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Interaction with Cystic Fibrosis Transmembrane Conductance Regulator-associated Ligand (CAL) Inhibits β1-Adrenergic Receptor Surface Expression*

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G protein-coupled receptors such as the β1-adrenergic receptor (β1AR) must be trafficked to the plasma membrane in order to bind with their extracellular ligands and regulate cellular physiology. By using glutathione *S*-transferase pull-down techniques, we found that the β1AR carboxyl terminus directly interacts with the cystic fibrosis transmembrane conductance regulator-associated ligand (CAL; also known as PIST, GOPC, and FIG), a protein known to be primarily localized to the Golgi apparatus. CAL contains two predicted coiled-coil domains and one PSD-95/Discs-large/ZO-1 homology (PDZ) domain. The β1AR carboxyl terminus (CT) binds to the PDZ domain of CAL, with the last few amino acids (ESKV) of the β1AR-CT being the key determinants for the interaction. Mutation of the terminal valine residue resulted in markedly reduced association of the β1AR-CT with CAL. Numerous other mutations to the ESKV motif also impaired the β1AR-CT/CAL interaction, suggesting that this motif is close to optimal for association with the CAL PDZ domain. In cells, full-length β1AR robustly associates with CAL, and this interaction is abolished by mutation of the terminal valine to alanine of the receptor (V477A), as determined by co-immunoprecipitation experiments and immunofluorescence co-localization studies. Consistent with observations that CAL is a Golgi-associated protein, overexpression of CAL reduces surface expression of β1AR. Interaction with CAL promotes retention of β1AR within the cell, whereas PSD-95, another β1AR-associated PDZ domain-containing protein, competitively blocks β1AR association with CAL and promotes receptor trafficking to the cell surface. These data reveal that CAL, a novel β1AR-binding partner, modulates β1AR intracellular trafficking, thereby revealing a new mechanism of regulation for β1AR anterograde trafficking through the endoplasmic reticulum-Golgi complex to the plasma membrane.

β-Adrenergic receptors (βARs)[1] are G protein-coupled receptors (GPCRs) that play critical roles in mediating physiological responses to the hormone epinephrine and the neurotransmitter norepinephrine. Noradrenergic stimulation of β1-adrenergic receptor (β1AR) in the brain potently regulates memory formation and synaptic plasticity (1–6), and stimulation of β1AR in the heart profoundly regulates both the rate and force of cardiac contractions (7–9). Neither norepinephrine nor epinephrine can easily cross the plasma membrane; thus, β1AR must be localized at the cell surface in order to be activated by its ligands. The regulation of the trafficking and surface expression of β1AR and other GPCRs is therefore a topic of considerable physiological importance.

Intracellular trafficking of GPCRs has been extensively studied, with most of this work focused on the events involved in receptor internalization and recycling. Within seconds or minutes of agonist stimulation, βARs are phosphorylated by G protein-coupled receptor kinases and cAMP-dependent protein kinases (10, 11). Subsequently, in the cases of β1AR and β2AR, this phosphorylation promotes the binding of the cytosolic protein β-arrestin, which inhibits the ability of the βARs to couple to G proteins and targets the phosphorylated receptors to clathrin-coated pits for endocytosis. In the cytoplasm, the internalized receptors are first accumulated in early endosomes, dephosphorylated, and subsequently recycled back via a perinuclear compartment to the plasma membrane in a fully resensitized state (10, 11).

PDZ domain-containing scaffold proteins have recently been shown to modulate βAR intracellular trafficking via specific interactions with the carboxyl termini of β1AR and β2AR (12–16). PDZ domains are named for the first three proteins in which they were identified (the post-synaptic density protein PSD-95, the *Drosophila* tumor suppressor protein Dlg, and the tight junction protein ZO-1), and are known to bind to their target proteins through specific carboxyl-terminal motifs (17). The Na^+/H^+ exchanger regulatory factor (NHERF-1), a PDZ domain-containing protein, is known to associate with the β2-adrenergic receptor (β2AR) to control β2AR-mediated regulation of Na^+/H^+ exchange (12) and also to modulate β2AR endocytic sorting (13). β1AR does not interact with NHERF-1

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[1] The abbreviations used are: βARs, β-adrenergic receptors; PDZ, PSD-95/Discs-large/ZO-1 homology; NHERF-1, Na^+/H^+ exchanger regulatory factor; CFTR, cystic fibrosis transmembrane conductance regulator; GST, glutathione *S*-transferase; CT, carboxyl-terminus; PSD-95, postsynaptic density protein of 95 kDa; MAGI, membrane-associated guanylate kinase-like protein with an inverted domain structure; HA, hemagglutinin; HEK, human embryonic kidney; ER, endoplasmic reticulum; GPCRs, G protein-coupled receptors; CAL, CFTR-associated ligand; PBS, phosphate-buffered saline.

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but rather associates with other PDZ proteins such as PSD-95, MAGI-2, GIPC, and CNrasGEF to regulate its intracellular trafficking and functional activity (14, 15, 18, 19). MAGI-2 and PSD-95 are structurally related PDZ proteins of the MAGUK subfamily, but nonetheless they exhibit diametrically opposing effects on agonist-induced β1AR internalization; MAGI-2 strongly promotes β1AR internalization (15), whereas PSD-95 markedly inhibits it (14).

Most studies on βAR trafficking have focused on the events involved in receptor internalization, recycling, and degradation (11), with much less attention devoted to how βARs are processed through post-ER pathways after their synthesis. A novel Golgi-associated PDZ protein termed CAL (for CFTR-associated ligand) has recently been found to modulate post-synthetic trafficking of CFTR (20). Most interestingly, CFTR exhibits a carboxyl-terminal motif similar to that of β1AR and β2AR (D/E(S/T)*X*(L/V)-COOH), and in fact CFTR and β2AR have both been found to associate avidly with NHERF-1 via this motif (12, 21–23). Given the similarity between the CFTR and βAR carboxyl termini, we considered the possibility that CAL might interact with one or both of the βARs to regulate the post-synthetic trafficking of the receptors. This idea was tested in the experiments described in the present report.

