Assembly of an SAP97-AKAP79-cAMP-dependent Protein Kinase Scaffold at the Type 1 PSD-95/DLG/ZO1 Motif of the Human β1-Adrenergic Receptor Generates a Receptosome Involved in Receptor Recycling and Networking*

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Appropriate trafficking of the β1-adrenergic receptor (β1-AR) after agonist-promoted internalization is crucial for the resensitization of its signaling pathway. Efficient recycling of the β1-AR required the binding of the protein kinase A anchoring protein-79 (AKAP79) to the carboxyl terminus of the β1-AR (Gardner, L. A., Tavalin, S. A., Goehring, A., Scott, J. D., and Bahouth, S. W. (2006) J. Biol. Chem. 281, 33537–33553). In this study we show that AKAP79 forms a complex with the type 1 PDZ-binding sequence (ESKV) at the extreme carboxyl terminus of the β1-AR, which is mediated by the membrane-associated guanylate kinase (MAGUK) protein SAP97. Thus, the PDZ and its associated SAP97-AKAP79 complex are involved in targeting the cyclic AMP-dependent protein kinase (PKA) to the β1-AR. The PDZ and its scaffold were required for efficient recycling of the β1-AR and for PKA-mediated phosphorylation of the β1-AR at Ser312. Overexpression of the catalytic subunit of PKA or mutagenesis of Ser312 to the phosphoserine mimic aspartic acid both rescued the recycling of the trafficking-defective β1-AR PDZ mutant. Thus, trafficking signals transmitted from the PDZ-associated scaffold in the carboxyl terminus of the β1-AR to Ser312 in the 3rd intracellular loop (3rd IC) were paramount in setting the trafficking itinerary of the β1-AR. The data presented here show that a novel β1-adrenergic receptosome is organized at the β1-AR PDZ to generate a scaffold essential for trafficking and networking of the β1-AR.

The sympathetic nervous system mediates its regulatory effects through G protein-coupled receptors (GPCR)3 related to the family of α- and β-adrenergic receptors. Among these receptors is the β1-AR, which is coupled to the Gs-cyclic AMP axis and plays a major role in transmitting sympathetic regulation to cardiac, renal, vascular, and other organs (2, 3).

Persistent activation of the β1-AR or other GPCR causes their desensitization and internalization via clathrin-coated pits or caveolae into early endosomes (4–6). Internalized GPCR are either recycled back to the cell surface for another round of signaling or retained for degradation by lysosomal or proteasomal pathways (7–9). Characterization of the players involved in these distinct outcomes is the purpose of this study.

Recycling and resensitization of the β1-AR are dependent upon two motifs; one is the ESKV sequence in the carboxyl-terminal tail, and the other is the region surrounding Ser312 in the 3rd IC of the β1-AR (10, 11). The ESKV tetrapeptide conforms to a type I (PDZ-95/DLG/ZO1) PDZ ligand (i.e. X(S/T)XX, where X at positions −1 and −3 is any amino acid, and X at position 0 is a hydrophobic amino acid) (12, 13). Mutagenesis of the type 1 PDZ or Ser312 to alanine prevented the recycling and resensitization of the β1-AR (10, 11). Concerning Ser312, we determined that this residue is specifically phosphorylated by PKA and that the activity of PKA was required for recycling and resensitization of the human β1-AR (11).

These results indicate that two distinct motifs are involved in recycling of the β1-AR, but they do not explain how they cross-talk to one another to coordinate the sequence of events involved in recycling of this GPCR. A major breakthrough in identifying the mechanism of cross-talk between these two motifs was the identification of AKAP79 as the AKAP involved in recycling of the β1-AR in HEK-293 and other cell lines (1). AKAP79 promoted the targeting of PKA to the β1-AR by binding to the carboxyl-terminal 53 amino acids (between residues 425 and 477) of the β1-AR (1). Here we report that the binding domain of AKAP79 to the β1-AR overlaps with its type 1 PDZ motif. However, the binding between the PDZ and AKAP79 is indirect and involves the MAGUK protein SAP97 that simultaneously binds to AKAP79 and type 1 PDZ to target PKA to the

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1 Investigator career of the American Lung Association.

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3 The abbreviations used are: GPCR, G protein-coupled receptors; β1-AR, β1-adrenergic receptor; WT, wild type; AKAP, A-kinase anchoring proteins; PDZ, PSD-95/DLG/ZO1; MAGUK, membrane-associated guanylate kinase; PKA, cyclic AMP-dependent protein kinase; cPKA, catalytic subunit of cyclic AMP-dependent protein kinase; β1-ARPDZ, β1-AR mutant in which the type 1 PDZ “ESKV” sequence is mutated to alanine; 3rd IC, 3rd intracellular loop; ICYP, [125I]iodocyanopindolol; FRET, fluorescence resonance energy transfer; FRETN, normalized FRET; GRK, G protein-coupled receptor kinase; HEK, human embryonic kidney; DMEM, Dulbecco’s modified Eagle’s medium; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; siRNA, small interfering RNA; PBS, phosphate-buffered saline; GST, glutathione S-transferase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; NMMA, N-methyl-D-aspartate; BSA, bovine serum albumin.
β1-AR. By scaffolding PKA to the β1-AR, SAP97 facilitates PKA-mediated phosphorylation of Ser112, which is critical for trafficking of the internalized β1-AR to membranes. These results indicate that a novel β1-adrenergic receptosome is involved in recycling and resensitization of the β1-AR as well as in its other physiological effects.

EXPERIMENTAL PROCEDURES

Construction of FLAG- or Myc-tagged Full-length and Truncated β1-AR—To allow rapid assessment of cell surface expression of the β1-AR, the amino-terminal initiator methionine was replaced either by the FLAG tag sequence (DYKDDDK) or by the Myc tag (EQKLISEEDL) sequence, resulting in N-FLAG/MyC-tagged WT β1-AR or the S312D mutant in the mammalian expression vector pcDNA3.1 (Invitrogen) (14). To generate the β1-ARΔ425–441 and β1-ARΔ425–463 constructs, the full-length β1-AR cDNA was cut with Smal, and the resulting 1.3-kb cDNA encoding β1-AR(1–424) was cloned into pcDNA3.1. The 162-bp Smal-EcoRI cDNA between bases 1272 and 1434 was used as a template for PCRs that generated cDNAs encoding the sequences between amino acids 441–477 and 463–477, which were then ligated in-frame into the β1-AR(1–424). The WT β1-AR or S312D was used as PCR templates with the sense primer described in Gardner et al. (1) and the anti-sense primer 5′-TGATTCCTACGTCTGCTGGCAGGCGAAGCCGAGGCCGCAC-3′ to generate the β1-ARΔAPDZ and the S312D-β1-ARΔPDZ, respectively. Sequences of all the epitope-tagged β1-AR constructs were verified by automated dyeoxy sequencing.

Construction of Fluorescently Tagged β1-AR, SAP97, AKAP79, and Riggs Site—Amino-terminal variants of the WT β1-AR and AKAP79 to CFP and YFP were described (1). CFP- or YFP-β1-ARΔPDZ was generated using the forward primer described earlier and the reverse primer (5′-TGATTCCTACGTCTGCTGGCAGGCGAAGCCGAGGCCGCAC-3′) to generate the β1-ARΔAPDZ and the S312D-β1-ARΔPDZ, respectively. Sequences of all the epitope-tagged β1-AR constructs were verified by automated dyeoxy sequencing.

