The Scaffold Protein Gravin (cAMP-dependent Protein Kinase-anchoring Protein 250) Binds the β2-Adrenergic Receptor via the Receptor Cytoplasmic Arg-329 to Leu-413 Domain and Provides a Mobile Scaffold during Desensitization*

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The cyclic AMP-dependent kinase-anchoring proteins (AKAPs) function as scaffolds for a wide-range of protein-protein interactions. The 250-kDa AKAP known as gravin plays a central role in organizing G-protein-coupled receptors to the protein kinases and phosphatases that regulate receptor function in desensitization, resensitization, and sequestration. Although gravin is critical for G-protein-linked receptor biology, the molecular features of the receptor necessary for interaction with this scaffold are not known. Herein, we map the regions of the β2-adrenergic receptor that are required for binding to gravin. Intracellular loops 1, 2, and 3 appear not to participate in the binding of the receptor to the scaffold. In contrast, the C-terminal cytoplasmic region of the receptor (Arg-329 to Leu-413) competes readily for the binding of the β2-adrenergic receptor by gravin, both using in vitro and in vivo assays. C-terminally truncated peptides with sequences ranging from Arg-329 to Leu-342 (13 aminoacyl residues), to Asn-352 (23 residues), to Tyr-366 (37 residues), to Asp-380 (51 residues), or to His-390 (61 residues), as well as N-terminally truncated peptides from Gln-391 to Leu-413 (23 residues) or Leu-381 to Leu-413 (33 residues) displayed no ability to block binding of receptor to gravin. The combination of Arg-329 to His-390 peptide and Gln-391 to Leu-413 peptide, however, reconstitutes a fragmented but full-length C-terminal region and also potently blocks the ability of gravin to bind the β2-adrenergic receptor. The gravin-receptor interaction was examined in response to agonist by confocal microscopy. Remarkably, the association of the receptor with gravin was not disrupted upon agonist-induced sequestration. The receptor-scaffold complex was maintained during agonist-induced sequestration. These data, in agreement with the biochemical data, reveal that gravin binds the receptor through the β2-adrenergic receptor C-terminal cytoplasmic domain and that this interaction is maintained as the receptor is internalized. This is the first report of an AKAP scaffold protein translocating with its receptor, in this case a G-protein-coupled receptor.

G-protein-coupled receptors (GPCRs)1 constitute a superfamily of membrane proteins that link ligand activation into activation of heterotrimeric G-proteins, such as the stimulatory G-protein, Gs, that activates adenylylcyclase (1, 3). Activation of the β2-adrenergic receptor, for example, leads to activation of adenylylcyclase, increased accumulation of intracellular cyclic AMP, and activation of protein kinase A. Although ubiquitously found in nature, this signaling cascade activates protein kinase A in unique manners that can only be explained by spatially distinct forms of the kinase. The discovery of protein kinase A-anchoring proteins (termed AKAPs) helped to solve the question of spatial specificity of protein kinase A responses (4). With at least 70 known sequences, the AKAP family appears to function in the targeting and localization of not just protein kinase A, but also other protein kinases and phosphatases (5). Members of the AKAP79/150 family and gravin (AKAP250) are examples of AKAPs that can bind protein phosphatases, protein kinase C, and protein kinase A. Key to our understanding of gravin was the observation that it associates in a complex with the β2-adrenergic receptor, the prototypic GPCR coupled to adenylylcyclase, and to protein kinase A, which acts both downstream (activation) and upstream (phosphorylation) of the β2-adrenergic receptor (6).

Gravin has been shown to act as a scaffold protein, capable of organizing protein kinase A, protein kinase C, and protein phosphatase-2B with the β2-adrenergic receptor (6). More recently, it has been shown that the gravin-β2-adrenergic receptor complex includes not only these kinases and phosphatase, but also G-protein-linked receptor kinase 2 (GRK2) and transiently with both β-arrestin and clathrin (7). The Ht31-peptide can compete for the binding of protein kinase A to AKAPs (8). Disruption of gravin-protein kinase A interaction with the Ht31-peptide disrupts the β2-adrenergic receptor signaling complex, as does suppression of gravin expression with antisense oligodeoxynucleotides (6). Similarly, suppression of the expression of protein kinase A or protein kinase C, as compared with simple block of activity with chemical inhibitors, leads to disruption of the β2-adrenergic receptor signaling complex (9, 10). Gravin localization has been defined in living cells by the