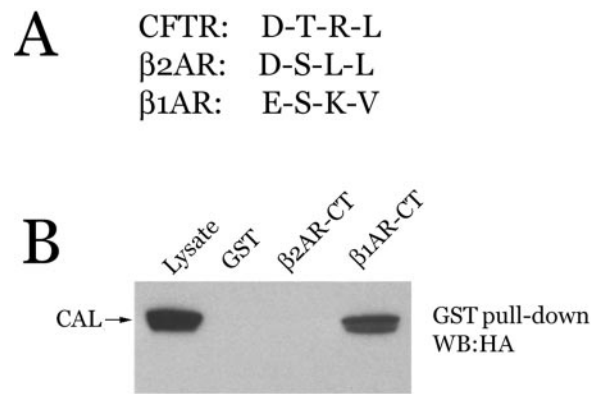


FIG. 1. **Identification of β1AR-CT association with CAL.** *A*, similarity in PDZ-binding motifs between CFTR and βARs. The PDZ-binding motif of CFTR is very similar to that of β2AR (D(S/T)*X*L), but somewhat different from that of β1AR (ESKV). *B*, β1AR-CT associates with CAL. Lysates from COS-7 cells transiently transfected with HA-CAL were utilized in pull-down experiments with beads loaded with GST, GST-β1AR-CT, or GST-β2AR-CT. The precipitates were run on 4–20% SDS-polyacrylamide gels, blotted, and visualized with an anti-HA antibody. Coomassie Blue staining revealed equal loading of the fusion proteins (not shown). *WB*, Western blot.

EXPERIMENTAL PROCEDURES

Preparation of Plasmids and Fusion Proteins—HA-tagged CAL was kindly provided by William B. Guggino (The Johns Hopkins University). The hexahistidine- and S-tagged PDZ domains of CAL and PSD-95 PDZ3 were created via insertion of PCR products derived from a human CAL cDNA (encoding amino acids 270–455) and mouse PSD-95 cDNA (encoding amino acids 307–446) into pET-30A, respectively (Novagen), followed by expression and purification. NHERF-1 fusion proteins and FLAG-β1AR were made by the methods described previously (24, 25). The β1AR carboxyl terminus (the last 30 amino acids of the human β1-adrenergic receptor, β1AR-CT) and β2AR carboxyl terminus (the last 90 amino acids of the human β2-adrenergic receptor, β2AR-CT) were amplified by PCR and then subcloned into pGEX-4T-1. Point mutations of both β1AR-CT and full-length FLAG-β1AR construct (V477A) were introduced by PCR and verified by sequencing. The myc-PSD-95 plasmid was a generous gift from Morgan Sheng (Massachusetts Institute of Technology).

Cell Culture and Transfection—All tissue culture medium and related reagents were purchased from Invitrogen. HEK-293 and COS-7 cells were maintained in complete medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37 °C, 5% CO_2 incubator. To express receptors in the absence or presence of PDZ proteins, 1 μg of total DNA was mixed with LipofectAMINE 2000 (15 μl) (Invitrogen) and added to 5 ml of complete medium in 10-cm tissue culture plates containing cells at ~50–80% confluency. Following 4 h of incubation, fetal bovine serum was added to the medium to 10%, and cells were then harvested after 48 h.

Western Blotting—Samples (30 μg per lane) were run on 4–20% SDS-polyacrylamide gels (Invitrogen) for 1 h at 150 V and then transferred to nitrocellulose. The blots were blocked in "blot buffer" (2% non-fat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM Hepes, pH 7.4) for at least 30 min and then incubated with primary antibody in blot buffer for 1 h at room temperature. The blots were then washed three times with 10 ml of blot buffer each and were incubated for 30 min at room temperature with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) in blot buffer. Finally, the blots were washed three more times with 10 ml of blot buffer each and were visualized via enzyme-linked chemiluminescence using the ECL kit (Pierce).

GST Fusion Protein Pull-down Assay—GST fusion proteins were purified from bacteria using glutathione-Sepharose 4B beads (Sigma), according to the manufacturer's protocol, and were resuspended in PBS containing 0.5% Nonidet P-40 and protease inhibitors. Equal amounts of GST fusion proteins (conjugated on beads) were incubated with 1 ml of clarified whole cell extracts from COS-7 cells transfected with HA-CAL. After incubation at 4 °C with gentle rotation for 4 h, the beads were extensively washed with ice-cold PBS containing 0.5% Nonidet P-40. Proteins were eluted from beads with SDS sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. CAL was detected via Western blotting with the monoclonal anti-HA antibody (12CA5, 1:3000) followed by horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:2000). Bands were visualized via chemiluminescence using the ECL kit (Pierce).

Overlay Assay—The binding of receptor carboxyl-terminal GST fusion proteins to hexahistidine-tagged/S-tagged PDZ domain fusion proteins was assayed via a blot overlay technique. The receptor CT-GST fusion proteins (1 μg/lane) were run on 4–20% SDS-polyacrylamide gels (NOVEX, San Diego), blotted, and overlaid with the hexahistidine-tagged/S-tagged PDZ domains (50 nM final concentration) in a buffer consisting of 2% milk, 0.1% Tween 20 in PBS (blot buffer) for 1 h at room temperature. The blots were then washed three times with blot buffer, incubated with S-protein horseradish peroxidase conjugate (Novagen, 1:4000) for 1 h at room temperature, and visualized via chemiluminescence as described above.