Acid Strip Confocal Recycling Microscopy Protocol—HEK-293 cells expressing the FLAG- or Myc-tagged WT β1-AR or β1-ARΔPDZ were grown on poly-1-lysine-coated glass coverslips and serum-starved at 37 °C for 1 h in DMEM supplemented with 25 mM HepES, pH 7.4. The receptors were labeled with fluorescein isothiocyanate-conjugated anti-FLAG M2 IgG (10 µg/ml) for 1 h at 37 °C. Cells were treated with 10 µM isoprotanol for 30 min at 37 °C to promote agonist-mediated β1-AR internalization. Then the cells were chilled to stop endocytosis and exposed to 0.5 M NaCl, 0.2 M acetic acid, pH 3.5, for 4 min on ice to remove antibody bound to extracellular β1-AR (1, 15, 16). Cultures were then incubated with culture medium supplemented with 100 µM of the β-agonist alprenolol at 37 °C for 10, 20, 30, or 45 min to establish the recycle time. After each time period, the coverslips were rinsed and fixed in 4% glutaraldehyde and 4% paraformaldehyde, permeabilized with 1% Triton X-100 in PBS, and stained with Cy3-conjugated anti-Myc 9E10 monoclonal antibody and anti-PSD-95 antibody was from Novus Biologicals (NB 600-1229); anti-human SAP97 monoclonal antibody was from StressGen (VAMPS005, Nventa Corp., San Jose, CA); anti-SAP97 polyclonal antibody was from Novus Biologicals (NB 600-1229); anti-human SAP97 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-PSD-95 antibodies (anti-pan-PDZ monocular antibody 05-427 directed against the PDZ domain (residues 77–299) of human PSD-95 and anti-PDZ antibody 05-494) were from Upstate Biotechnology, Inc. The siRNA to AKAP79 5′-AAagagacugacagagguau-3′ and its scrambled control AAagacacagagguauac were described (1). The siRNA sequence to human SAP97, 5′-GATATCCAGGAACATAAAT-3′, or its control, 5′-CCATAATAACGGTATAA-3′, were cloned into the pSuper™ plasmid (OligoEngine Corp., Seattle, WA).

Cell Cultures and Radioligand Binding Parameters—HEK-293 cells cultured in DMEM supplemented with 10% fetal bovine serum until they were ~90% confluent. The WT β1-AR or its point mutants in pcDNA 3.1 were transiently transfected into HEK-293 cells using the Cytofectene reagent (Bio-Rad) as follows. Plasmid DNA (1 µg) was diluted into 200 µl of DMEM and then mixed with an equal volume of DMEM containing 12 µl of Cytofectene at room temperature for 20 min. Then 4 ml of DMEM was added, and the DNA-lipid complex was layered over the cells for 5 h at 37 °C, followed by the addition of an equal volume of DMEM + 10% fetal bovine serum. G-418-stable cell lines for the constructs described in Table 1 were generated and used where indicated. Binding of [125I]iodocyanopindolol (ICYP) to 0.5 µg of membranes prepared from the cells described in Table 1 was measured in 50 mM Tris-HCl, pH 7.4, plus 10 mM MgCl2 binding buffer containing 0.1 mM ascorbic acid for 2 h at 25 °C. For saturation binding experiments, ICYP concentrations ranging between 5 and 300 pm were used to calculate the Kd and the Bmax values for ICYP binding by parametric fitting of the data by using the Prism 4 software (GraphPad Corp.).

Antibodies, siRNA, Peptides, and Additional Reagents—The monoclonal antibodies against FLAG (M2) and Myc (9E-10) epitopes were purchased from Sigma and Upstate (Charlottesville, VA), respectively. The antibodies to human AKAP79 and to the various subunits of PKA were from Clontech; anti-human SAP97 monoclonal antibody was from StressGen (VAMP-PS005, Nventa Corp., San Jose, CA); anti-SAP97 polyclonal antibody was from Novus Biologicals (NB 600-1229); anti-human β1-AR antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-PDZ-95 antibodies (anti-pan-PDZ monocular antibody 05-427 directed against the PDZ domain (residues 77–299) of human PSD-95 and anti-PDZ antibody 05-494) were from Upstate Biotechnology, Inc. The siRNA to AKAP79 5′-AAagagacuagacagagguau-3′ and its scrambled control AAagacacagagguauac were described (1). The siRNA sequence to human SAP97, 5′-GATATCCAGGAACATAAAT-3′, or its control, 5′-CCATAATAACGGTATAA-3′, were cloned into the pSuper™ plasmid (OligoEngine Corp., Seattle, WA).
Trafficcking of the β₁-AR by a PDZ-anchored Receptosome

...ected with the desired plasmids using the Lipofectamine reagent (Invitrogen) for 24–36 h. After transfection, cells were plated on poly-l-lysine-covered coverslips for 24 h, fixed with 4% paraformaldehyde, pH 7.4, and mounted onto glass slides in Fluoromount G mounting media (Electron Microscopy Sciences, Hatfield, PA). Coverslips were sealed with clear nail polish and imaged using the sensitized emission or the acceptor photobleaching methods described in Gardner et al. (1). After image acquisition, the LSM 510 FRET macro tool was used to calculate FRETN values. FRETN is a measure of FRET that is normalized for the concentrations of donor and acceptor fluorophores and therefore represents a fully corrected measure of FRET (17–19). In this method the corrected FRET value for each pixel is calculated and then divided by concentration values for donor and acceptor (18–20). FRETN was calculated on a pixel-by-pixel basis for the entire image.

In addition to FRETN microscopy, we performed acceptor photobleaching FRET microscopy. This method measures changes in the intensity of the donor channel that are observed upon complete photobleaching of the acceptor (YFP) by a 514 nm argon laser (1, 16–18). From each photobleaching session, an image set consisting of time-lapse recordings of donor and acceptor channel intensities was obtained. FRET was recorded by examining the loss of quenching of CFP during YFP photobleaching, followed by an analysis of these images by the LSM FRET tool version 1.5 (AIM software release 3.2) to calculate the FRET efficiencies using selected area averages for donor CFP before and after bleaching. FRET efficiencies (%) are presented as the means ± S.E. from 3 to 10 separate acquisition experiments on 5–10 images per experiment.

Co-immunoprecipitations and Pulldown Assays—Co-immunoprecipitations between FLAG- or Myc-tagged β₁-AR, SAP97, AKAP79, or the RII α-subunit of PKA were performed as follows. Cells stably expressing the indicated FLAG-tagged or Myc-tagged β₁-AR were lysed in radioimmune precipitation assay buffer (1), and the insoluble cellular debris was removed by centrifugation at 14,000 × g_{av} for 15 min at 4 °C. After equalizing protein concentrations across all samples, lysates were added to M2 anti-FLAG- or anti-Myc-agarose beads at 4 °C with gentle rotation for 4 h. Control experiments were performed by incubating lysates with preimmune IgG at the same concentration for 4 h at 4 °C. The immune complexes were washed three times in radioimmune precipitation assay buffer and eluted from the beads with 40 μl of 2× Laemmli sample buffer containing 20 mM dithiothreitol. Resolved proteins and lysate inputs were separated by SDS-PAGE under denaturing conditions and electroblotted to nitrocellulose. Identical gels were run and transferred for separate detection of receptor, AKAP79, SAP97, or the RIIα subunit of PKA by Western blotting.

