout of Iso, gravin-KD cells failed to recycle internalized β2AR (Fig. 4B).

The ability of expression of WT gravin versus that of the Δ1–51 mutant gravin to rescue the recycling of internalized β2AR that is absent in gravin-KD cells was investigated. Expression of gravin, but not the Δ1–51 gravin truncate, reconstitutes the ability of the gravin-deficient cells to recycle the internalized β2AR (Fig. 4B). These observations are in agreement with the results obtained in the direct assay of accumulation of intracellular cyclic AMP for resensitization of the β-adrenergic responsiveness (Fig. 4A). Thus, the functional capacity of this AKAP (resensitization and recycling of β2AR) requires at least one or perhaps both PRSs in the N-terminal region.

Src Binds to and Functions through a Type I Class of SH3 Ligand Sequence of Gravin—The presence of two potential sites of Src docking in the N-terminal region of gravin fostered further analysis aimed at establishing if the 12PlXXP15 (site 1), the 22PlXXP25 (site 2), or both, were bona fide ligands for the SH3
domain of Src. We engineered mutant forms of gravin with Pro to Ala substitutions at Pro^{15-16} and Pro^{22}, employing a double mutation on site 1 (P15A,P16A) to ensure that the site was disrupted. A431 cells were transfected with expression constructs harboring either the P15A,P16A HA-tagged gravin or the P22A HA-tagged gravin or the full-length wild-type HA-gravin (Fig. 5A). Immune complexes of the HA-tagged proteins were prepared from whole cell lysates, subjected to SDS-PAGE, and the resolved proteins transferred to blots. Staining of the blots of the immune complexes of HA-gravin with anti-Src antibody revealed, as shown earlier, positive staining for Src. Those immune complexes prepared from the HA-tagged P22A mutant also stained positive for Src. Immune complexes prepared from the HA-tagged P15A,P16A mutant, in contrast, did not stain positive for Src. The most N-terminal PRS in gravin, a class I type ligand for SH3 interactions, binds Src. We further tested these observations by direct assays of gravin function.

As was observed for the gravin mutants devoid of the N terminus harboring^{12}PXXP^{15}, targeted mutation of the class I type ligand for SH3 docking demonstrates no capacity to rescue either agonist-induced resensitization of the cyclic AMP

subjected to SDS-polyacrylamide gel electrophoresis. The resolved proteins were transferred to nitrocellulose blots and the blots probed with antibodies against Src or the HA tag. The data presented are representative of three or more separate determinations performed with as many separate cell lysates.