1 The abbreviations used are: GPCR, G-protein-coupled receptor; AKAP, cyclic AMP-dependent protein kinase-anchoring protein; CHO, Chinese hamster ovary; eBFP, enhanced blue fluorescent protein; GFP, green fluorescent protein; GRK, G-protein-linked receptor kinase; GST, glutathione S-transferase; HA, hemagglutinin antigen; 125I[CYP, 125Iiodocyanopindolol; iL, intracellular loop; PAGE, polyacrylamide gel electrophoresis; protein kinase A, cyclic AMP-dependent protein kinase; PP2A, protein phosphatase type 2B.
use of green fluorescent protein (GFP)-tagged gravin and epifluorescence microscopy. Gravin is localized to the membrane and also is found distributed in the cytosol (7). Here we report on the results of studies designed to address two significant questions about the gravin-β2-adrenergic receptor complex: what is the structural basis of the β2-adrenergic receptor that is responsible for its interaction with the gravin scaffold protein, and whether the gravin-β2-adrenergic receptor complex is retained following agonist treatment. The results from these studies reveal the C-terminal cytoplasmic region of the β2-adrenergic receptor as the site of receptor-gravin interaction and that the β2-adrenergic receptor remains associated with gravin following agonist activation and agonist-induced sequestration. These are the first data to reveal an AKAP as a “mobile” scaffold that appears to retain the receptor-gravin complex during sequestration and internalization (further treated under “Discussion”).

EXPERIMENTAL PROCEDURES

Glutathione S-Transferase (GST) Fusion Proteins—To construct GST fusion proteins, DNA sequences corresponding to the indicated regions of human β2-adrenergic receptor (Fig. 1) were amplified by polymerase chain reaction (PCR) from pCDNA3-β2-adrenergic receptor-WT/GFP. Primers (sequences of all primers used available from the authors upon request) were designed to generate PCR products with 5′ BamHI and 3′ EcoRI restriction sites. PCR products were gel-purified, double-digested with BamHI and EcoRI, and subcloned into the BamHI/EcoRI sites of the GST fusion protein vector pGEX2T (Amersham Pharmacia Biotech). Each construct was confirmed by DNA sequencing, transformed into Escherichia coli BL21, and screened for isopropyl-1-thio-β-galactopyranoside-induced expression of fusion proteins. Selected clones were used to inoculate 1000-ml aliquots of LB medium, and the inoculated mixture was incubated at 30 °C overnight with vigorous shaking. Expression of fusion protein was induced at 30 °C for another 4 h in the presence of 0.2 mM isopropyl-1-thio-β-galactopyranoside. Cells were collected by centrifugation at 5000 × g for 10 min. The collected pellets were resuspended in 36 ml of ice-cold phosphate-buffered saline containing 0.5 mM of protease inhibitor mixture Set III (Roche Molecular Biochemicals), and distributed into 5-ml aliquots, and each was then sonicated for 1 min. Fusion proteins were extracted by adding 4 ml of 10% Triton X-100 (final concentration, 1%) and mixing at 4 °C for 30 min. Insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4 °C. The supernatant was transferred to a fresh container. A 0.8-ml 50% slurry of glutathione-Sepharose 4B was added to the supernatant, and the slurry was then incubated at room temperature for 30 min. The beads were collected by centrifugation at 500 × g for 2 min and washed four times with 30 ml of ice-cold phosphate-buffered saline. Fusion proteins bound to glutathione-Sepharose 4B were released by treatment with 100 mM reduced glutathione in phosphate-buffered saline.

Cell Culture—Human epidermoid carcinoma cells (A431) 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 by Antibodies against β2-Adrenergic Receptor or Gravin—For most studies, A431 cells were either untreated or stimulated with 10 μM isoproterenol for 30 min. Cells were harvested and lysed in a lysis buffer (1% Triton X-100, 0.5% Nonidet-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. After centrifugation of the cell debris at 10,000 × g for 15 min, the lysate were precleared with protein A/G-agarose for 90 min and then incubated with or without GST fusion proteins for 2 h at 4 °C. The mixture were then subjected to immunoprecipitation for 2 h with antibodies specific to the β2-adrenergic receptor or the primary antibodies to the primary antibodies were linked covalently to a protein A/G-agarose matrix. Immunocomplexes were washed three times with radioimmune precipitation buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, 1% Triton X-100, pH 8.0) and separated on 4–12% linear gradient SDS-acrylamide Laemmli gels. Immunoblotting and detection of gravin and of the β2-adrenergic receptor by immunostaining was performed as previously described (4). In several experiments, wild-type and mutated versions of a β2-adrenergic receptor that was tagged with the hemagglutinin antigen (HA) was expressed in A431 cells. Anti-HA antibodies were employed to immunoprecipitate the HA-tagged receptors from whole-cell extracts prepared from transiently transfected clones, as described above.