Pulldown Assays—The human β₁-AR cDNA was digested with Smal and XhoI to isolate the carboxy-terminal fragment encoding the amino acids between 425 and 477. This fragment and the corresponding carboxy-terminal tail fragment of the β₂-AR were cloned into the pGEX-4T-2 GST vector (GE Healthcare) and amplified in BL-21 Escherichia coli cells. HEK-293 cells that were transfected either with empty or with myc-SAP97 expressing vector were lysed with in 0.2% Triton X-100 in PBS supplemented with protease inhibitors. After 16,000 × g_{av} centrifugation of cell lysates, GST or β₁-AR_c-tail or β₂-AR_c-tail-GST fusion proteins were added to aliquots of the supernatants. Twenty μl of glutathione-agarose beads (50% slurry in H_2O) were added after mixing for 30 min at 4 °C. The mixture was mixed for another 2 h at 4 °C. After washing three times with the same lysis buffer, the proteins were eluted from beads with sample buffer (containing 2.5% β-mercaptoethanol). Eluates were separated on a 4–15% gel and analyzed for SAP97 by immunoblotting. Far Western blots were performed to detect the interaction between FLAG-β₁-AR (WT and ΔPDZ2) and SAP97 according to Hall (21) with a few modifications. In brief, the purified receptor was slot-blotted onto a dry nitrocellulose membrane in a volume of 100 μl (20 μl at a time was added) under vacuum (manifold II). The membrane was wetted in 5 ml of Tris-buffered saline containing 0.2% Tween 20 (TBST) and then blocked with TBST containing 10 mg/ml bovine serum albumin (TBST-BSA) for 1 h at 22 °C by gentle shaking. Affinity purified Myc-SAP97 was hybridized with the membranes in TBST-BSA for 16 h at 4 °C with gentle rocking. The membrane was washed five times (5 min each with TBST) and probed with 1:1000 dilution of anti-Myc IgG (9E-10 monoclonal antibody) for 1 h at 22 °C in TBST-BSA. After washing the blot five times with TBST, it was probed with horseradish peroxidase-conjugated anti-mouse IgG (Pierce) in TBST-BSA for 20 min at room temperature. The blot was washed five times in TBST (5 min each) and then developed using enhanced chemiluminescence.

Cyclic AMP Accumulation and Adenylyl Cyclase Assays—HEK-293 cells stably expressing the various β₁-AR constructs in 6-well plates were switched to DMEM + 25 mM HEPES for 2 h. Appropriate drugs in DMEM/HEPES, supplemented with 300 μM of the phosphodiesterase inhibitor isobutylmethylxanthine, were added to the cells for 10 min at 37 °C. The reaction was stopped, and 1 ml of 0.1 N cold HCl was added followed by freezing of the entire plate in liquid nitrogen. Frozen plates were quickly thawed at 65 °C to break the cells, and the cell extract was lyophilized. The dry pellet was resuspended in assay buffer, and cyclic AMP was quantified by radioimmunoassay (RIANEN Assay System; PerkinElmer Life Sciences). For the determination of adenylyl cyclase activity, membranes were prepared from cells without phenylmethylsulfonyl fluoride, and the activity of adenylyl cyclase in response to increasing concentrations of isoproterenol was determined (14, 16). The concentration-response curves to isoproterenol were fitted by nonlinear regression using Prism 4.1 software (GraphPad Corp.) in order to determine the concentration of isoproterenol that generated 50% of the maximal response (EC_{50}) for each β₁-AR construct.

Adenylyl Cyclase Assays for β-AR Desensitization and Resensitization—HEK-293 cells stably expressing the various β₁-AR constructs were divided into four sets. The first and second sets were used as control for desensitization and the third and fourth sets for resensitization assays. Cells for desensitization were exposed to 1 mM ascorbic acid (control) or 10 μM isoproterenol for 10 min at 37 °C and then processed for the preparation of membranes. The third set was used as the control for resensitization and the fourth set for resensitization assays. Cells for resensitization were exposed either to 1 mM ascorbic acid (control) or to 10 μM isoproterenol for 3 h at 37 °C.
and then incubated with 100 μM alprenolol for 1.5 h at 37 °C, followed by the preparation of membranes. Adenylyl cyclase activities in these membranes were determined (14,16), and the K_{act} ± S.E. for each β₁-AR was calculated using the Prism 4 program, and statistical comparisons were analyzed using Prism 4 and Instat programs (GraphPad Corp.).

**Phosphorylation and Phosphopeptide Mapping of the β₁-AR—**

To determine the effect of disrupting the β₁-AR PDZ or down-regulation of SAP97 on isoproterenol-mediated phosphorylation of the β₁-AR, HEK-293 cells expressing the WT β₁-AR were transfected with the SAP97 siRNA vector or its scrambled control. On the day of the experiment, the ATP pools were labeled with 200 μCi of 32PO₄/ml for 1.5 h, and the cells were stimulated with either 1 mM ascorbic acid or 10 μM isoproterenol in 1 mM ascobic acid for 10 min at 37 °C. After cell lysis, equivalent amounts of proteins in each supernatant were incubated with M2 anti-FLAG-agarose beads at 4 °C for 5 h. The resins were washed in radioimmuneprecipitation assay buffer, and the eluted proteins were resolved by SDS-PAGE. The gels were transferred to nitrocellulose, and amounts of 32P incorporated into the β₁-AR were determined by electronic counting with Packard Instamager™. The bands corresponding to phosphorylated β₁-AR protein on the filter were cut out and submerged in 70% (v/v) formic acid containing 100 mg per ml of cyanogen bromide (Science Lab Chemicals, Kingswood, TX) for 1.5 h at room temperature (1). At the end of the digestion, the samples were lyophilized and dissolved in Tricine sample buffer. Then 5 μl from each sample was spotted onto a GF/C filter pre-moistened with 10% trichloroacetic acid. The filters were mounted on a filtration manifold and washed three times with 5 ml of 10% trichloroacetic acid to remove the free 32P. After drying, the counts/min of 32P/filter were determined by liquid scintillation spectrometry. Equal counts/min (1,200 ± 50) of 32P were loaded per lane and subjected to electrophoresis on 16% acrylamide gels in Tricine cathode buffer. At the end of the run the gel was electroblotted to nitrocellulose, and the proteins were transferred to nitrocellulose, and amounts of 32P were determined (1,200 ± 50) of 32P were loaded per lane and subjected to electrophoresis on 16% acrylamide gels in Tricine cathode buffer. At the end of the run the gel was electroblotted to nitrocellulose, and the filters were counted by the Instamager™ and then exposed to an x-ray film overnight.

**Biotinylation Assay of β₁-AR Recycling with Cleavable Biotin—**

Cells expressing the WT β₁-AR with siRNA to SAP97 or its control were surface-biotinylated with 1.5 mg/ml sulfo-NHS-SS-biotin (Pierce) in Hanks’ balanced salt solution with Ca²⁺ and Mg²⁺ at 4 °C (1). Biotinylated cells were exposed to isoproterenol for 30 min and then cooled to 4 °C to stop membrane trafficking, and the remaining surface biotin was quantitatively cleaved with glutathione. After cleavage, warm DMEM was added, and cells were incubated at 37 °C for 15, 30, and 60 min to allow internalized receptor to recycle before the cells were cooled to 4 °C and incubated with glutathione cleavage buffer for a second time to ensure complete cleavage of any newly appearing surface biotin. At the end of each time point, the cells were scraped into detergent-free lysis buffer, sonicated, and then centrifuged at 100,000 × g_{av} for 20 min at 2 °C. The membrane pellet was dissolved in lysis buffer supplemented with detergents and recentrifuged at 100,000 × g_{av} for 20 min at 2 °C. The supernatant was collected, and equal amounts of protein from all samples were mixed with 50 μl of BSA-blocked ultralink-neutravidin beads (Pierce) to isolate the biotinylated proteins. The resin was extracted, and the extracts were subjected to immunoblotting with anti-FLAG antibody to determine the density of β₁-AR.