B, resensitization of β2AR-mediated cyclic AMP accumulation in A431 cells desensitized by 30 min treatment with Iso was examined as described in the legend to Fig. 4. A431 cells treated with either control, scrambled sequence morpholinos (Control) or with morpholinos antisense to gravin to knock down (KD) the expression of endogenous gravin. The antisense morpholino-treated gravin KD cells were untreated further (gravin-KD) or transiently transfected with an expression vector harboring an HA-tagged P15A,P16A gravin mutant (gravin KD + P15A,16A gravin), HA-tagged P22A gravin mutant (gravin KD + P22A gravin), or an HA-tagged full-length, wild-type gravin (gravin KD + WT gravin). Isoproterenol-stimulated cyclic AMP accumulation was measured in untreated cells (0), cells were first challenged with 10 μM Iso for 30 min (30), and in cells first treated with Iso and then washed free of agonist and incubated for 60 min (W60), as detailed in the legend to Fig. 4. Isoproterenol-stimulated cyclic AMP accumulation was measured in these cells and is reported as picomoles of cyclic AMP accumulated per 10^6 cells. The results, displayed as mean ± S.E., are derived from at least three separate experiments performed with as many separate cultures of A431 cells. *, p < 0.01 for the difference from the amount of cyclic AMP accumulation measured in the control cells at 60 min following a washout of the agonist (W60). Panel C, cell surface β2AR in A431 cells were measured in untreated cells (0), cells desensitized by 30 min treatment with 10 μM Iso (30), and cells treated with agonist, washed free of agonist, and incubated for 60 min to permit resensitization (W60), as assayed using [3H]CGP-12177 radioligand binding. A431 cells treated with either a control, scrambled sequence morpholino (Control) or with morpholinos antisense to gravin to knock down (KD) the expression of endogenous gravin. The antisense morpholino-treated gravin KD cells were either not transfected (gravin-KD) or transiently transfected with an expression vector harboring an HA-tagged P15A,P16AAla gravin mutant (gravin KD + P15A,16AAla gravin), HA-tagged P22A gravin mutant (gravin KD + P22A gravin), or an HA-tagged full-length, wild-type gravin (gravin KD + WT gravin). Cells were challenged with a β2-adrenergic agonist (Iso, 10 μM) for 30 min to provoke agonist-induced internalization of β2AR. The recovery from agonist-induced internalization of β2AR, termed recycling, was measured in cells at 60 W60 min following the washout. Cell surface β2ARs were quantified by [3H]CGP-12177 radioligand binding at 0 min (0, set as 100%), in cells following a 30-min challenge with 10 μM Iso (30, desensitized), and in cells challenged with Iso for 30 min, washed-free of agonist, and incubated for 60 min following the washout (W60, resensitized). The results, displayed as mean ± S.E., are derived from at least three separate experiments performed with as many separate cultures of A431 cells. *, p < 0.01 for the difference from the amount of receptor binding measured in the control cells at 60 min following a washout of the agonist (W60), reflecting the resensitization and recycling of the receptors to the cell membrane. IB, immunoblot; IP, immunoprecipitate.
FIGURE 6. Src activity in vitro is increased by addition of the proline-rich N-terminal fragment of AKAP gravin. A, time course of Src docking to AKAP gravin in response to stimulation with Iso. Wild-type A431 cells were either untreated (time 0) or stimulated with the β-adrenergic agonist Iso (10 μM) for up to 60 min and sampled at the times indicated for the binding of Src to the AKAP gravin. The assay of Src binding to gravin was performed by analysis of pulldowns of gravin for Src binding as described in the legend to Figs. 2 and 4 (above). The amount of Src bound was determined by absorbance scanning densitometry of the blots, setting the zero time as 1.0. These data are presented as the mean ± S.E. from at least three separate experiments. B, Src activity was assayed in whole cell lysates of A431 cells supplemented either without or with purified fragments of AKAP gravin, as described under “Experimental Procedures.” The assay reaction buffer for Src-catalyzed phosphorylation contained a sample of whole cell lysates plus a sample (2.5 μg of protein) of either purified gravin-(1–231) fragment (harboring PXXP sites 1 and 2) or purified the gravin-(52–231) fragment (harboring no PXXP sites). The AKAP fragments were expressed in E. coli and purified. The assay was performed without (Basal) and with the lysates (+ lysates), supplemented with or without the purified gravin fragment, and with or without Src inhibitor PP2 (50 nM). The Src activity obtained in the assay in the absence of gravin peptides or PP2 inhibitor was set as 100%. The results are displayed as mean ± S.E. from three separate experiments, each performed with different cell lysates. The values of the Src activity (cpm) measurements were as follows: lane 1, 3,515 ± 800; lane 2, 71,496 ± 780; lane 3, 34,646 ± 2,418; lane 4, 113,287 ± 3,676; lane 5, 45,998 ± 5,997; lane 6, 72,568 ± 2,399; lane 7, 36,680 ± 2,273. C, assay of gravin-associated Src activity in pull-downs from A431 cells. A431 cells were transiently transfected with HA-tagged full-length gravin. The cell extracts were subjected to immune precipitation (IP) pull-down assays with antibody against the HA-tag (+) or A/G-agarose beads (−), as a control. The immune precipitates were then washed with Src activity assay buffer, then incubated in a phosphorylation reaction at 30 °C for 30 min in assay buffer containing 10 μCi of ATP (0.5–2.0 × 10^15 cpm/mol) and with or without 1 unit of purified human Src. At the end of the phosphorylation reaction, the immunoprecipitated proteins were washed 2 times, collected, denatured, and subjected to SDS-PAGE and autoradiography. Separate immune precipitation complexes were subjected to SDSPAGE and the resolved proteins transferred to blots that were stained with antibodies against either HA or Src. *, p ≤ 0.05 for the difference from the zero time (A), from the Src activity in the whole cell lysates (B), from the Src activity in the absence of gravin immunoprecipitated from cells not treated with Iso (C), and from the basal level of phosphorylation of gravin in the absence of added, exogenous purified Src (D), respectively.
response (Fig. 5B) or the recycling of agonist-induced internalization β2AR back to the cell membrane (Fig. 5C) in gravin-KD cells. Whereas gravin deficiency blocks both responses (Fig. 4, A and B), the expression of the P22A gravin, like that of gravin, but not that of the P15A mutant (Fig. 5, B and C), was able to rescue each of these functional responses absent in gravin-KD cells.