Construction of an Enhanced Blue Fluorescent-β2-Adrenergic Receptor Fusion Protein Expression Construct—To create a Flag epitope-tagged, β2-adrenergic receptor-enhanced blue fluorescent protein (EBFP) fusion protein, primers were designed to amplify the eBFP fragment from the eBFP-N1 plasmid vector (CLONTECH, Palo Alto, CA) with an XbaI site at the N terminus, CCGGCTCTAGAATTCGACGAAGGCGAGG, and a NotI site at the C terminus, CCGGGCGGGCGCTTTAATGTTACAGCCTGTC. The XbaI/NotI-digested PCR product was combined with the Flag-tagged, β2-adrenergic receptor fragment that was excised from β2-adrenergic receptor-GFP construct (kindly provided by Dr. Jeffrey Benovic, Department of Pharmacology, Thomas Jefferson Medical School) by digesting the vector with HindIII/XbaI and subcloning this fragment into HindIII/NotI-digested pCDNA3.1/Zeo (+) vector (Invitrogen, Carlsbad, CA).

Co-transfection of GFP-tagged Gravin and eBFP-tagged β2-Adrenergic Receptor into A431 Cells—A431 cells were transfected with gravin-GFP using Lipofectin® (Life Technologies, Inc.) according to the manufacturer’s protocol, and viable clones were selected in 400 μg/ml of the neomycin analogue G418. Resistant colonies were subcloned and screened for GFP fusion protein expression by epifluorescence microscopy. Selected stable transfected cell lines were then transfected with β2-adrenergic receptor-eBFP-pcDNA3.1/Zeo (+), selected in 100 μg/ml Zeocin, and screened for eBFP fusion protein expression by epifluorescence microscopy.

Epifluorescence Imaging—Microscopy of live cells was performed on the Eclipse TE300 (Nikon) inverted microscope equipped with 40 × objective and set of filters. Images were acquired using MicroMAX imaging system (Princeton Instruments Inc.) and WinView/32 software. Fluorescent dyes were imaged sequentially in frame-interface mode to eliminate spectral overlapping between the channels.

Confocal Microscopy—A431 cells stable expressing β2-adrenergic receptor growing on glass slides were either left untreated or stimulated with 10 μM isoproterenol for 30 min and then washed twice with HBBS, fixed (2% paraformaldehyde, pH 7.2), and permeabilized. Affinity-purified rabbit polyclonal anti-gravin antibodies were used in combination with Alexa 633 anti-rabbit antibodies (Molecular Probes). Stained ob-
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RESULTS

Creation and Expression of GST Fusion Proteins Containing the Cytoplasmic Domains of the β2-Adrenergic Receptor.—In view of the central role of gravin in the biology of GPCRs, we sought to define the site(s) of the β2-adrenergic receptor through which it interacts with the gravin scaffold protein. The strategy was to express each of the cytoplasmic domains of the β2-adrenergic receptor and employ these as probes for disrupting the interaction between gravin and the receptor. The intracellular loops (iLs) 1, 2, and 3, as well as the cytoplasmic C-terminal tail (termed the BAC1 domain) of the β2-adrenergic receptor, were selected as the major starting points. These domains have been established by immunochemical means as cytoplasmic (11), providing a logical place to initiate the interaction with gravin (Fig. 1). The three intracellular loops (Ile-58 to Ile-72, Asp-130 to Arg-151, and Ser-220 to Leu-275) and C-terminal BAC1 (Arg-329 to Leu-413) were designed as probes for disrupting the interaction between the receptor and the gravin scaffold protein (Fig. 2). The β2-adrenergic receptor and gravin scaffold protein: analysis by pull-downs with antibodies to the β2-adrenergic receptor. A, A431 cell lysates preincubated with protein A/G were incubated with or without 100 nM GST fusion proteins GST-iL1, -iL2, -iL3, and -BAC1 for 2 h and then immunoprecipitated with antibodies against β2-adrenergic receptor (CM-04), which was covalently conjugated to protein A/G-agarose beads. The immune complexes were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with antibody specific to gravin. The immune complexes were made visible by the chemiluminescence method. The relative densities of the protein bands were determined by use of an imaging densitometer (GS-700, Bio-Rad) and Multi Analyst densitometer software (Bio-Rad). A representative immunoblot and the results of the densitometry (mean ± S.E. of three independent experiments) are displayed. B, A431 cell lysates preincubated with protein A/G protein were incubated with or without increasing concentrations (1–1000 nM) of GST fusion protein GST-BAC1 for 2 h and then immunoprecipitated with antibodies against β2-adrenergic receptor (CM-04), which was covalently conjugated to protein A/G-agarose beads. The samples were processed and the data analyzed as described above. β2AR, β2-adrenergic receptor; IP, immunoprecipitation; IB, immunoblotting.