Immunoprecipitation—The method used for immunoprecipitation has been described previously (26). Briefly, cells were harvested and lysed in lysis buffer (10 mM Hepes, 50 mM NaCl, 5 mM EDTA, 1 mM benzamidine, 0.5% Triton X-100). After the lysate was solubilized and clarified, it was incubated with 30 μl of anti-FLAG M2 affinity gel (Sigma) for 2 h with end-over-end rotation at 4 °C. After washing three times, the immunoprecipitated proteins were visualized by Western blot analysis with antibodies to the FLAG epitope (anti-FLAG monoclonal antibody from Sigma) and/or antibodies to the HA epitope (anti-HA monoclonal antibody, 12CA5 from Roche Applied Science).

Receptor Cell Surface Expression Assay—Transfected cells were grown in 35-mm dishes and incubated in the absence and presence of agonist. The cells were then rinsed in PBS, fixed with 4% paraformaldehyde in PBS for 30 min, and then rinsed three more times in PBS and blocked with blocking buffer (2% non-fat dry milk in PBS) for at least 30 min. The fixed cells were then incubated with primary antibody in blocking buffer for 1 h at room temperature. The dishes were subsequently washed three times with 2 ml of blocking buffer and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody in blocking buffer. Finally, the dishes were washed three times with 2 ml of blocking buffer and one time with 2 ml of PBS and incubated with 2 ml of ECL reagent (Pierce) for exactly 15 s. The luminescence, which corresponds to the amount of receptor on the cell surface, was determined by using a TD 20/20 luminometer (27).

RESULTS

CAL Is Identified as a Novel Binding Partner of β1AR-CT—β2AR and CFTR possess very similar carboxyl-terminal motifs (Fig. 1*A*) and are known to associate via these motifs with some of the same PDZ proteins, such as NHERF-1/EBP50 and NHERF-2/NHE3 kinase A regulatory protein (12, 21–23, 28). Given the recent report (20) that CFTR can interact via its carboxyl-terminal motif with another PDZ protein, CAL, it seemed a reasonable possibility that β2AR might also associate with CAL. To test this hypothesis, we employed a fusion pro-