**Gravin Catalyzes Activation of Src in vitro**—A time course for the docking of Src to gravin was conducted (Fig. 6A). In the basal state, a significant amount of Src is bound to gravin. Isoproterenol treatment of the A431 cells promotes enhanced Src association with the AKAP, peaking at about 30 min following challenge with agonist (Fig. 6A).

If Src docks to the class 1 ligand sequence of gravin, then one might expect a change in the activity of Src reflecting release from the autoinhibitory control of this tyrosine kinase. We probed this premise by testing the ability of purified gravin fragments to activate Src in vitro employing whole cell lysates of A431 cells. An N-terminal fragment of gravin that harbors the N-terminal 12PXXP site (i.e. gravin 1–231) as well as a gravin fragment devoid of the N-terminal 51 residues (i.e. gravin 52–231) were expressed in bacteria, purified, and employed in these experiments. Each of the fragments was added directly to whole cell lysates and the Src activity measured using a Src-specific substrate (amino acids 6–20 of p34cdc2, Fig. 6B). The peptides were added to the lysates at equivalent molar concentrations and the amount of Src activity determined. Src activity was observed (set to 100% control) in the whole cell lysates (Fig. 6B). Inclusion of the Src family inhibitor PP2 suppresses the activity of Src by ~50%. Addition of purified gravin-(1–231) fragment harboring the N-terminal 12PXXP site stimulates increased Src activity (Fig. 6B). This increased Src activity stimulated by the addition of the gravin fragment harboring the N-terminal 12PXXP also was sensitive to inhibition by PP2. Addition of the purified 1–51 truncate of the gravin-(1–231) fragment (i.e. 52–231) to the whole cell lysates, in contrast, did not alter Src activity (Fig. 6B). Although competing with endogenous gravin present in these whole cell lysates, the purified gravin-(1–231) fragment added was able to stimulate Src increased activity, providing support for the notion that docking of Src to the AKAP scaffold leads to activation of Src. This observation is in agreement with the mechanism by which class 1 ligands of SH3 domains would release Src from autoinhibition (28).

We also sought to determine whether the pulldowns from A431 cells of gravin that bind Src display Src activity (Fig. 6C). Immune complexes of gravin were prepared from A431 cells and the Src activity measured using the Src-specific substrate. Pulldown reactions performed with A/G-agarose chemically coupled with anti-HA antibody (+) or with uncoupled A/G-agarose (as a control (−)) from A431 whole cell lysates were assayed for Src activity. Increased Src activity was observed in the gravin pulldowns from A431 cells (Fig. 6C). This increased Src activity associated with gravin also was effectively inhibited by PP2.

An in vitro phosphorylation reaction was employed to examine if addition of purified Src to pulldowns of HA-tagged gravin from whole cell lysates catalyzed phosphorylation of gravin itself (Fig. 6D). Purified Src was added to the pulldowns of gravin from whole cell lysates, the phosphorylation reaction was performed, and the reaction mixture subjected to SDS-PAGE, electrophoretic blotting, and autoradiography. Phosphorylation of gravin was readily increased by the addition of purified Src (Fig. 6D), suggesting that gravin docks and activates the Src. The gravin scaffold itself docks Src and also appears to act as a substrate for Src, in a manner very similar to that in which gravin docks PKA and also acts as a substrate for PKA (3).