**RESULTS**

**Characterization of AKAP79 Binding to the β₁-AR—**

Myc-tagged β₁-AR constructs with progressive deletions within their carboxyl termini were co-expressed with FLAG-AKAP79 in order to localize by co-immunoprecipitations the sequence in the β₁-AR that bound AKAP79 (Fig. 1A). Deletion of the amino acids between 425 and 441 in the carboxyl-terminal tail of the β₁-AR had little effect on the immunoprecipitation of AKAP79 by this β₁-AR mutant. Deletion of the sequence between 425 and 463 significantly reduced the immunoprecipitation of AKAP79, indicating that the binding site between AKAP79 and the β₁-AR partially overlapped with this sequence. Mutagenic inactivation (Δ) of each residue in the type 1 PDZ sequence (ESKV) between amino acids 474 and 477 to alanine (β₁-ARΔPDZ) completely inhibited the interaction between the β₁-AR and AKAP79, confirming that the AKAP79 interaction site overlapped with the type 1 PDZ sequence. The interactions between AKAP79 and the β₁-AR are involved in recycling of the agonist-internalized β₁-AR back into the cell membrane in a process termed “resensitization” (1, 11). This process is involved in trafficking of the agonist-internalized β₁-AR back to the cell membrane (Fig. 1B, images a–e). Inhibition of the binding of AKAP79 to the β₁-AR by inactivating the PDZ pre-
TABLE 1

| Construct | $B_{\text{max}}$ (pmol β1-AR/mg) | $K_D$ for ICYP (µM) | EC50 value for isoproterenol-mediated activation of adenylyl cyclase (µM) |
|-----------|-------------------------------|---------------------|------------------------------------------------------------------|
| FLAG-tagged WT β1-AR                | 1.05 ± 0.12               | 21 ± 1              | 0.46 ± 0.08                                                        |
| FLAG-tagged β1-ARΔPDZ               | 1.2 ± 0.1                 | 29 ± 2              | 0.7 ± 0.1                                                         |
| Myc-tagged WT β1-AR                 | 1.4 ± 0.3                 | 24 ± 1              | 0.5 ± 0.1                                                         |
| Myc-tagged β1-ARΔPDZ                | 0.9 ± 0.15                | 29 ± 2              | 0.7 ± 0.1                                                         |
| β1-AR-CFP                           | 0.8 ± 0.1                 | 30 ± 2              | 1 ± 0.2                                                          |
| β1-AR-YFP                           | 1.1 ± 0.2                 | 31 ± 2              | 0.8 ± 0.2                                                         |
| FLAG-tagged S312D                   | 1.85 ± 0.1                | 27 ± 2              | 0.7 ± 0.1                                                         |

To characterize the mechanism by which AKAP79 binds to the β1-AR, full-length WT β1-AR or β1-AR ΔPDZ was hybridized to immobilized AKAP79 (Fig. 3, A and B). In these far Western assays, no direct binding of AKAP79 to either β1-AR construct was observed, indicating that these proteins did not interact directly. An alternative mechanism that can account for indirect binding of AKAP79 to the β1-AR is through their mutual association with MAGUK proteins (22). MAGUK proteins related to the PSD/SAP family (PSD-95/SAP90, SAP97/hdlg, Chasyn-110/PSD-93, and SAP102) share a common domain organization consisting of three PDZ domains in their amino-terminal half that bind to type 1 PDZs and Src homology 3 and guanylate kinase-like domains at their carboxyl terminus that bind to AKAP79 and other proteins (22-24). MAGUK proteins related to PSD-95 and MAGI-II have been shown to bind to the β1-AR PDZ (25, 26). Therefore, HEK-293 cells were probed for the expression of these proteins, but none of them was detected in this cell line. Consequently, we explored whether other MAGUK-related proteins were expressed in HEK-293 cells by probing cell extracts with a pan-PDZ antibody (Upstate Biotechnology, Inc.), which identified a prominent immunoreactive species with an apparent molecular mass of 110–116 kDa (data not shown). This protein was confirmed as human SAP97 by Western blotting with a monoclonal antibody to human SAP97 (Fig. 3C) and with a polyclonal anti-
After the removal of isoproterenol, the internalized control cells, Cy-3-labeled SAP97 (Fig. 3) terminus of the WT isoproterenol caused the internalization of the other intracellular compartments (24). Exposing these cells to cells (Fig. 3) altering the cellular distribution of SAP97 (Fig. 3). 

**FIGURE 3. Identification of SAP97 as an interacting partner at the carboxyl-terminal type 1 PDZ of the β₁-AR.** A and B, β₁-AR does not interact with AKAP79 in far Western blotting assays. FLAG-WT β₁-AR and FLAG-β₁-ARΔPDZ (~50 ng) were immobilized onto nitrocellulose filters. The filters were hybridized with 100 ng/ml FLAG-AKAP79, washed, and then probed with the anti-AKAP79 monoclonal antibody. C, HEK-293 cell lysates express SAP97. D, immobilized Myc-SAP97 (~50 ng) interacts with FLAG-β₁-AR but not with FLAG-β₁-ARΔPDZ in far Western assays. E, purified GST-β₁-AR-carboxy-terminal tail (between amino acids 425–477, left panel) binds preferentially to SAP97 in pulldown assays that were conducted as described under “Experimental Procedures.” F, Myc-SAP97 was transiently transfected into a HEK-293 cell line stably expressing the WT β₁-AR-YFP. After 2 days, the cells were exposed to ascorbic acid (No ISO) or to 10 μM isoproterenol for 30 min, acid-washed, and treated with 100 μM alprenolol for 0, 10, 20, 30, and 45 min. At the end of each time period, the cells were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100 in PBS, stained with Cy-3 conjugated to anti-Myc 9E-10 monoclonal antibody, and visualized by dual confocal microscopy (YFP, λ_em = 514 nm, λ_exc = 530 nm; Cy3, λ_em = 543 nm, λ_exc = 560 nm) using LSM-510 multitracking configuration. IB, immunoblot; CBB, Coomassie Brilliant Blue; ISO, isoproterenol.

SAP97 antibody (data not shown). Far Western assays between the β₁-AR and SAP97 showed direct binding between SAP97 and the full-length β₁-AR, which was abrogated when the PDZ domain in the β₁-AR was mutated (Fig. 3D). Pulldown assays between SAP97 and glutathione S-transferase (GST) fusions of full-length carboxyl termini of the β₁-AR and the β₂-AR indicated that SAP97 preferentially associated with the carboxyl terminus of the human β₁-AR (Fig. 3E).

To further confirm that the β₁-AR could bind to SAP97, we determined whether they were co-localized. Myc-SAP97 was transiently transfected into a HEK-293 cell line stably expressing the WT β₁-AR and the β₁-AR indicated that SAP97 preferentially associated with the carboxyl terminus of the human β₁-AR (Fig. 3E).

The organization of this scaffold was further studied using siRNAs to SAP97 (Fig. 4, panel B). We predicted that if SAP97 was a bridging molecule between the β₁-AR and AKAP79/PKA, consequently knockdown of SAP97 should destabilize the binding between the β₁-AR and AKAP79. Toward this, cells expressing 1.4 pmol/mg Myc-β₁-AR and FLAG-AKAP79 were transfected with SAP97 siRNA or its scrambled control (Fig. 4, panel B, h–m). In cells expressing the scrambled siRNA, β₁-AR precipitates co-immunoprecipitated the β₁-AR, AKAP79, and PKA assembles at the type 1 PDZ in the carboxyl-terminal tail of the β₁-AR. The organization of this scaffold was further studied using siRNAs to SAP97 (Fig. 4, panel B). We predicted that if SAP97 was a bridging molecule between the β₁-AR and AKAP79/PKA, consequently knockdown of SAP97 should destabilize the binding between the β₁-AR and AKAP79. Toward this, cells expressing 1.4 pmol/mg Myc-β₁-AR and FLAG-AKAP79 were transfected with SAP97 siRNA or its scrambled control (Fig. 4, panel B, h–m). In cells expressing the scrambled siRNA, β₁-AR precipitates co-immunoprecipitated the β₁-AR, AKAP79, and PKA assembles at the type 1 PDZ in the carboxyl-terminal tail of the β₁-AR. The organization of this scaffold was further studied using siRNAs to SAP97 (Fig. 4, panel B). We predicted that if SAP97 was a bridging molecule between the β₁-AR and AKAP79/PKA, consequently knockdown of SAP97 should destabilize the binding between the β₁-AR and AKAP79. Toward this, cells expressing 1.4 pmol/mg Myc-β₁-AR and FLAG-AKAP79 were transfected with SAP97 siRNA or its scrambled control (Fig. 4, panel B, h–m). In cells expressing the scrambled siRNA, β₁-AR precipitates co-immunoprecipitated the β₁-AR, AKAP79, and PKA assembles at the type 1 PDZ in the carboxyl-terminal tail of the β₁-AR. The organization of this scaffold was further studied using siRNAs to SAP97 (Fig. 4, panel B).
knocked down SAP97 levels (Fig. 4m). Therefore, because the SAP97 siRNA abolished the ability of the β1-AR to co-immunoprecipitate AKAP79 and SAP97, it indicates that SAP97 is likely to serve as a bridging molecule between the β1-AR and the AKAP79-PKA complex.