Fig. 2. Expression of GST fusion proteins containing iL1, iL2, and iL3 and the C-terminal cytoplasmic domain (BAC1) of the β2-adrenergic receptor. GST fusion proteins were created and then expressed in E. coli. The expressed proteins were isolated, purified, and subjected to SDS-PAGE. The gels were stained for protein with Coomassie Brilliant Blue, destained, and photographed. The Mr values (in thousands) of protein standards are displayed at the left. The expressed proteins were purified by affinity chromatography to apparent homogeneity, as deduced by polyacrylamide gel electrophoresis (PAGE) in SDS (Fig. 2). The calculated Mr values (in thousands) of protein standards are displayed at the left. The expressed proteins were purified by affinity chromatography to apparent homogeneity, as deduced by polyacrylamide gel electrophoresis (PAGE) in SDS (Fig. 2). The calculated Mr values (in thousands) of protein standards are displayed at the left.
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resonance analysis (not shown).

**Analysis of the Ability of Cytoplasmic Loops 1–3 and the C-terminal Cytoplasmic Tail of the β2-Adrenergic Receptor to Block Receptor Interaction with the Gravin Scaffold**—The ability of the GST fusion proteins to compete with the β2-adrenergic receptor for gravin binding was tested in whole-cell extracts prepared from human epidermoid carcinoma A431 cells. The whole-cell extracts were supplemented with GST fusion protein to a final concentration of 100 nM, and the ability of immunoprecipitates of the β2-adrenergic receptor to co-precipitate gravin was measured by SDS-PAGE analysis of the immunoprecipitates of the receptor that were immunoblotted with antibodies specific for gravin (Fig. 3A). The antibody against the β2-adrenergic receptor used in this study for immune precipitation pull-down reactions was CM-04, a polyclonal antibody directed against the first exofacial loop epitope of the β2-adrenergic receptor (11). In the absence of competing peptide, a strong signal representing gravin bound by the β2-adrenergic receptor was observed. This interaction of β2-adrenergic receptor and gravin has been detected by several independent means earlier (6, 7). At 100 nM concentrations, only one of the GST fusion proteins, GST-BAC1, harboring the C-terminal cytoplasmic domain, demonstrated the ability to block the interaction of the β2-adrenergic receptor with gravin. Fusion proteins with intracellular loops 1, 2, and 3 all failed to block the receptor-gravin interaction. Even at concentrations up to 1 μM, or in combination with each other, these loop fusion proteins failed to alter the receptor-gravin interaction (not shown).

The ability of the GST-BAC1 fusion protein to block the interaction of β2-adrenergic receptor with gravin was explored further in competition experiments in which the amount of GST-BAC1 protein included in the whole-cell lysate was varied from nil to 1 μM (Fig. 3B). Increasing the concentration of BAC1 fusion protein from 1 to 1000 nM in the whole-cell extract produced a dose-dependent competition curve for the inhibition of gravin-β2-adrenergic receptor interaction. Based upon multiple competition curves, the concentration of the cytoplasmic C-terminal tail-GST fusion protein at which 50% of the receptor-gravin binding was lost is ~5 nM GST-BAC1, under these conditions. Concentrations at 100 nM or higher of GST-BAC1 fusion protein nearly abolished the ability of the β2-adrenergic receptor to interact with gravin. These data suggest that the cytoplasmic C-terminal domain of the β2-adrenergic receptor is the dominant domain for interaction with the gravin scaffold protein. The intracellular loops, in contrast, do not appear to play a significant role in the gravin-receptor interaction, based upon their inability to block this interaction as GST fusion proteins.

**Expression and purification of the GST fusion proteins containing C-terminally truncated fragments of the β2-adrenergic receptor cytoplasmic tail**. The truncated β2-adrenergic receptor C-terminal tail peptides were engineered as fusion proteins with GST protein (see Fig. 1). Shown is Coomassie Blue staining of SDS-PAGE gels of the expressed and purified GST fusion proteins containing the C-terminally truncated fragments of the β2-adrenergic receptor. Approximately 2 μg of GST fusion protein was loaded per lane of the SDS-PAGE gel shown.