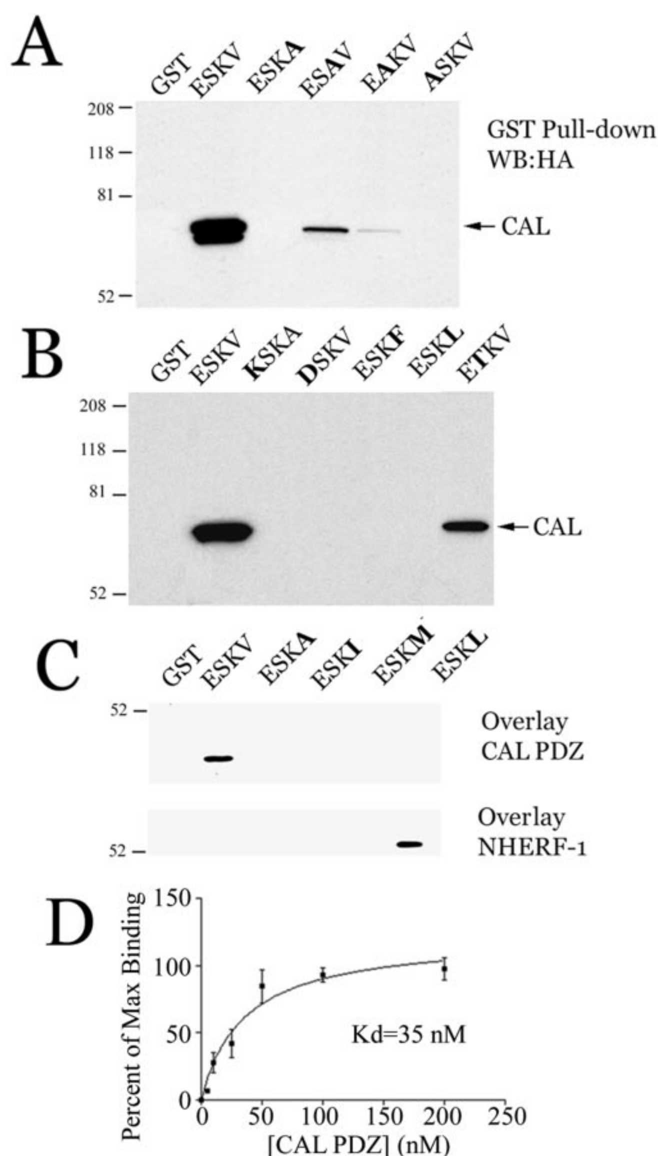


FIG. 2. **Structural determinants of the β1AR-CT interaction with CAL.** *A*, CAL associates with β1AR-CT via the ESKV motif of the receptor. Purified GST fusion proteins corresponding to either the wild-type β1AR-CT (denoted by its last four amino acids, "ESKV") or point-mutated versions of the β1AR-CT (denoted by sequential replacement of each of the last four amino acids with alanine) were adsorbed to glutathione-agarose beads, and used to pull down from lysates of COS-7 cells that had been transiently transfected with HA-tagged CAL. The precipitates were run on 4–20% SDS-PAGE gels, transferred to nitrocellulose, and probed with an anti-HA antibody (12CA5). Coomassie staining confirmed equivalent amounts of the various GST fusion proteins in each sample (not shown). *WB*, Western blot. *B*, further mutations to the ESKV motif of the β1AR-CT. Additional point mutations of β1AR-CT (denoted by sequential replacement of each of the last four amino acids as indicated) were also analyzed in GST pull-down experiments. *C*, fusion protein overlay assay confirms the terminal valine of β1AR-CT as a key determinant for interaction with CAL. Wild-type GST-β1AR-CT as well as GST-β1AR-CT with different mutations to the terminal Val residue, as indicated in the panel, were run on a 4–20% SDS-PAGE gels (1 μg per lane), transferred to nitrocellulose membranes, and overlaid with either S tag-CAL-PDZ or S tag-NHERF-1 fusion protein. Binding of the PDZ proteins was then visualized with anti-S-protein conjugate horseradish peroxidase. *D*, the interaction between β1AR-CT and CAL PDZ is of high affinity. Nitrocellulose strips containing 2 μg of GST-β1AR-CT were incubated with His/S-tagged CAL PDZ at six different concentrations between 0 and 200 nM. Specific binding of CAL PDZ did not increase between 100 and 200 nM, and thus the binding observed at 200 nM was defined as "maximal" binding. The binding observed at the other concentrations was expressed as a percentage of maximal binding within each experiment. The *bars* and *error bars* shown on this graph indicate mean ± S.E. ($n = 3$). The K_D for CAL PDZ binding to β1AR −CT was estimated at 35 nM. The data shown in this figure are representative of 3–5 independent experiments for each panel.

tein pull-down approach. The carboxyl termini of the human β2-adrenergic receptor (β2AR-CT) and human β1-adrenergic receptor (β1AR-CT) were prepared as GST fusion proteins. Equal amounts of these GST fusion proteins, along with GST alone, were adsorbed onto glutathione-Sepharose beads and incubated with cell extracts from COS-7 cells that had been transfected with HA-tagged CAL. As shown in Fig. 1*B*, GST-β2AR-CT did not detectably bind to CAL. Surprisingly, GST-β1AR-CT, which possesses a PDZ interaction motif less similar to that of CFTR, was found to robustly pull down CAL from the cell lysates under the same conditions. Thus, these studies revealed that β1AR-CT, but not β2AR-CT, can associate with CAL.

The Carboxyl-terminal ESKV Motif of β1-AR Is Close to the Optimal Binding Sequence for the CAL PDZ Domain—We next explored the structural determinants of the β1AR-CT/CAL association. First, we individually mutated the last four residues of β1AR-CT to alanine and expressed these mutants as GST fusion proteins. As shown in Fig. 2*A*, three of the four mutations, including Val at position 0, Ser at the −2 position, and Glu at the −3 position, resulted in almost complete elimination of β1AR-CT binding to CAL. Mutation of Lys at the −1 position to Ala also resulted in a markedly reduced β1AR/CAL interaction. Because little is presently known about the target binding specificity of the CAL PDZ domain, we further prepared a number of additional β1AR-CT point mutations. As shown in Fig. 2*B*, most of the mutations examined resulted in sharp reductions in the amount of CAL pulled down by the β1AR-CT. The conservative mutation of Ser at the −2 position to Thr was an exception, having only a modest effect on the interaction, but even this change resulted in a slight but consistent reduction in CAL pull-down by β1AR-CT. Mutation of Val at position 0 to either Phe or Leu also strongly reduced the β1AR-CT/CAL association. These results demonstrate that the last four residues of β1AR-CT are critical determinants for association with CAL, with the ESKV motif most likely being close to an ideal binding motif for the CAL PDZ domain.

In addition to the fusion protein pull-down experiments, we also examined the interaction between β1AR-CT and CAL via a second technique, a blot overlay. A representative experiment of this type, examining more extensive mutations to the terminal amino acid of the β1AR-CT, is shown in Fig. 2*C*. Consistent with the GST pull-down results, overlaid fusion proteins corresponding to the CAL PDZ domain bound very well to blotted wild-type β1AR-CT, with mutation of the terminal Val to structurally similar amino acids such as Met, Ile, Leu, and Ala resulting in strong reductions in CAL binding to the β1AR-CT. In matching experiments, we also overlaid the blots with a fusion protein corresponding to NHERF-1, a PDZ protein that strongly prefers to bind to carboxyl-terminal motifs terminating in leucine (12, 21, 22). As expected, NHERF-1 did not detectably associate with wild-type GST-β1AR-CT, but interestingly it robustly associated with the β1AR-CT mutant that had its terminal Val changed to Leu (ESKL). These data confirmed the specificity of the interactions of the CAL *versus* NHERF-1 PDZ domains with particular carboxyl-terminal motifs, and further established that CAL had a strong preference for binding to carboxyl termini such as β1AR-CT that terminate in Val. To assess the affinity of the β1AR/CAL interaction, saturation binding studies were performed (Fig. 2*D*). The binding affinity of CAL PDZ and β1AR-CT was estimated at 35 nM, revealing that this interaction is of relatively high affinity.