Characterization of the Association between the β1-AR, SAP97, and AKAP79 by FRET Microscopy—Fluorescence confocal and FRET microscopy provide data relevant to the cellular distribution and proximity of the proteins under study (1, 17–19). To determine whether SAP97 and the β1-AR interacted with one another, WT β1-AR-CFP or -YFP and SAP97-YFP or -CFP were generated. The binding and Gβγ-coupling parameters of β1-AR-CFP and β1-AR-YFP were determined by radioligand binding and adenylyl cyclase assays (Table 1 and Fig. 2C). These chimera bound ICFP with affinities (Kd) comparable with that of the WT β1-AR (Table 1). β1-AR-CFP and β1-AR-YFP chimera displayed basal activities of adenylyl cyclase that were comparable with that of the WT β1-AR (Fig. 2C). Moreover, isoproterenol generated a graded escalation in the activity of adenylyl cyclase in chimeric receptors that culminated in an ~6-fold increase in its activity, with EC50 values that were comparable with those of the WT β1-AR (Table 1).

Next, WT β1-AR-CFP and SAP97-YFP were cotransfected into HEK-293 cells, and their interaction was assessed by acceptor photobleaching FRET microscopy (Fig. 5A). The imaging data indicated that the interactions between the WT β1-AR and SAP97 were strong and displayed FRET efficiencies of 24 ± 5.0% (Table 2). To assess the influence of the PDZ site on the distribution of the β1-AR and its interaction with SAP97, FRET interaction efficiencies between the β1-ARΔPDZ-CFP and SAP97-YFP were analyzed by FRET microscopy (Fig. 5D). Data from several experiments did not reveal FRET interactions between the β1-ARΔPDZ and SAP97, confirming that the interaction between SAP97 and the β1-AR was mediated through the PDZ. Similarly, AKAP79 interacted with the full-length WT β1-AR with a moderate FRET efficiency of 12 ± 1.1% (Fig. 5E), and this interaction was abolished when the PDZ site was mutated (Fig. 5E). Finally, SAP97 and AKAP79 interacted with a FRET efficiency of 19–21%, indicative of high affinity interactions (Fig. 5C and Table 2). Previously we have shown by FRET microscopy strong interactions between the RIIα subunit of PKA and AKAP79 and between the RIIα and the WT β1-AR (1). These data complement the immunoprecipitation results in Fig. 4 that have shown binding between the SAP97-AKAP79-PKA complex and the WT β1-AR.

Additionally, we used FRET microscopy to determine the efficiency of molecular interactions between the WT β1-AR and the SAP97-AKAP79 complex in live cells (Fig. 6). This method generates high resolution, real time images of sensitized emission FRET using image subtraction, which allows comparison of FRET from multiple cells for a given acceptor-donor pair (18, 19). FRET showed strong association between the WT β1-AR and SAP97 (FRET efficiency of 23 ± 5%). Furthermore, strong interactions between SAP97 and AKAP79 (FRET efficiency = 21 ± 2%) and between the WT β1-AR and AKAP79 (FRET efficiency = 12 ± 1.1%) were observed (Fig. 6, B and C). Finally, in agreement with Nakagawa et al. (20), we confirmed the existence of SAP97 dimers because SAP97-CFP interacted with SAP97-YFP with a high FRET efficiency of 19.7 ± 3% (Fig. 6D).

Effect of the β1-AR PDZ and SAP97 on Agonist-mediated Phosphorylation of the β1-AR—To study the role of the β1-AR PDZ and its associated scaffold in signaling by the β1-AR, we determined whether inactivation of the β1-AR PDZ or knockdown of SAP97 produced comparable effects on β-agonist-mediated phosphorylation of the β1-AR. Cell lines stably expressing comparable levels of FLAG-tagged WT β1-AR (1.05 ± 0.12 pmol/mg) and of FLAG-tagged β1-ARΔPDZ (1.2 ± 0.1 pmol/
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TABLE 2

FRET efficiencies in (%) as recorded by the acceptor photobleaching method in fixed cells

| CFP donor          | YFP acceptor | WT $\beta_1$-AR | SAP97 | AKAP79 |
|--------------------|--------------|-----------------|-------|--------|
| WT $\beta_1$-AR-CFP | SAP97        | 24 ± 5          | 10 ± 2|
| AKAP79             | 24 ± 5       | 19.6 ± 3        | 19 ± 3|

* ND indicates not determined.

mg) were used. Isoproterenol-mediated phosphorylation of the WT $\beta_1$-AR or $\beta_1$-AR PDZ increased total phosphorylation of the WT $\beta_1$-AR by 6-fold and that of the $\beta_1$-AR PDZ by 2-fold (Fig. 7A). These experiments were also performed in cells expressing the FLAG-tagged WT $\beta_1$-AR with scrambled or SAP97 siRNAs. Isoproterenol increased total phosphorylation of the WT $\beta_1$-AR by 5.7-fold in control or in cells expressing the scrambled siRNA (Fig. 7A, compare lanes 1 to 2 and 5 to 6). However, in cells expressing the $\beta_1$-AR PDZ or in those co-expressing the WT $\beta_1$-AR and SAP97 siRNA, isoproterenol increased the phosphorylation of the $\beta_1$-AR by ~2.5-fold (Fig. 7A, compare lanes 3 to 4 and 7 to 8). Thus, inactivation of the $\beta_1$-AR PDZ or knockdown of SAP97 was roughly equivalent in inhibiting the phosphorylation of the $\beta_1$-AR by isoproterenol.

The two major kinases that are involved in agonist-mediated phosphorylation of the $\beta_1$-AR are the GRK and PKA (27). Thus, the underlying cause for reduced phosphorylation of the $\beta_1$-AR PDZ might be due either to inhibition of GRK, PKA, or both. The preferred substrates for phosphorylation by PKA are serine/threonine residues that are preceded by RX- or RRX- (where $X$ is any amino acid and $R$ is arginine). This organization is found solely around Ser212 in the 3rd IC, which corresponds to RRRP312 (11). The preferred substrates for phosphorylation by GRK are serine/threonine residues that are preceded by an acidic amino acid (28, 29). This organization is found around four serine residues that reside exclusively in the carboxyl terminus of the $\beta_1$-AR (27). To identify the kinase affected by knockdown of SAP97 or by the inactivation of the PDZ, the phosphorylation of the carboxyl terminus versus that of the 3rd IC should be independently determined. Therefore, the phosphorylated $\beta_1$-AR was cleaved with cyanogen bromide that cleaves the full-length $\beta_1$-AR into a 10-kDa 32P-labeled peptide that encompasses the 3rd IC and into a 15-kDa 32P-labeled peptide that encompasses the carboxyl terminus of the $\beta_1$-AR (1). Previously we have shown that isoproterenol increased the phosphorylation of the 10- and 15-kDa peptides by ~6-fold (1). However, the counts/min derived from cyano-

![Image](image-url)
gen bromide cleavage of an equivalent amount of receptor protein from cells pre-exposed to ascorbic acid were insufficient to accurately estimate the incorporation of $^{32}$P into these peptides under basal conditions (1). Therefore, the $^{32}$P-labeled $\beta_1$-ARs on nitrocellulose filters were digested with cyanogen bromide, and the amounts of $^{32}$P incorporated into the resulting peptides were determined. Then an equal number $^{32}$P counts/min derived from isoproterenol- and ascorbic acid-treated cells were subjected to electrophoresis on 16% acrylamide/Tricine gels (Fig. 7B). In the samples derived from cells pre-exposed to ascorbic acid, we observed that the 10- and 15-kDa peptides were phosphorylated at a ratio of $\sim$1:3 (Fig. 7B, lane 1). Isoproterenol increased the total phosphorylation of each peptide by $\sim$6-fold (1) but did not alter their phosphorylation ratios, indicating that both the 10- and 15-kDa peptides were substrates for isoproterenol-mediated phosphorylation (Fig. 7B, lane 2). Cleavage of $^{32}$P-$\beta_1$-ARΔPDZ from control or isoproterenol-treated cells, followed by loading the same number of $^{32}$P counts/min as in Fig. 7B, lanes 1 and 2, generated the 15-kDa phosphopeptide only (Fig. 7B, lanes 3 and 4). Thus, mutagenesis of the PDZ abrogated basal and isoproterenol-mediated phosphorylation of the 10-kDa peptide.