**FIG. 4.** GST fusion proteins containing the cytoplasmic C-terminal domain, but not those containing intracellular loops 1–3, of the β2-adrenergic receptor inhibit the interaction between the receptor and the gravin scaffold protein: analysis by pull-downs with anti-gravin antibodies. A, A431 cell lysates precleared by protein A/G protein were incubated with or without 100 nM GST fusion proteins GST-iL1, -iL2, -iL3, and -BAC1 for 2 h and then immunoprecipitated with antibodies against gravin, which was covalently conjugated to protein A/G-agarose beads. The immune complexes were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with antibody against the β2-adrenergic receptor (CM-02). The immune complexes were made visible by the chemiluminescence method. The relative densities of the protein bands were determined by use of an imaging densitometer (GS-700, Bio-Rad) and MultiAnalyst densitometer software (Bio-Rad). A representative immunoblot and the results of the densitometry (mean ± S.E. of three independent experiments) are displayed. B, A431 cells lysates precleared by protein A/G protein were incubated with or without increasing concentrations (1–1000 nM) of GST fusion protein GST-BAC1 for 2 h and then immunoprecipitated with antibodies against gravin, which was covalently conjugated to protein A/G-agarose beads. The samples were processed and the data analyzed as described above. β2AR, β2-adrenergic receptor. IP, immunoprecipitation; IB, immunoblotting.
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Full-length C-terminal cytoplasmic domain, but not C-terminally truncated fragments of this domain, of the β2-adrenergic receptor effectively blocks the interaction of the receptor with the gravin scaffold protein. A431 cell lysates precleared by protein A/G protein were incubated with or without 100 nM GST fusion proteins for 2 h and then immunoprecipitated with antibody against β2-adrenergic receptor (CM-04) (A) or antibody against gravin (B), which were covalently conjugated to protein A/G-agarose beads. The immune complexes were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with antibody specific to antibody against gravin (A) or antibody against β2-adrenergic receptor (B). The immune complexes were made visible by the chemiluminescence method. The relative densities of the bands (A and B, top panels) were determined by use of an imaging densitometer (GS-700, Bio-Rad) fitted with MultiAnalyst densitometer software (Bio-Rad) (A and B, bottom panels). The blot displays the mean values ± S.E. of three independent experiments. β2AR, β2-adrenergic receptor; IP, immunoprecipitation; IB, immunoblotting.

To test further the results obtained with the immunoprecipitations of the β2-adrenergic receptor, complementary experiments were performed in which the receptor-gravin complexes were isolated via immunoprecipitations with antibodies to gravin (Fig. 4). The immune complexes of gravin were precipitated with anti-gravin antibodies, and the immune complexes were subjected to SDS-PAGE and immunoblotting with antibodies specific for β2-adrenergic receptor. The gravin-receptor interaction was observed in the absence of any competing peptide (Fig. 4A), confirming parallel experiments performed with immunoprecipitations obtained with the anti-β2-adrenergic receptor antibodies (Fig. 3A). In the presence of 100 nM concentrations of competing GST fusion proteins for intracellular loops 1, 2, and 3, the β2-adrenergic receptor-gravin interaction was not influenced. The competition experiments performed with 100 nM C-terminal fusion protein GST-BAC1, in sharp contrast, markedly attenuated the receptor-gravin interaction, as observed in Fig. 3A. Thus, irrespective of the manner in which the receptor-gravin complexes are pulled down for analysis, the C-terminal cytoplasmic domain is the only β2-adrenergic receptor domain tested that blocks the ability of the β2-adrenergic receptor to bind to gravin. A dose-response experiment was performed for the receptor-gravin interaction with increasing amounts of competing GST-BAC1 fusion protein (Fig. 4B). Increasing the concentration of GST-BAC1 from 1 to 1000 nM resulted in a dose-dependent loss of receptor-gravin interaction. The concentration of GST-BAC1 that yields 50% inhibition of β2-adrenergic receptor-gravin binding was less than 5 nM GST-BAC1, whereas concentrations of intracellular loops 1, 2, and 3 to 1 μM were without effect (not shown).

Detailed Analysis of the Ability of C-terminally and N-terminally Truncated Fragments of the β2-Adrenergic Receptor to Block Receptor-Gravin Interactions—Based upon the data demonstrating the C-terminal cytoplasmic tail of the β2-adrenergic receptor to be the site of interaction with gravin, we prepared
GST fusion proteins in which defined fragments of the C-terminal region were engineered (Fig. 1). Five fragments (designated C2–C6), varying in length from 61 to 13 aminoacyl residues and representing truncations from the C-terminal end of the region, were designed. Two additional peptides, 14 and 31 aminoacyl residues in length (designated CT1 and CT2, respectively), were prepared synthetically, each retaining the C-terminal sequence and truncated from the N-terminal region of BAC-1. As before, the BAC1 and C2–C6 series of GST fusion proteins containing these regions of the C-terminal cytoplasmic tail of the β2-adrenergic receptor were expressed in E. coli and purified to homogeneity, as judged by SDS-PAGE and protein staining (Fig. 5). The calculated Mr values (in thousands) for the GST fusion proteins of the cytoplasmic C terminus and each of the C-terminal truncation fragments were as follows: BAC1, 38.4; C2, 36.2; C3, 35.1; C4, 32.6; C5, 31.2; and C6, 30.9. The synthetic peptides corresponding to CT1 and CT2 were purified to homogeneity by HPLC. The GST fusion proteins and synthetic peptides displayed dynamic light scattering values, suggesting that each behaved as a mono-dispersion in solution. GST fusion proteins harboring other β-adrenergic receptor sequences were found to display significant proteolytic processing that precluded their use (not shown).