We investigated if gravin played an essential role in tyrosine phosphorylation of the β2AR (Tyr150) observed in response to stimulation by Iso (29, 30). Suppression of Src as well as treatment with PP2 can suppress tyrosine phosphorylation of the β2AR that occurs rapidly in response to stimulation with Iso (31). Unlike the effects of Src on resensitization/recycling of β2AR observed 30–60 min after the desensitization and internalization response, tyrosine phosphorylation of the β2AR in response to Iso peaks within 15 min (Fig. 7A). To test whether or not the AKAP gravin (which binds Src) participates in agonist-stimulated tyrosine phosphorylation of the β2AR, we performed the same experiments in gravin-KD cells. The agonist-stimulated increase in the phosphotyrosine content of the β2AR was unaffected by the deficiency of gravin (Fig. 7B). Src functioning in the early phase of β2AR activation/phosphorylation thus may be docked elsewhere in the complex, not at gravin.

**Expression of the Src-binding Domain of AKAP Gravin Acts as a Dominant-Negative of β2AR Resensitization and Recovery**—We explored whether or not expression of the Src-biding domain of gravin would influence the ability of the scaffold to function in β2AR resensitization. HA-tagged gravin-(1–51) fragment was expressed transiently in A431 cells and
resensitization of β2AR-mediated. Iso-stimulated cyclic AMP accumulation was investigated following agonist-induced desensitization (Fig. 8A). Expression of gravin-(1–51) yields a dominant-negative effect on the resensitization process of β2AR-mediated cyclic AMP accumulation following Iso-induced desensitization. Resensitization of the β2AR-mediated response was essentially blocked in cells expressing the HA-tagged gravin-(1–51) fragment. Similarly, we tested the effect of expression of the gravin-(1–51) fragment on the ability of desensitized, internalized β2AR to recycle to the cell membrane (Fig. 8B). Expression of the gravin-(1–51) fragment likewise abolished recycling to the cell membrane of β2AR internalized in response prior to agonist treatment. These two independent sets of data argue for a critical role of the N-terminal Src-binding domain of gravin for the proper resensitization and recycling of β2AR. We also explored if the expression of the gravin RBD (554–938, Fig. 2A), like that of the Src-binding domain (Fig. 3), would act as a dominant-negative in assays of β2AR resensitization (Fig. 8A) and recycling (Fig. 8B), post-agonist-induced desensitization and internalization. Expression of the RBD of gravin was found to block the resensitization of the desensitized, β2AR-mediated cyclic AMP response as well as the recycling of the internalized β2AR.

**DISCUSSION**

In the current work we uncover a new dimension of the complex role for Src in the biology and trafficking of G protein-coupled receptors (12). The demonstration of a prominent role of AKAP gravin in resensitization and recycling of internalized β2AR (11) fostered our thinking that the enigmatic effects of Src inhibitors on resensitization/recycling of β2AR (16, 31, 32) might reflect a role of the scaffold in this aspect of the overall trafficking of receptors. Inspection of the protein sequence of receptor binding domain (554–938, Fig. 2) of gravin (HA-gravin RBD). Cells were challenged with a β-adrenergic agonist (Iso, 10 μM) for 30 min to provoke agonist-induced desensitization and internalization of β2AR. The recovery from agonist-induced desensitization, termed resensitization, was measured in cells after a 30-min challenge with Iso (30), and in Iso-treated cells that were washed free of agonist for 60 min (W60). Isooproterenol-stimulated cyclic AMP accumulation was measured in these cells and is reported as picomoles of cyclic AMP accumulated per 10^6 cells. The results, displayed as mean ± S.E., are derived from at least three separate experiments performed with as many separate cultures of A431 cells. *p ≤ 0.01 for the difference from the amount of cyclic AMP accumulation measured in the control cells at 60 min following a washout of the agonist (W60). A representative immunoblot of stained, HA-tagged fragments is displayed. B, recycling of β2AR internalized in response to agonist-induced desensitized (30 min, 10 μM Iso) was examined. A431 cells were either untreated or transiently transfected with an expression vector harboring either an HA-tagged gravin-(1–51) mutant (HA-gravin 1–51) or the HA-tagged Figure 8. Expression of Src binding peptide (1–51) of gravin acts as a dominant-negative for AKAP gravin-mediated resensitization/recycling of internalized β2AR. A, cyclic AMP accumulation of β2AR-mediated cyclic AMP accumulation in A431 cells that were desensitized by 30 min treatment with Iso and then washed free of agonist for 60 min (W60) to permit resensitization was examined. Agonist-stimulated (Iso, 10 μM) cyclic AMP accumulation was measured in untreated cells (0), in cells following a 30-min prior stimulation with Iso (30), and in cells treated with Iso for 30 min, washed free of agonist, incubated for 60 min, and then assayed (W60). A431 cells were either untreated or transiently transfected with an expression vector harboring either an HA-tagged gravin-(1–51) mutant (HA-gravin 1–51) or the HA-tagged gravin RBD domain (554–938). Cells were challenged with a β-adrenergic agonist (Iso, 10 μM) for 30 min to provoke agonist-induced internalization of β2AR (30). The recovery from agonist-induced internalization of β2AR, termed recycling, was measured in cells at 60 min following the washout of agonist (W60). Cell surface β2ARs were quantified by [3H]CGP-12177 radioligand binding at 0 min (0), set as 100%, in cells following a 30-min challenge with 10 μM Iso (30, desensitized), and in cells challenged with Iso for 30 min, washed free of agonist, and incubated for 60 min following the washout (W60, resensitized). The results, displayed as mean ± S.E., are derived from at least three separate experiments performed with as many separate cultures of A431 cells. *p ≤ 0.01 for the difference from the amount of receptor binding measured in the control cells at 60 min following a washout of the agonist (W60). Reflecting the resensitization and recycling of the receptors to the cell membrane. A representative immunoblot of stained, HA-tagged fragments is displayed.
AKAP Gravin, Src, and Scaffold Function