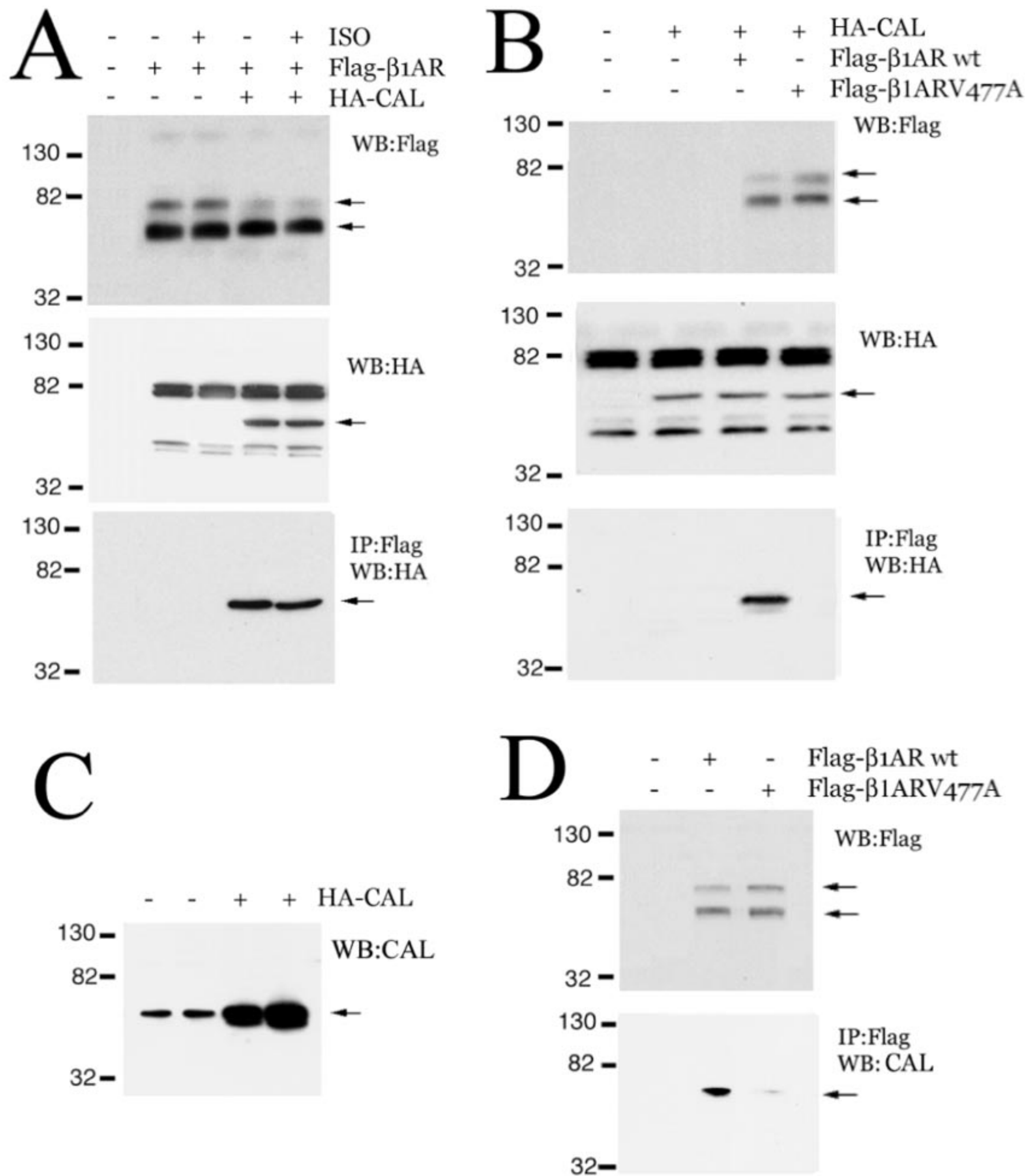


FIG. 3. **Cellular association of CAL with full-length β1AR.** *A*, co-immunoprecipitation of CAL with β1AR. COS-7 cells were transfected with FLAG-β1AR in the absence and presence of HA-CAL and incubated before harvesting in the absence or presence of the β1AR-selective agonist, isoproterenol (50 μM, 10 min), as indicated in the figure. All lysates were probed with a FLAG monoclonal antibody to quantify the total amount of receptor (*top panel*) and an anti-HA monoclonal antibody (*middle panel*) to visualize expression of CAL, and also incubated with anti-FLAG antibody coupled to beads in order to immunoprecipitate (*IP*) the FLAG-tagged β1AR (*lower panel*). *WB*, Western blot; *wt*, wild type. *B*, the V477A mutation to full-length β1AR disrupts association with CAL. Full-length wild-type FLAG-β1AR, or a β1AR mutant with the terminal valine residue changed to alanine (V477A), was co-transfected with HA-CAL into COS-7 cells. All lysates were probed with an anti-FLAG or anti-HA antibody to visualize the expression of the total amount of receptors (*top panel*) or CAL (*middle panel*). CAL was efficiently co-immunoprecipitated with wild-type β1AR but not the V477A mutant (*lower panel*), indicating that the last amino acid of β1AR is critical for its association with CAL in cells. *C*, CAL expressed endogenously in COS-7 cells. COS-7 cells were transiently transfected in the presence and absence β1AR. All cells were harvested and run on 4–20% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with an anti-CAL antibody (1:1000, Abcam Inc.). *D*, wild-type β1AR associates with endogenous CAL. COS-7 cells were transfected with full-length wild-type FLAG-β1AR or the FLAG-β1AR V477A mutant. The total lysates were blotted with anti-FLAG antibody to visualize the receptor expression (*top panel*). All lysates were solubilized and incubated with anti-FLAG antibody coupled to beads in order to immunoprecipitate the FLAG-β1AR. The immunoprecipitates were probed with an anti-CAL antibody (*lower panel*). The data shown in all panels of this figure are representative of three to five independent experiments, with molecular mass standards (in kDa) shown on the *left* of each panel.