The C-terminally truncated GST fusion proteins of the β2-adrenergic receptor tail were used as probes to test whether they would disrupt the interaction of receptor with gravin. When added at 100 μm to the whole-cell extracts of A431 cells, none of the C-terminally truncated fusion proteins displayed the ability to influence the binding of β2-adrenergic receptor to gravin (Fig. 6A). The BAC1 fusion protein at the same concentration essentially abolished the gravin-receptor interaction. Increasing the concentration of GST-C2, -C3, -C4, -C5, or -C6 to 1 μm failed to alter the gravin-receptor interaction (data not shown), as determined in immunoprecipitation pull-down reactions performed with antibodies to the β2-adrenergic receptor, followed by SDS-PAGE and immunoblotting of the immunoprecipitates with anti-gravin antibody. When the pull-downs were performed with antibodies to gravin and the immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with antibodies to the β2-adrenergic receptor, essentially identical results were obtained (Fig. 6B). The BAC1 fusion protein, but none of the C-terminally truncated versions of BAC1, was able to block effectively the gravin-β2-adrenergic receptor interaction. Thus, the data demonstrated that minimally, the C-terminal region of the β2-adrenergic receptor cytoplasmic tail is required for interaction with gravin.

We explored whether fragments of the furthest C-terminal reach of the β2-adrenergic receptor cytoplasmic tail were sufficient to block the interaction of gravin with the receptor. Synthetic peptides corresponding to the region Gln-391-Leu-413-COOH (CT1) and region Leu-381-Leu-413-COOH (CT2) were purified and employed in these experiments (Fig. 1). At a final concentration of 100 nm in whole-cell extracts, neither CT1 nor CT2 peptides alone influenced the ability of β2-adrenergic receptors to bind to gravin in pull-down assays with antibodies to the receptor and immunoblotting of the immune complexes stained with anti-gravin antibodies (Fig. 7A). The GST fusion protein with the full-length, cytoplasmic C-terminal region of the β2-adrenergic receptor (GST-BAC1) sharply reduced the receptor-gravin interaction. Next, we tested the ability of the CT1 fragment to act in tandem with GST fusion proteins con-
containing the C2, C3, C4, C5, and C6 regions of the β2-adrenergic receptor (Fig. 1) to inhibit gravin-receptor binding. The N-terminal fragment GST-C2 and the CT1 C-terminal fragment, when used in combination, reconstituted the inhibition of the gravin-β2-adrenergic receptor interaction (Fig. 7A). Similar reconstitutions of CT1 with GST-C3, -C4, -C5, and -C6, in contrast, failed to yield any significant ability to compete for the binding of the receptor to the gravin scaffold protein. Complementary experiments were conducted in which the pull-downs were performed with anti-gravin antibodies and the immunoblots of the immune complexes were stained with antibodies to the β2-adrenergic receptor (Fig. 7B). The results obtained when the receptor-gravin complexes are pulled down with antibodies to gravin were identical to those when the pull-downs were performed with antibodies to the receptor. These data suggest that the determinants for the binding of the β2-adrenergic receptor to gravin are polytopic and require both N-terminal and C-terminal domains of the cytoplasmic tail of the receptor.

Analysis of the Ability of Cytoplasmic Fragments of the β2-Adrenergic Receptor to Block Receptor-Gravin Interactions in Vivo—The results obtained from in vitro experiments in which peptides compete for binding sites on gravin provided compelling data that the cytoplasmic C terminus of the β2-adrenergic receptor is the major site of protein-protein interactions with the gravin scaffold protein. To test the conclusions in vivo, we engineered a mammalian expression vector into which the BAC1 was inserted and examined the ability of the BAC1 to compete for gravin binding with the β2-adrenergic receptor (Fig. 8). A431 cells were transiently transfected with the empty expression vector (pcDNA3, control) and vector expressing either BAC1 or iL3. The protein-protein interactions between the β2-adrenergic receptor and gravin were not altered by the expression of iL3 or by transfection with the empty vector. Expression of BAC1, in contrast, abolished the interaction between the β2-adrenergic receptor and the gravin scaffold protein (Fig. 8A).