gravin revealed nine proline-rich domains with a PXXP motif (33). Although these PRS are distributed largely along the length of this AKAP molecule, analysis of Src docking revealed that only the most N-terminal PRS was essential for Src binding. Targeted mutagenesis of this Src docking site on gravin confirmed the docking data in functional terms through study of the resensitization of the cyclic AMP response and recycling of internalized β₂AR. In the basal state, Src binding to AKAP gravin is observed and in response to activation of the β₂AR by β-adrenergic agonist increased Src binding to the scaffold is observed. The most N-terminal Src binding site displays a sequence, 10RXPXPX15, flanked on the C-terminal side by an additional prolyl residue, constituting a class I ligand for SH3 domains, as that found in Src (25). C-terminal to this class 1 ligand site is a second PXXP site that, in sharp contrast, does not bind Src. The 10RXPXPX15 docking site was shown by two independent functional assays to be essential for the ability of AKAP gravin to resensitize and to recycle the internalized β₂AR, a prototypic GPCR.

Several features of Src deserve mention in the context of the current studies. Src, like gravin, is N-myristoylated and harbors short positively charged domains (positively charged domain, see Fig. 2A) that target this non-receptor tyrosine kinase to the cell membrane (35). Src is catalytically least active in the “basal” state, reflecting autoinhibition by phospho-Tyr350, which neutralizes the SH2 domain (36) and a proline-rich domain adjacent to the kinase domain that neutralizes the SH3 domain. Src is shown to associate with the AKAP gravin in the unstimulated state, in a manner that requires PRS in the far N terminus of the scaffold. Stimulating cells with β-adrenergic agonist leads to the well known desensitization/internalization of β₂AR (9, 37), a process that includes participation by PKA, G protein-coupled receptor kinases, and the adaptor molecule Grb2 (34) and regulatory subunit (p85) of phosphatidylinositol 3-kinase (4). This Tyr350 residue of the β₂AR is directly phosphorylated by the insulin receptor kinase in response to insulin, both in vivo and in vitro (21, 29) and is obligate for the counterregulatory effects of insulin on β-catecholamine action. It has been shown that Src can be activated by interaction with the GTP-ligated form of α-subunits of specific heterotrimeric G proteins (e.g. Gs (20, 40)). Taken together, the results of the current study and those of earlier studies provide insight into the rich repertoire of functions that are catalyzed by Src on GPCR-based signaling, both in the desensitization and internalization of GPCRs, but also the newly discovered role of catalyzing (via an AKAP scaffold) the resensitization and recycling of β₂AR.

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AKAP Gravin, Src, and Scaffold Function

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