\(\alpha\)-Dystrobrevin-1 recruits \(\alpha\)-catulin to the \(\alpha_1D\)-adrenergic receptor/dystrophin-associated protein complex signalosome

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\(\alpha_1\)D-adrenergic receptors (ARs) are key regulators of cardiovascular system function that increase blood pressure and promote vascular remodeling. Unfortunately, little information exists about the signaling pathways used by this important G protein-coupled receptor (GPCR). We recently discovered that \(\alpha_1\)D-ARs form a “signalosome” with multiple members of the dystrophin-associated protein complex (DAPC) to become functionally expressed at the plasma membrane and bind ligands. However, the molecular mechanism by which the DAPC imparts functionality to the \(\alpha_1\)D-AR signalosome remains a mystery. To test the hypothesis that previously unidentified molecules are recruited to the \(\alpha_1\)D-AR signalosome, we performed an extensive proteomic analysis on each member of the DAPC. Bioinformatic analysis of our proteomic data sets detected a common interacting protein of relatively unknown function, \(\alpha\)-catulin. Commumophagocytosis and blot overlay assays indicate that \(\alpha\)-catulin is directly recruited to the \(\alpha_1\)D-AR signalosome by the C-terminal domain of \(\alpha\)-dystrobrevin-1 and not the closely related splice variant \(\alpha\)-dystrobrevin-2. Proteomic and biochemical analysis revealed that \(\alpha\)-catulin supersensitizes \(\alpha_1\)D-AR functional responses by recruiting effector molecules to the signalosome. Taken together, our study implicates \(\alpha\)-catulin as a unique regulator of GPCR signaling and represents a unique expansion of the intricate and continually evolving array of GPCR signaling networks.

G protein-coupled receptors (GPCRs) are seven-transmembrane spanning proteins that are responsible for communicating information in the form of extracellular stimuli across lipid membranes into distinct intracellular signals with precise accuracy. After ligand binding, GPCRs signal through the canonical heterotrimeric G protein signaling pathway to activate a diverse array of downstream effectors (1). Recently, it has become evident that most GPCRs collaborate with one or more additional proteins at specific points in their lifecycle. These GPCR interacting proteins (or GIPs) are largely receptor subtype and cell context specific, include both membrane and cytosolic proteins, and typically play a highly specific supporting role for GPCR function (i.e., trafficking, ligand binding, enhancing signaling, signal termination, and/or degradation) (2–4).

Recently, we used yeast two-hybrid and proteomic screens to identify GIPs for a clinically important GPCR, the \(\alpha_1\)D-adrenergic receptor (AR) (5). A member of the adrenergic family (\(\alpha_1\), \(\beta_1\), \(\alpha_1\)D-ARs are ubiquitously expressed on blood vessels and are responsible for increasing blood pressure during exercise, injury, stress, or cardiovascular disease (6). \(\alpha_1\)D-AR knockout mice are hypertensive and resistant to high salt diet-induced hypertension (7, 8), yet this GPCR has been largely ignored over the past 20y because after transfection into cell culture \(\alpha_1\)D-ARs are sequestered in the endoplasmic reticulum (9, 10). Clinical interest in the \(\alpha_1\)D-AR as a drug target has recently increased with the discoveries that \(\alpha_1\)D-ARs are the predominant subtype expressed in epicardial coronary arteries (11) and that \(\alpha_1\)D-AR prostate expression increases in patients with benign prostatic hypertrophy (12). Through proteomic screening, we discovered that \(\alpha_1\)D-ARs are scaffolded to the dystrophin-associated protein complex (DAPC) via the anchoring protein syntrophin (10). Coexpression with syntrophins increases \(\alpha_1\)D-AR plasma membrane expression, drug binding, and activation of Goq/11 signaling after agonist activation. Moreover, syntrophin knockout mice lose \(\alpha_1\)D-AR-stimulated increases in blood pressure, demonstrating the importance of these essential GIPs for \(\alpha_1\)D-AR function in vivo (10).

Proper organization of signaling molecules within cells by the DAPC is essential for the maintenance of cellular homeostasis at synaptic junctions (13). Mutations in DAPC result in severe muscle wasting diseases, such as Duchenne muscular dystrophy/Becker muscular dystrophy, and as a result, the role of this complex for proper skeletal muscle function has been thoroughly studied (14). However, the DAPC performs many other functions: it facilitates proper water transport across the blood–brain barrier by anchoring aquaporin (15, 16), clusters nicotinic acetylcholine receptors to ensure signal transmission at parasympathetic synapses (13), and anchors neuronal NOS at the cell membrane in cardiac myocytes to permit cardiodiastole (17). We previously demonstrated that \(\alpha_1\)D-ARs form a complex with the DAPC (10), but why this interaction is necessary for \(\alpha_1\)D-AR functional coupling is unknown.