The next series of experiments was conducted in cells expressing the WT $\beta_1$-AR with scrambled or SAP97 siRNA (Fig. 7, lanes 5–8). We have shown that siRNA-mediated knockdwon of AKAP79 inhibited the phosphorylation of the 10-kDa peptide in response to isoproterenol (1). However, we were not able to estimate the effect of the AKAP79 siRNA on the peptides derived from ascorbic acid-treated cells. Thus, the $^{32}$P-$\beta_1$-AR with scrambled (Scr, lanes 5 and 6) or SAP97 siRNAs (lanes 7 and 8) generated 15-kDa phosphopeptides (Fig. 7B, lanes 5 and 6). Electronic counting of the $^{32}$P incorporated into the $\beta_1$-AR was as follows: lane 1, 135; lane 2, 768; lane 3, 138; lane 4, 330; lane 5, 153; lane 6, 780; lane 7, 137; and lane 8, 326. B. phosphorylated WT $\beta_1$-AR or $\beta_1$-ARΔPDZ cleaved with cyanogen bromide, hyphosphorylated, and then redissolved in Tricine sample buffer, and $^{32}$P incorporated into their peptides was determined as described under “Experimental Procedures.” Equal counts/min (1,200 ± 50) of $^{32}$P from control-treated cells (ascorbic acid) or isoproterenol-treated cells, harboring WT $\beta_1$-AR (lanes 1 and 2) or the $\beta_1$-ARΔPDZ (lanes 3 and 4), were subjected to electrophoresis on 16% acrylamide gels in Tricine cathode buffer. These experiments were repeated in cells expressing the WT $\beta_1$-AR with scrambled (Scr, lanes 5 and 6) or SAP97 siRNAs (lanes 7 and 8). Electronic counting of $^{32}$P incorporated in lane 2 indicated that the % of the counts/min in the 10- versus the 15-kDa band was $\sim$20% (190–571 cpm, respectively). In lanes 3 and 4, $\sim$635 cpm were counted in each lane that was exclusively located in the 15-kDa band. In lanes 5 and 6, the % counts/min in the 10- versus the 15-kDa band was $\sim$15% (202–623 cpm, respectively). In lanes 7 and 8, $\sim$693 cpm were counted in each lane that was exclusively located in the 15-kDa band.
**Trafficking of the β₁-AR by a PDZ-anchored Receptosome**

### A.

| Time after the removal of isoproterenol | 
|----------------------------------------|
| No Iso | Iso 30’ | Iso 30’ A/W |
| β₁-ARΔPDZ + pcDNA | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| β₁-ARΔPDZ + cPKA | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) |
| S312D β₁-ARΔPDZ | ![Image](image17.png) | ![Image](image18.png) | ![Image](image19.png) | ![Image](image20.png) | ![Image](image21.png) | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) |

**FIGURE 8. Roles of the ESKV sequence in the carboxyl-terminal tail of the β₁-AR and Ser³¹² in the 3rd IC in regulating the recycling and resensitization of the β₁-AR in response to isoproterenol.**

A. recycling of the β₁-ARΔPDZ in response to 10 μM isoproterenol (Iso). HEK-293 cells stably expressing the β₁-ARΔPDZ were mock-transfected (pcDNA) or transfected with an expression vector for the catalytic subunit of PKA (cPKA). In addition, HEK-293 cells were transiently transfected with the point mutant of β₁-ARΔPDZ in which the serine residue at position 312 was mutated to aspartic acid (S312D). Recycling of the β₁-AR in response to 10 μM isoproterenol (n = 3) for 30 min followed by an acid wash (A/W) to remove the antibody bound to extracellular β₁-AR were conducted as described under “Experimental Procedures.” Each scale bar represents 5 μm. B, summary of the results of the experiment in A are presented as line graphs of n = 3. The isoproterenol-internalized β₁-ARΔPDZ did not recycle (images d–h), whereas the β₁-ARΔPDZ with cPKA and the S312D β₁-ARΔPDZ recycling was rapid with a t₀.₅ of 25 ± 5 and 18 ± 4 min, respectively. C, comparison of adenylyl cyclase activities in response to short term isoproterenol (short term desensitization) and in response to isoproterenol followed by antagonist (desensitization followed by resensitization) treatments in HEK-293 cells expressing WT, ΔPDZ, S312D, and S312ΔPDZ constructs of the β₁-AR. These experiments were replicated (n = 3) each in triplicate.

Cross-talk between the β₁-AR PDZ Scaffold and Ser³¹²—Two distinct domains in the β₁-AR, namely Ser³¹² in the 3rd IC and the PDZ in the carboxyl-terminal tail, participate in imparting a recycling signal to the β₁-AR. If, as suggested earlier, one of the functions of the PDZ domain is to target PKA to the β₁-AR, then we hypothesized that overexpression of PKA might overcome the effect of inactivating the PDZ on recycling of the β₁-AR. To address this question, the recycling experiment for β₁-ARΔPDZ was conducted under conditions of increased PKA activation (Fig. 8A). HEK-293 cells expressing 1.2 ± 0.1 pmol/mg of FLAG-β₁-ARΔPDZ were transfected with the empty vector or with the vector expressing cPKA. In cells expressing the empty vector, the agonist-internalized β₁-ARΔPDZ did not recycle (Fig. 8A, images d–g). In cells expressing cPKA, the β₁-ARΔPDZ recycled back to the cell surface within 45 min from the removal of isoproterenol (Fig. 8A, images i–p). A boundary was drawn around the inner circumference of the cells in Fig. 8A in order to determine the distribution of pixels between membranous and intracellular compartments. The density of the pixels residing inside the boundary versus those residing outside the boundary was used as an index for internalized and membranous β₁-AR, respectively. The pixel data were plotted as a function of time after the removal of isoproterenol in order to calculate the recycling kinetics of the β₁-AR (Fig. 8B). The data indicate that in cells expressing the β₁-ARΔPDZ with the empty pcDNA vector, the internalized β₁-ARΔPDZ did not recycle (Fig. 8A, images d–g). However, in cells expressing the β₁-ARΔPDZ and cPKA, the agonist-internalized β₁-ARΔPDZ recycled with a t₀.₅ = 20 ± 5 min, indicating that super induction of cPKA overcame the
effect of the PDZ mutation on recycling of the β1-AR. If the recycled β1-ARΔPDZ was inserted properly into the cell membrane, then Cy3-conjugated anti-FLAG IgG bound to the amino-terminal FLAG epitope should be oriented extracellularly. In this case, a second acid wash would strip Cy3-IgG from the recycled receptor population. In agreement with these assumptions, we observed reduced cell fluorescence in the recycled β1-ARΔPDZ in cPKA expressing cells, but not in pcDNA expressing cells (Fig. 8A, compare image p with h). It should be emphasized however, that a 30-min pretreatment with forskolin, which activates all the isomers of adenyl cyclase and markedly activates PKA, did not restore the recycling phenotype to β1-ARΔPDZ (data not shown), suggesting that chronic activation of PKA might be necessary to restore the recycling of the β1-ARΔPDZ.