Agonist treatment results in increased association of β2-adrenergic receptor with gravin (7). We explored the possible role of the protein kinase A sites as a partial explanation for the association of the β2-adrenergic receptor with the AKAP250 gravin. Because the results from the in vitro and in vivo competition studies establish the dominant cytoplasmic domain for gravin-β2-adrenergic receptor interactions as the C-terminal tail, we compared the ability of native BAC1 and the ability of BAC1 with mutated protein kinase A sites (S345A/S346A) to compete with native β2-adrenergic receptor for binding to gravin. Both native BAC1 and the mutant form of BAC1 lacking the protein kinase A site displayed the ability to block β2-adrenergic receptor-gravin interactions. (Fig. 8A). These data suggest that the protein kinase A site of the C-terminal tail of the β2-adrenergic receptor does not play a dominant role in the protein-protein interactions between gravin and the β2-adrenergic receptor.

We approached the same question using a complementary strategy, expression of the wild-type β2-adrenergic receptors, as well as mutant forms of the receptor in which various protein kinase A sites have been eliminated and examining stably expressing both the GFP-tagged gravin and the eBFP-tagged version of the human β2-adrenergic receptor were analyzed by epifluorescence microscopy in the unstimulated, basal state, as well as following stimulation for 30 min with the β-adrenergic agonist isoproterenol (10 μM) for 30 min. The microscopy was performed on living cells using a Nikon inverted epifluorescence microscope. The images (confocal (B) and epifluorescence (A)) shown are representative of those acquired in at least five separate experiments, sampling several dozen images per experiment.

**Fig. 10.** Treatment with the β-adrenergic agonist isoproterenol leads to enhanced association of receptor with the scaffold protein gravin during agonist-induced sequestration: analysis by epifluorescence microscopy of the autofluorescently tagged version of gravin (gravin GFP) and of the eBFP-tagged β-adrenergic receptor (β-AR-eBFP) and by confocal microscopy. A431 clones
was important to establish whether GRK2 and b-adrenergic receptor (βAR) with the scaffold protein gravin. See under “Discussion” for full details.

FIG. 11. Schematic working model of the role of AKAPs in the regulation of G-protein-linked receptors based upon the interactions of the β2-adrenergic receptor (βAR) with the scaffold protein gravin. See under “Discussion” for full details.

Gravin and Signaling Complexes
tagged Versions of Gravin and the Receptor—In order to probe further the interaction of the β2-adrenergic receptor with gravin, we designed experiments in which autofluorescent versions of each of the partners could be employed. Previously, we reported on the analysis of GFP-tagged versions of the β2-adrenergic receptor as well as gravin (7). In order to evaluate the interaction, an eBFP-tagged version of the β2-adrenergic receptor was created. The eBFP tag was engineered into the C-terminal region of the human β2-adrenergic receptor, at the same site in which the GFP tag was inserted. We evaluated the ability of the expressed eBFP-tagged version of the β2-adrenergic receptor to function. The eBFP-tagged β2-adrenergic receptor was expressed in CHO-K cells that lack β-adrenergic receptors. The radioligand binding capability of the eBFP-tagged receptor was assayed using the radiolabeled, high affinity β2-adrenergic antagonist ligand [125I]CYP. CHO clones stably transfected to express the eBFP-tagged receptor displayed normal levels of binding to gravin. Taken together, these data demonstrate that gravin-β2-adrenergic receptor interactions are not dependent upon the availability of an intact protein kinase A site for phosphorylation but do require the cytoplasmic C-terminal tail of this G-protein-coupled receptor.

Association of GRK2 and β-Arrestin with the β2-Adrenergic Receptor-Gravin Scaffold Complex in Response to Agonist—Previously we reported interactions between the β2-adrenergic receptor and gravin as well as between the receptor and the G-protein-coupled receptor kinase GRK2 and β-arrestin (6). It was important to establish whether GRK2 and β-arrestin are found in complex with gravin and the β2-adrenergic receptor. A431 cells were transiently transfected for 48 h with expression vector harboring HA-tagged wild-type receptor or β2-adrenergic receptor lacking the C-terminal site for protein kinase A (S345A/S346A). At the end of the 48 h, the cells were challenged with 10 μM isoproterenol. The HA-tagged receptors were subjected to immunoprecipitation with anti-HA antibodies. Wild-type β2-adrenergic receptor and the receptor lacking the C-terminal protein kinase A site displayed co-immunoprecipitation with gravin AKAP250. In addition, a mutant version of the β2-adrenergic receptor was expressed in which both the C-terminal site (Ser-345 and Ser-346) and the I3L site (Ser-261 and Ser-262) for protein kinase A were substituted with alanine (β2-adrenergic receptor (S261A/S262A/S345A/S346A)). The mutated versions of the β2-adrenergic receptor lacking the C-terminal or the C-terminal and I3L sites for protein kinase A displayed normal levels of binding to gravin. Taken together, these data demonstrate that gravin-β2-adrenergic receptor interactions are not dependent upon the availability of an intact protein kinase A site for phosphorylation but do require the cytoplasmic C-terminal tail of this G-protein-coupled receptor.