In this study, we postulated that molecules necessary for \(\alpha_1\)D-AR signaling are recruited by the DAPC. Using a sequential proteomic screening approach, we identified \(\alpha\)-catulin as a unique member of the \(\alpha_1\)D-AR signalosome. The goal of these experiments was to understand how \(\alpha\)-catulin integrates into this growing GPCR protein complex and to decipher the purpose of this relatively unstudied protein in GPCR signaling networks.

Results

\(\alpha\)-Catulin: Unique Member of the \(\alpha_1\)D-AR/DAPC Signalosome. We previously demonstrated that syntrophins are required for \(\alpha_1\)D-AR function in vitro and in vivo by anchoring \(\alpha_1\)D-ARs to the DAPC (10). Our working hypothesis is that the DAPC facilitates \(\alpha_1\)D-AR function by acting as a multiprotein scaffold to arrange signaling molecules in close proximity to the receptor. To test this hypothesis, we fused tandem-affinity purification (TAP) epitopes...
containing streptavidin/calmodulin-binding proteins to the α1D-AR and all members of the DAPC, including, α-syntrophin, β1-syntrophin, β2-syntrophin, α dystrobrevin-1 (αDB1), and αDB2. HEK293 cell lines stably expressing each individual clone were created. TAP-tagged and associated proteins were purified from lysates of each stable cell line, digested with trypsin, and the tryptic peptides subjected to liquid chromatography (LC) tandem mass spectrometry (MS/MS).

Analysis of the peptides identified by LC-MS/MS revealed both predicted and unique components of the scaffold (Fig. L4 and Table S1). The core components of the scaffold (dystrophin, utrophin, α/β-DB, and α/β1/β2-syntrophin) were cross-identified in each of the individual pulldowns. Our experiments identified known interactors [i.e., CASK and LIN7C with β2-syntrophin, Gα subunits with α-syntrophin (18, 19)] for each bait protein, confirming the validity of our experimental data sets. One protein, α-catenin (CTNNAL1) was consistently identified in the majority of our proteomic screens (Table S1).

Next, we confirmed these findings using immunoprecipitation experiments of full-length proteins in living cells. Shown in Fig. 1B, lysates from HEK293 cells stably expressing TAP-α1D-ARs were precipitated and probed for α-catenin, αDB1, and syntrophin. Both endogenous α-catenin and transfected flag-tagged α-catenin bound to TAP-α1D-ARs, along with other members of the α1D-AR/DAPC signalosome. Immunoprecipitation and TAP/MS analysis revealed that α-catenin does not interact with the α1A-AR subtype (Fig. S1 and Dataset S1). Combined with our TAP/MS and biochemical analysis, these findings implicate α-catenin as a unique member of the α1D-AR/DAPC signalosome.

α-Catenin Is Recruited to the α1D-AR Signalosome by αDB1. We detected α-catenin as a positive initiator in the α1D-AR, αβ1/β2-syntrophin, and αDB1 proteomic screens, making it difficult to predict which protein directly binds and recruits α-catenin to the α1D-AR/DAPC signalosome. An important clue was that α-catenin was not detectable in the α2D-AR screen (Fig. L4 and Table S1). αDB1 and αDB2 are C-terminal splice variants of the αDB gene (20). Following their common 504-aa core, αDB1 has a unique 184-aa C terminus, whereas αDB2 has a 9-aa unique C terminus. Because α-catenin interacts with αDB1 but not with αDB2, we suspected that α-catenin is most likely recruited by association with the αDB1 C-terminal domain.

To test this possibility, we used HEK293 cells stably expressing TAP-αDB1 or TAP-αDB2. Coimmunoprecipitation experiments revealed that TAP-αDB1 interacts with both endogenous and transiently transfected α-catenin-flag, whereas TAP-αDB2 failed to coimmunoprecipitate either protein (Fig. 2A). These results were corroborated by experiments demonstrating that α-catenin-flag coimmunoprecipitates specifically with endogenous αDB1 but not αDB2 (Fig. 2B).

Next, we used nitrocellulose blot overlay assays to test whether the interaction between αDB1 and α-catenin is direct. TAP-αDB1, TAP-αDB2, or untransfected HEK293 cell lysates were separated by SDS/PAGE, transferred to nitrocellulose, and incubated with GST-α-catenin purified from Escherichia coli. Membranes were probed with antibodies directed against GST or dystrobrevins. Secondary antibodies conjugated to IRDye 680 and 800 permitted coanalysis of blot data using a LiCor Odyssey system. As shown, GST-α-catenin bound endogenous and TAP-αDB1 (Fig. 2C, Top). GST-α-catenin failed to bind either endogenous or TAP-αDB2 (Fig. 2C, Middle), consistent with our TAP/MS analyses and coimmunoprecipitations. However, we observed a direct interaction between GST-α-catenin and β-DB (Fig. 2C, Bottom), which shares considerable homology with αDB1 in the C-terminal region (Fig. S2). GST-α-catenin was unable to bind GST and other members of the DAPC, indicating that α-catenin directly and selectively interacts with αDB1 and β-DB (Fig. 3). Thus, we conclude from our TAP/MS, coimmunoprecipitation, and nitrocellulose blot overlay analyses that α-catenin is recruited to the α1D-AR signalosome through a direct interaction with the C-terminal domain of αDB1.