If one of the functions of the β1-AR PDZ is to facilitate the phosphorylation of Ser312 by targeting PKA to the β1-AR PDZ. This hypothesis was tested directly by generating a β1-ARPDZ construct in which the serine at position 312 was mutated to aspartic acid (S312D PDZ). Indeed the agonist-internalized S312D PDZ recycled efficiently (Fig. 8A, images t–w), with kinetics comparable with those of the WT-β1-AR (t0.5 15 ± 4 min) (Fig. 8B). Because recycling of the agonist-desensitized and internalized GPCR is a priori for its resensitization, we determined whether β1-AR-mediated activation of adenyl cyclase was functionally resensitized in those β1-ARΔPDZ constructs that were capable of recycling (Fig. 8C). Rapid desensitization of adenyl cyclase in membranes expressing all the four β1-AR constructs described in Fig. 8C was observed after 10 min of exposing the cells to isoproterenol, indicating that mutagenesis of either the PDZ or Ser312 alone or in combination did not affect short term desensitization of the receptor. The resensitization assay involves the desensitization of the β1-AR by exposing cells to isoproterenol for 3 h, followed by incubating the cells with 100 μM of the β1-antagonist alprenolol to induce the recycling of the β1-AR and subsequent resensitization of its adenyl cyclase activity (1, 11). In this assay, we observed the resensitization of adenyl cyclase activity in the WT β1-AR, but the activity of adenyl cyclase of the β1-ARΔPDZ was significantly desensitized (Fig. 8C). Functional resensitization of adenyl cyclase activity of the β1-ARΔPDZ was restored in the context of S312D PDZ construct, indicating that the modification of Ser312 to its phosphoserine mimic “aspartic acid” resuscitated the recycling and resensitization of β1-ARΔPDZ.

Characterization of the Role of SAP97 in Recycling of the Human β1-AR—Thus far, we have shown that cross-talk between the β1-AR PDZ domain and Ser312 was involved in regulating the recycling and resensitization of the β1-AR in HEK-293 cells. To determine the role of SAP97 in this phenomenon, the effects of SAP97 knockdown and overexpression on recycling of the WT β1-AR were assessed (Figs. 9 and 10). In cells stably expressing 1.1 pmol/mg protein of WT β1-AR-YFP, knockdown of SAP97 inhibited the recycling of the WT β1-AR as determined by the confocal recycling assay (Fig. 9A, images i–l). However, knockdown of SAP97 had variable effects on agonist-induced internalization of the β1-AR as well, whereas in some cells SAP97 had no effect on internalization, and in others it reduced the internalization by ~35% (compare internal pixels in Fig. 9A, images h versus b). Therefore the effect of knockdown of SAP97 on trafficking of the β1-AR was determined by surface biotinylation because in this assay the internalization and recycling data are derived from the entire cell population rather than from few imaged cells (1). HEK-293 cells stably expressing FLAG-tagged WT β1-AR with scrambled or SAP97 siRNA were surface-biotinylated with cleavable biotin followed by quenching of excess biotin with glycine. The amount of biotin incorporated into the β1-AR under this condition indexed total cellular β1-AR biotinylation (Fig. 9B, lanes 1 and 6). The cells were then exposed to isoproterenol for 30 min, followed by cleavage of the remaining cell surface biotin (Fig. 9B, lanes 2 and 7). The amount of biotin recovered in this step indexed the amount of biotinylated β1-AR that was internalized in response to isoproterenol, whereas the ratio of internal to total biotin indexed the percentile of total receptors that were internalized. Isoproterenol induced the internalization of ~60% of total β1-AR in control cells, whereas the internalization of the β1-AR in SAP97 siRNA-treated cells was reduced by ~20 (n = 4). To initiate recycling, isoproterenol was replaced with the β-antagonist alprenolol, and the cells were warmed to 37 °C for an additional 15, 30, or 60 min (Fig. 9B, lanes 3–5 and 8–10). After each time period, the cells were cooled to 4 °C, and biotin was cleaved for the second time to ensure cleavage of any newly appearing “recycled” surface biotin. Thus, the loss of biotin from the second cleavage step indexed the recycling of the β1-AR. The data indicate that by 60 min, more than 90% of the biotin was lost from the β1-AR in cells expressing the scrambled siRNA, reflecting membrane recycling and subsequent biotin cleavage (Fig. 9B, lanes 3–5). In contrast, the internalized (biotinylated) β1-AR in cells expressing SAP97 siRNA was not changed even after 1 h from the removal of isoproterenol, reflecting their internal distribution (Fig. 9B, compare lanes 9 and 10 with lanes 4 and 5). Next, we quantified the amount of biotin remaining as a function of time after the removal of isoproterenol and determined that the β1-AR recycled with a t0.5 of 25 ± 5 min (Fig. 9C).

In follow-up experiments, the effect of overexpression of Myc-SAP97 or Myc-PSD-95 in cells stably expressing 1.1 ± 0.2 pmol/mg protein of WT β1-AR-CFP on isoproterenol-mediated β1-AR internalization and recycling was determined by confocal microscopy (Fig. 10A). The rationale for analyzing the effect of PSD-95 is that PSD-95 binds to the β1-AR PDZ with high affinity and interferes with the internalization of the receptor, but its effect on recycling is unknown (26). However, because HEK-293 cells do not express PSD-95 (Fig. 10B), PSD-95 was overexpressed along with SAP97. Overexpression of SAP97 did not affect the rate or magnitude of isoproterenol-mediated internalization of the β1-AR (t0.5 = 5 min ± 1 min). On the other hand, overexpression of PSD-95 markedly inhibited the magnitude (~50%) and rate of isoproterenol-mediated internalization of the WT β1-AR (Fig. 10A, images u–z, and C). Overexpression of SAP97 did not affect the rate or magnitude of β1-AR recycling, but PSD-95 reduced both the rate and the magnitude of β1-AR recycling (Fig. 10C). Thus, these MAGUK
proteins exerted different effects on internalization, recycling, and resensitization of the $\beta_1$-AR. Finally, knockdown of AKAP79 or SAP97 in conjunction with FRET microscopy was conducted to determine the organization of the scaffolding complex that binds to the $\beta_1$-AR PDZ (Fig. 11). Knockdown of AKAP79 did not prevent the association between the WT $\beta_1$-AR-YFP and SAP97-CFP as assessed by acceptor photobleaching FRET microscopy (Fig. 11A). Knockdown of SAP97, however, abolished the interaction between WT $\beta_1$-AR and AKAP79, indicating that SAP97 served as bridging molecule between the $\beta_1$-AR and the AKAP79-PKA complex (Fig. 11, B and C).

**DISCUSSION**

Type 1 PDZ domains in the tails of GPCR such as the $\beta_1$- or the $\beta_2$-AR are necessary for efficient recycling of these receptors (10, 30–32). The type 1 PDZ in the human $\beta_1$-AR has attracted wide attention because it interacted with numerous proteins related to the MAGUK family, and these interactions exerted different effects on its functions. The association of the $\beta_1$-AR with the MAGUK protein PSD-95, for example, inhibited the internalization of the $\beta_1$-AR but facilitated its interaction with N-methyl-D-aspartate (NMDA) receptors (26). Another family of PDZ-interacting proteins was the MAGI-related proteins, which increased the magnitude of agonist-induced internalization of the $\beta_1$-AR (25, 33). Two additional PDZ-binding proteins were shown to interact with the type 1-PDZ in the carboxyl-terminal tail of the $\beta_1$-AR. The first was a protein called the $\beta_1$-AR-binding partner, which is involved in regulating $\beta_1$-AR-mediated activation of extracellular signal-regulated kinases 1/2 (34). The other was the cystic fibrosis transmembrane conductance regulator-associated ligand, which is involved in surface expression of the $\beta_1$-AR (35).