β1AR Associates with CAL in Cells—To determine whether full-length β1AR might associate with CAL in a cellular context, COS-7 cells were co-transected with HA-CAL and FLAG-β1AR. Immunoprecipitation of β1AR followed by Western blotting for CAL revealed robust co-immunoprecipitation of a CAL-β1AR complex (Fig. 3*A*). Following a10-min stimulation with the selective β-adrenergic receptor agonist, isoproterenol, the amount of co-immunoprecipitated CAL was not found to be significantly altered (Fig. 3*A*). The GST pull-down and blot overlay results indicated that Val-477, the last amino acid of the β1AR polypeptide, is a critical determinant of the CAL/β1AR association. To examine whether or not this was also the case in cells, we prepared the same mutation in the context of full-length β1AR, and we performed further co-immunoprecipi-

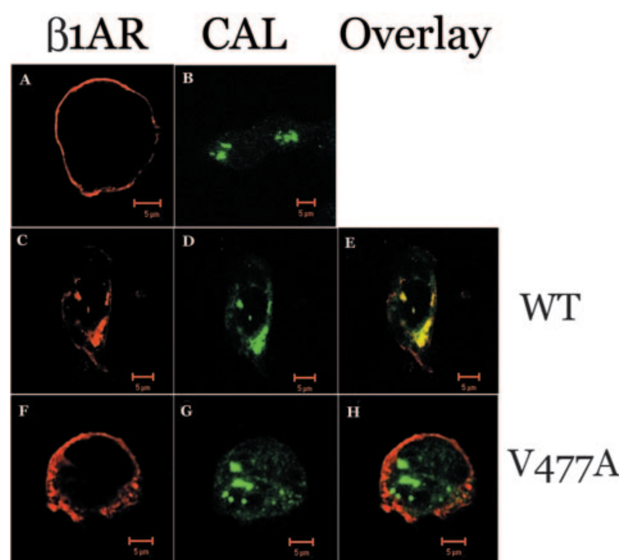


FIG. 4. **CAL co-localizes with wild-type (*WT*) β1AR but not the V477A mutant receptor in HEK-293 cells.** HEK-293 cells were transiently transfected with FLAG-tagged-β1AR alone (*A*), HA-tagged CAL alone (*B*), FLAG-β1AR wild-type plus HA-CAL (*C–E*), or FLAG-β1AR mutant (V477A) plus HA-CAL (*F–H*). After fixation and permeabilization, cells were stained with a mouse anti-FLAG M2 antibody followed by a Texas Red-conjugated anti-mouse IgG and/or stained with a rabbit anti-HA antibody followed by a fluorescein isothiocyanate-conjugated anti-rabbit IgG.

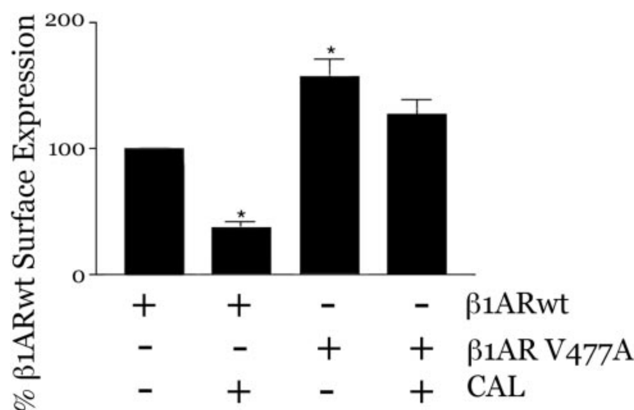


FIG. 5. **CAL regulates β1AR surface expression.** COS-7 cells transfected with either wild-type (*wt*) FLAG-β1AR or the V477A mutant, in the absence or presence of HA-CAL, were split into 35-mm dishes 24 h after transfection. Receptor expression on the cell surface was quantified via a luminometer-based assay as described under "Experimental Procedures." The *bars* and *error bars* represent mean ± S.E. for three independent experiments, and the values within each experiment were normalized to total receptor expression as determined by Western blot (not shown). Total expression levels of the wild-type *versus* mutant receptors were generally similar, so only a modest amount of normalization was required within each experiment. * indicates a significant difference from β1ARwt alone ($p < 0.05$).

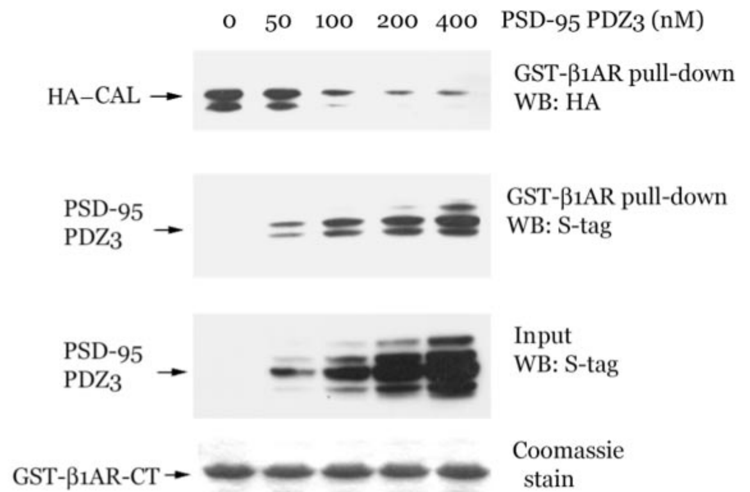
tation experiments. As shown in Fig. 3*B*, the V477A mutation sharply reduced the ability of the receptor to co-immunoprecipitate CAL from cells. To explore whether the CAL/β1AR interaction could still be detected at endogenous levels of CAL expression, the experiments were repeated using the lysates from COS-7 cells that were transfected only with FLAG-β1AR. Endogenous CAL was detected using a commercially available anti-CAL antibody. As shown in Fig. 3*C*, endogenous CAL expression was detected in COS-7 cells, consistent with previous reports (29). Wild-type β1AR was able to co-immunoprecipitate endogenous CAL, but the V477A mutant was not, indicating that the wild-type receptor but not the mutant associated with endogenous CAL in COS-7 cells. Moreover, Western blots of the post-immunoprecipitation lysates (not shown) revealed a significant depletion of CAL following immunoprecipitation of wild-type β1AR, indicating that a substantial fraction of cellular CAL was associated with β1AR in these studies.