Exploring the Interaction of the β2-Adrenergic Receptor-Gravin Scaffold Interaction by Use of Autofluorescent Protein-
\( \beta_2 \)-adrenergic receptor, we were able to conduct experiments designed to evaluate the localization of both the \( \beta_2 \)-adrenergic receptor and the gravin scaffold molecule.

Epifluorescence microscopy was employed to examine the cellular distribution of GFP-tagged gravin and eBFP-tagged \( \beta_2 \)-adrenergic receptor that were co-expressed in human epidermoid carcinoma A431 cells (Fig. 10). The phase contrast images of the living cells subjected to the epifluorescence analysis are shown in Fig. 10A, panels a and b. Detection of the GFP-derived signal (green) revealed the gravin scaffold protein to be distributed in the cytoplasm throughout the entire cell and excluded in the nucleus (Fig. 10A, panel c). The eBFP-derived signal (pseudo-red) revealed the localization of the \( \beta_2 \)-adrenergic receptor, largely on the cell membrane (Fig. 10A, panel e). Merging the signal revealed (yellow) that the receptor and scaffold protein appear to colocalize at the cell membrane in the unstimulated, basal state (Fig. 10A, panel g). When this analysis was performed with A431 cells stimulated with isoproterenol (1 \( \mu \)M) for 30 min, the signal for the \( \beta_2 \)-adrenergic receptor demonstrated large-scale sequestration of the receptor from the cell surface to intracellular compartments (Fig. 10A, panel f). The distribution of the gravin in the isoproterenol-stimulated cells remained largely unchanged (Fig. 10A, panel d). Merging of the gravin and \( \beta_2 \)-adrenergic receptor epifluorescence signals from the cells treated with agonist demonstrated enhanced colocalization of the receptor with the scaffold (Fig. 10A, panel h). Equivalent data were obtained in maximum projection images from confocal microscopy performed with antibodies to gravin and the GFP-\( \beta_2 \)-adrenergic receptor (Fig. 10B). The pattern of intracellular distribution for gravin alters in response to agonist treatment, and the merging of the signals from gravin with that from the receptor indicates that the sequestered receptor is still associated with gravin. This conclusion is supported by recent observation made using an independent approach, the pull-down assays of gravin-receptor interaction with a working template, this model accommodates the current knowledge, illuminates an expanded role of AKAPs such as AKAP79 in the biology of GPCRs, and can be tested as to gravin (as shown here) remains to be tested. The current work reveals that the gravin-receptor complex appears to be mobile (Figs. 10 and 11). In the absence of agonist, gravin can be found in complex with the \( \beta_2 \)-adrenergic receptor, providing a basis for plasma membrane localization of this protein observed in several compartments in the cell (7). The localization of the \( \beta_2 \)-adrenergic receptor, a well known substrate for protein kinase A, with gravin and an ensemble of protein kinases and phosphatases positions the complex for signaling, desensitization, and resensitization. Upon agonist treatment, we observed sequestration of the receptor and a similar distribution of gravin molecules. These data suggest that rather being released off from the AKAP250 to clathrin, as one might expect (13), the \( \beta_2 \)-adrenergic receptor remains associated with gravin, keeping the ensemble of protein kinases and phosphatases organized through the process of desensitization and resensitization. Not surprisingly, suppression of gravin expression has been shown to disrupt signaling and resensitization of the \( \beta_2 \)-adrenergic receptor (7). Similarly, expression of the full-length cytoplasmic C-terminal domain of the \( \beta_2 \)-adrenergic receptor (BAC1) also leads to a loss of agonist-induced desensitization and resensitization (2). This would be the first indication that AKAPs are mobile, affording the molecules that bind to them a scaffold that can translocate from the plasma membrane to vesicles involved in trafficking the receptor-gravin complex during desensitization and resensitization. Thus, the composition of the gravin-receptor complex that includes protein kinase A, protein kinase C, and PP2B provides a scaffold to enable desensitization by protein kinase A and GRKs, as well as association of \( \beta_2 \)-arrestin. Agonist-induced sequestration targets the gravin-receptor complex through clathrin association with \( \beta_2 \)-arrestin, maintaining PP2B in close proximity to the phosphorylated receptor. Resensitization follows the desensitization and sequestration, allowing for the PP2B-catalyzed dephosphorylation, subsequent loss of \( \beta_2 \)-arrestin and its interaction with clathrin, and finally trafficking of the receptor-laden scaffold back to the plasma membrane (Fig. 10). Although only a working template, this model accommodates the current knowledge, illuminates an expanded role of AKAPs such as gravin and AKAP79 in the biology of GPCRs, and can be tested further to explore the nature of this fascinating protein ensemble.

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