These MAGUK proteins, however, are static entities because they lack ATP-binding and catalytic core motifs and are thought to function as adaptor proteins (23). Therefore, in addition to identifying SAP97 as a novel MAGUK protein that interacts with the $\beta_1$-AR PDZ, we have confirmed that an AKAP79-PKA complex binds to SAP97. This novel organiza-
A. Time after the addition of isoproterenol

| Time (min) | WT β₁-AR-YFP + pcDNA 3.1 | WT β₁-AR-YFP + Myc-SAP97 | WT β₁-AR-YFP + Myc-PSD95 |
|-----------|--------------------------|--------------------------|--------------------------|
| 0         | ![image](117x591 to 158x623) | ![image](117x631 to 158x663) | ![image](117x671 to 159x703) |
| 2         | ![image](118x671 to 159x703) | ![image](118x671 to 159x703) | ![image](118x671 to 159x703) |
| 3         | ![image](118x671 to 159x703) | ![image](118x671 to 159x703) | ![image](118x671 to 159x703) |
| 5         | ![image](118x671 to 159x703) | ![image](118x671 to 159x703) | ![image](118x671 to 159x703) |
| 10        | ![image](118x671 to 159x703) | ![image](118x671 to 159x703) | ![image](118x671 to 159x703) |
| 30        | ![image](118x671 to 159x703) | ![image](118x671 to 159x703) | ![image](118x671 to 159x703) |

B. pcDNA Myc-SAP97

IB:SAP97

C. pcDNA Myc-PSD95

IB:PSD95

C. Membrane β₁-AR (100) vs. Time (min)

FIGURE 10. Effect of overexpression of SAP97 or PSD95 on isoproterenol-mediated internalization and recycling of the WT-β₁-AR. A, cells stably expressing 1.1 pmol/mg protein of WT β₁-AR-YFP were transfected with the empty pcDNA 3.1 vector (upper panel), Myc-SAP97 (middle panel), or Myc-PSD95 (lower panel) to determine the effects of MAGUK proteins on the internalization and recycling of the WT β₁-AR. B, blots of HEK-293 cell extracts (5 μg in each lane) prepared from mock (pcDNA 3.1), Myc-SAP97, or Myc-PSD95 transfected cells. IB, immunoblot. C, quantification of WT β₁-AR internalization and recycling in control HEK-293 cells or in cells overexpressing SAP97 or PSD95. The effect of isoproterenol on the distribution of YFP pixels outside versus those inside a 300-nm boundary in images a–f, k–p, and u–z, was determined. Because the number of pixels inside the boundary progressively increased after the addition of isoproterenol, this was reflected as a reduction in the percentile of membranous receptors in the + isoproterenol images. After the initiation of recycling (−isoproterenol), there was a progressive decline in the pixels inside the 300-nm boundary and a corresponding increase in the pixels outside this boundary in images g–j, q–t, and a′–d′. The data are derived from n = 2 experiments each utilizing 5–7 cell images per time point.
Ser$^{312}$, thereby providing a vivid example of cross-talk between a domain in the extreme carboxy terminus and another in the 3rd IC of the GPCR (Figs. 7 and 11C). If the phosphorylation of Ser$^{312}$ lies downstream from the PDZ in setting the trafficking itinerary for the $\beta_1$-AR, then the PDZ-generated recycling signal, which is phospho-Ser$^{312}$, could be replicated either by super-induction of PKA or by mutagenesis of Ser$^{312}$ in the 3rd IC of the $\beta_1$-AR to the phosphoserine mimic aspartic acid (S312D). Our data in Fig. 8 showed that the $\beta_1$-ARΔPDZ would recycle under conditions where the catalytic activity of PKA was chronically elevated or when its putative target Ser$^{312}$ in the $\beta_1$-ARΔPDZ mutant was replaced by its phosphoserine mimic aspartic acid. These findings imply that Ser$^{312}$ is downstream from the PDZ and apparently occupies a more dominant position than the type 1 PDZ in setting the trafficking itinerary of the $\beta_1$-AR. We arrived at this conclusion because our model extended beyond the binary MAGUK-PDZ model into a quaternary model that incorporates an AKAP, which is involved in targeting PKA to the $\beta_1$-AR microdomain (Fig. 11C).

The role of PKA targeting in setting the trafficking itinerary of GPCR is a nascent field with relatively few reports. The role of PKA in translocating aquaporin-2 from intracellular compartments into cell membranes in response to vasopressin and in recycling of agonist-internalized NMDA receptors is well documented (15, 37). Nevertheless, beyond these few examples, the significance of PKA and its targeting to the PDZ motif in trafficking of the agonist-internalized GPCR remains to be substantiated. Recently, the EBP-50-binding protein ezrin was identified as an AKAP-like protein, which binds to the RI subunit of PKA, instead of the more common RII subunit (38). These data suggest that EBP-50, which binds to the $\beta_2$-AR PDZ and is required for the recycling of the $\beta_2$-AR, targets an AKAP-like protein to the $\beta_2$-AR PDZ. Similarly, aquaporin-2 has a type 1 “G(S/T)KA” PDZ sequence at its extreme carboxy terminus, and its recycling requires EBP-50 and involves AKAP/PKA-mediated phosphorylation of Ser$^{256}$ (37). These observations suggest that PDZ-mediated targeting of PKA to the GPCR might be more common than expected and could be involved in the recycling of these and other proteins.

The identification of SAP97 as an organizer of a scaffold composed of AKAP79 and PKA converts the PDZ-binding domain from a static multiprotein-binding complex into a dynamic network with increased number, range, and intensity of signals that are transmitted via the $\beta_1$-AR. The PDZ domains of the PSD/SAP family of MAGUK proteins interact with the carboxy-terminal type 1 PDZ motif found in a variety of membrane and intracellular proteins, including Shaker K$^+$ channels, NMDA receptors, and the $\beta_1$-AR, which binds to PDZ-3 domain of SAP97 (33, 39, 40). The Src homology 3 and guanylate kinase domains are involved in the clustering activity of SAP97 because they bind to the GKAP/PAPAP/DAP family of postsynaptic density proteins as well as to many other signaling/scaffolding proteins, including AKAP79 and other PKA-binding proteins (22, 41). SAP97 contains an additional L27 domain in its amino terminus that is involved in dimerization of SAP97 and in binding to other MAGUK proteins such as mLIN-2/CASK and DLG3 (20, 23). These protein-protein interactions can therefore diversify the signaling of the $\beta_1$-AR and may explain some of the neuronal and cardiovascular functions attributed to the $\beta_1$-AR. For example, myocardial $\beta_1$-ARs selectively phosphorylate via PKA many substrates that are involved in regulating myocardial contractility through AKAP-dependent mechanisms (42). In neurons, SAP97 and AKAP79 both interact with the GluR1 $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunit, and the interaction between SAP97 and AKAP79 might recruit PKA to ionotropic glutamate receptors (17, 22). Thus it is conceivable that multiplexing between a $\beta_1$-AR-MAGUK-AKAP79 complex might scaffold $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors, L-type Ca$^{2+}$ channels, and other signaling molecules to the $\beta_1$-AR microdomain to facilitate their phosphorylation by the $\beta_1$-AR signaling pathway as has been observed in hippocampal neurons (43). These scaffold-mediated connections between the $\beta_1$-AR and voltage-gated channels, for exam-
ple, might underlie the observed effects of the β1-AR on synaptic plasticity (44, 45) and in the formation of emotionally charged memories that could result in post-traumatic stress disorders (46–48). The mechanism of β1-selective β-blockers in blocking the reconsolidation of traumatic memories, especially those following acts of terror, might be explained by the mélangé of proteins that are scaffolded directly or indirectly with the β1-AR through this novel receptosome (49, 50).

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