CAL Promotes Intracellular Retention of β1AR—To examine the subcellular localization of β1AR and CAL, fluorescence confocal microscopy studies were performed on HEK-293 cells transiently transfected with FLAG-β1AR and/or HA-CAL. In cells transfected with β1-adrenergic receptors alone, the receptors were located exclusively in the plasma membrane, as expected, forming a smooth rim around the cell (Fig. 4*A*). When HA-tagged CAL was expressed alone, it was observed predominantly in the cytoplasm as multiple spots (Fig. 4*B*), overlapping with the expression of the Golgi-associated protein GM 130 (data not shown). These data are consistent with observations reported previously (20, 29, 30) that CAL is primarily associated with the ER-Golgi complex. When β1AR was co-expressed with CAL, a significant fraction of total β1AR was retained in the cytoplasm (Fig. 4*C*), where it exhibited excellent co-localization with CAL (Fig. 4*E*). Conversely, when the β1AR V477A mutant was co-expressed with CAL, the mutant receptors remained almost exclusively distributed along the cell surface (Fig. 4*F*), exhibiting little overlap with CAL immunolabeling (Fig. 4*H*). Thus, CAL did not exhibit significant co-localization with the β1AR V477A mutant.

To study the effects of CAL on β1AR trafficking to the plasma membrane in a more quantitative fashion, we transfected FLAG-β1AR in the absence and presence of HA-CAL into COS-7 cells, and we examined β1AR surface expression using a luminometer-based assay, which has been described previously (27). As shown in Fig. 5, β1AR surface expression was reduced by more than 60% upon co-expression with CAL, suggesting that the interaction between CAL and β1AR promotes retention of β1AR within the cell. Surface expression of the V477A β1AR mutant, in contrast, was not significantly reduced by co-expression with CAL. Most interestingly, surface expression of the V477A mutant alone was significantly higher than surface expression of the wild-type β1AR, These data are consistent with the idea that endogenous CAL can regulate β1AR expression on the cell surface, with this effect being magnified by overexpression of exogenous CAL.

PSD-95 Competes with CAL to Interact with β1AR and Promotes β1AR Surface Expression—CAL associates with β1AR via interaction with the carboxyl-terminal ESKV motif of the receptor, which is the same region of β1AR that interacts with other PDZ-containing proteins, such as PSD-95, MAGI2, GIPC, and CNrasGEF (14, 15, 18, 19). It seems reasonable to speculate that PDZ proteins that bind to the same target motif should bind in a competitive fashion, but this idea remains primarily theoretical for the vast majority of known PDZ domain-mediated interactions. We directly tested the possibility that the association between PSD-95 and β1AR might block the β1AR/CAL interaction by examining the pull-down of HA-tagged CAL from COS-7 cell lysates by GST-β1AR-CT beads in the presence of increasing amounts of purified S protein-tagged PSD-95 PDZ3, which is the region of PSD-95 that binds to the β1AR-CT. As shown in Fig. 6*A*, the binding of CAL to the β1AR-CT was markedly reduced by increasing the concentrations of PSD-95 PDZ3. These data reveal that PSD-95 PDZ3 and CAL associate with β1AR-CT in a competitive fashion.

PSD-95 in cells is known to be mainly plasma membrane-associated because of lipid modifications that help anchor it to the plasma membrane (31). Because CAL is found predominantly in the Golgi (20, 29, 30), we further examined whether PSD-95 and CAL might compete in cells to regulate β1AR intracellular trafficking and cell surface expression. Wild-type



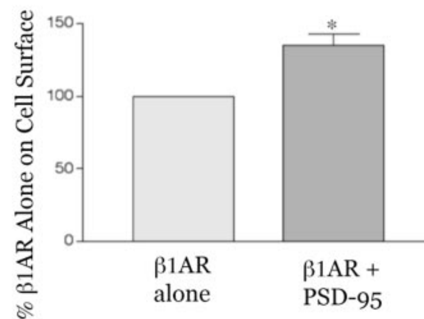


FIG. 6. **PSD-95 competes with CAL for association with β1AR.** *A*, PSD-95 and CAL competitively bind to β1AR. Lysates from COS-7 cells transfected with HA-CAL were included with different concentrations of S tag-PSD-95 PDZ3 fusion protein, as indicated in the panel, and then pulled down with GST-β1AR-CT. The data are representative of three independent experiments, with molecular mass standards (in kDa) shown on the *left* of each panel. *WB*, Western blot. *B*, overexpression of PSD-95 restores β1AR expression on the cell surface. COS-7 cells were transfected with FLAG β1AR in the absence and presence of Myc-PSD-95 co-transfection. Receptor expression on the cell surface was quantified via a luminometer-based assay as described previously. All lysates were probed with a FLAG monoclonal antibody to quantify the total amount of receptor and an anti-Myc monoclonal antibody to visualize expression of PSD-95 (not shown). The *bars* and *error bars* represent mean ± S.E. for three independent experiments, with the values for each experiment normalized to total receptor expression as determined via Western blot. Total expression levels of the wild-type *versus* mutant receptors were generally similar, so only a modest amount of normalization was required within each experiment. * indicates a significant difference from β1AR alone ($p < 0.05$).

β1AR was transfected in the absence and presence of full-length Myc-PSD-95 into COS-7 cells, and β1AR surface expression was quantified using the luminometer-based assay described earlier. As shown in Fig. 6*B*, overexpression of PSD-95 resulted in a significant enhancement in receptor expression at the cell surface relative to transfection of β1AR alone. These data are consistent with the idea that PSD-95 can compete with endogenous CAL to promote β1AR surface expression.

DISCUSSION

We have identified CAL as a novel binding partner of the β1-adrenergic receptor. The interaction between CAL and β1AR is highly specific and dependent on the PDZ domain of CAL and the carboxyl terminus of β1AR. It is well known that PDZ domain binding specificity is primarily determined by the last few amino acids on the carboxyl termini of target proteins (17). CAL has been found to associate with several proteins other than β1AR including CFTR (20), ClC-6b (32), GRID2R (33), and frizzled protein 5 and 8 (29). The last four amino acids of these proteins are DTRL (CFTR), STTL (ClC-6b), GTSI (GRID2R), and LSQV (frizzled protein 5 and 8), respectively. All of these motifs conform to the canonical class I PDZ-binding motif, which is composed of the consensus sequence (S/T)$X\Phi$ (where Φ is a hydrophobic amino acid at the extreme carboxyl terminus, and X is any amino acid). β1AR terminates in the motif ESKV, which is similar to the motifs found in the other proteins that associate with CAL. Most interestingly, all mutations that we made to the ESKV motif, with the exception of Ser to Thr at the −2 position, resulted in sharp reductions in CAL association, indicating that ESKV is probably close to the ideal binding motif for the CAL PDZ domain.

We observed that overexpression of CAL resulted in the intracellular accumulation of β1AR and a consequent reduction in the amount of β1AR on the cell surface. We have found previously that PDZ proteins can modulate β1AR internalization. Specifically, we observed that interaction with PSD-95 inhibits β1AR internalization, whereas MAGI-2 association with β1AR enhances the internalization of the receptor (14, 15). In the present work, however, we found no effect of CAL on the rate of β1AR internalization (data not shown), but rather only the aforementioned striking effect on the constitutive level of β1AR surface expression. Our studies revealed that β1AR expression at the cell surface was reduced by more than 60% upon co-expression with CAL. In contrast, no significant decrease in the receptor surface expression was observed for the β1AR V477A mutant, indicating that the effect required the direct interaction between CAL and β1AR. Because there is a low level of endogenous CAL expression in COS-7 cells (Fig. 3*C*), one might predict that endogenous CAL should also retard β1AR in the cytoplasm. Consistent with this idea, we found that the V477A mutant receptor was trafficked to the cell surface significantly more efficiently than the wild-type β1AR when the two receptors were expressed at equivalent levels. Most interestingly, CAL is known to be predominantly located in the Golgi apparatus and to interact with syntaxin 6, a Q-SNARE protein, via its coiled-coil domain (30). Syntaxin 6 interacts with the Rab5 effector EEA1 (early endosomal au-

toantigen) and is believed to function in the tethering of post-Golgi vesicles to early endosomes as well as the regulation of early endosome fusion (34). Thus, we speculate that the retention and accumulation of β1AR in the cytoplasm is because of CAL modulation of β1AR anterograde trafficking from the ER-Golgi to the cell surface.

Similar to our findings that CAL affects β1AR trafficking, CAL has been reported to play a role in the vesicle transport of both CFTR (20, 35) and frizzled 5 (29) from the Golgi apparatus to the plasma membrane. Frizzled 5, a transmembrane receptor for Wnt, was found to exhibit co-localization in the Golgi apparatus with CAL (29). In the case of CFTR, a transmembrane chloride channel, CAL was found to decrease the cell surface expression of CFTR in COS-7 cells by reducing the rate of anterograde CFTR trafficking to the plasma membrane (20). The Golgi apparatus is an organelle in the secretory pathway required for the processing of complex sugar structures on many proteins and lipids and for the sorting of these proteins and lipids to their correct subcellular destinations (36). Thus, CAL may play a role in the regulation of the post-translational modification of transmembrane proteins, thereby regulating the formation of mature transmembrane proteins competent for subsequent transport to the cell surface. Along these lines, we have found that glycosylation of β1AR in the ER/Golgi strongly regulates delivery of the receptor to the plasma membrane (27), an observation that has been made for numerous other transmembrane proteins (37, 38). Golgi-associated PDZ proteins such as CAL may act as gatekeepers to regulate the post-translational processing and surface expression of specific transmembrane proteins such as β1AR.

The β1AR carboxyl-terminal PDZ-binding motif associates not only with CAL but also with previously reported PDZ partners such as PSD-95 (14), MAGI2 (15), GIPC (18), and CNrasGEF (19). It is interesting to consider why the same site of β1AR might be able to associate with so many different PDZ proteins. One answer to this question is that competition between PDZ proteins for binding to the same motif provides a mechanism by which the trafficking of a receptor such as β1AR may be regulated. For example, we found that PSD-95 can compete with CAL for binding to β1AR, thereby promoting β1AR trafficking to the cell surface. It is not clear what molecular events might cause β1AR to switch from binding one PDZ partner to another, but it is known that PDZ interactions can be profoundly regulated by reversible post-translational modifications such as phosphorylation (17). A second answer to the question as to why β1AR might be able to associate with multiple PDZ partners is that all of these partners are known to exhibit distinct patterns of expression in different tissues (39–42). The interaction of β1AR with different PDZ partners in distinct tissues may contribute heavily to cell type-specific regulation of β1AR trafficking and function.

In summary, we have found that β1AR specifically interacts via its carboxyl terminus with the PDZ domain of CAL, a Golgi-associated protein that is known to interact with proteins involved in the regulation of vesicular transport. In both co-immunoprecipitation and co-immunofluorescence experiments, the association of β1AR with CAL was found to be dependent on the carboxyl-terminal motif of β1AR, especially the terminal valine residue. The interaction of β1AR with CAL resulted in the retention and accumulation of β1AR in the cytoplasm. These observations provide evidence for the importance of PDZ interactions in β1AR trafficking through the ER-Golgi complex to the plasma membrane and have implications for the regulation of post-translational processing for many other transmembrane proteins that are known to interact with PDZ-containing partners.

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