Protein domain analysis revealed three regions in α-catenin homologous to vinculin (Fig. S4). We tested whether these regions represent potential αDB1 interaction domains by introducing stop codons along the borders of each vinculin homology domain in α-catenin (Fig. 2D). Truncation mutants were expressed in and purified from HEK293 cells to ensure equivalent expression levels and proper predicted size of each construct (Fig. 2D). Biochemical analysis revealed that αDB1 retained the ability to coimmunoprecipitate with each C-terminal α-catenin mutant (T1–4), indicating that vinculin type domains 2 and 3 are not necessary for interactions with αDB1. However, TAP-α-catenin lacking N-terminal amino acids 1–277 resulted in a dramatic loss in αDB1 association, indicating that vinculin homology domain 1 is essential for this interaction.

To determine whether α-catenin cellular localization overlaps with DAPC members, we immunostained HEK293 cells for endogenous α-catenin and either syntrophins (Fig. S5) or α-dystrobrevins (Fig. 3). As shown, α-catenin codistributes with dystrobrevins in HEK293 cells (Pearson coefficient, 0.38) but displays little codistribution in the cytoplasm. Because the localization seen might be caused by artifacts from folded or perturbed membranes, we also examined cross-sections of murine quadriceps. α-Catenin displays strong codistribution with α-dystrobrevins in nerve bundles and blood vessels in these slices (Fig. 3). Taken together with our MS, coimmunoprecipitation, and blot overlay results, these data suggest that α-catenin and αDB1 associate together, presumably in complex with the
DAPC and membrane receptors [i.e., α₁D,-AR, SLO-K1 (21), and 5-hydroxytryptamine-2 receptor (22)].

α-Catulin Supersensitizes α₁D-AR Gqa/11 Signaling. What is the functional purpose of α-catenin in the α₁D-AR/DAPC signalosome? Previous studies indicate that α-catenin may facilitate intracellular signaling mechanisms by scaffolding essential downstream effectors in close proximity to one another (23–25). To determine whether α-catenin performs a similar function in the α₁D-AR/Gqa/11 signaling pathway, we examined the effects of overexpressing or knocking down α-catenin on α₁D-AR signaling. In agreement with previous studies, α₁D-AR displays low levels of functional activity with rate-limiting concentrations of downstream effectors, before being sequestered by reassociation with Gβγ through signal termination by enhanced GTP hydrolysis by RGS proteins (27). To test this, HEK293 cells stably expressing TAP-α-catenin were created for TAP/MS proteomic analysis. α-Catenin and associated proteins were purified from cell lysates, digested with trypsin, and subjected to LC-MS/MS. Bioinformatic analysis of the resulting data sets revealed ~30 α-catenin interacting proteins (Fig. 5C and Table S2). As expected, we identified all known members of the α₁D-AR signalosome, including dystrophin, utrophin, α-dystrobrevin-1, and all three syntrophin isoforms previously shown to bind the α₁D-AR C-terminal PDZ domain: α₁, β₁, and β₂-syntrophins (5). Our attention was drawn to one protein of particular interest: phospholipase C isomorph β₂ (PLCβ₂), which is a key effector molecule in the canonical Gqa/11 signaling pathway (28). After agonist stimulation, GTP-bound Gaq binds PLCβ₁1/11 binds and activates PLCβ₂, which then cleaves the membrane phospholipids phosphatidyl-inositol-4,5-bisphosphate into signaling molecules IP3 and diacylglycerol (29). Comunoprecipitation (Fig. 4D) and blot overlay (Fig. 4E) experiments reveal that α-catenin directly associates with PLCβ2, whereas TAP-α-D1 immunoprecipitation confirmed that PLCβ2 is recruited to the DAPC/α-catenin signalosome (Fig. 4F). Thus, these data clearly identified α-catenin as a unique member of the α₁D-AR/DAPC signalosome, provided a functional purpose for α-catenin in the complex, and revealed a previously unknown and unique mechanism by which PLCβ₂ is recruited to GPCRs.

Discussion
Recent studies show that GPCRs are tightly organized in cells through interactions with a highly diverse and selective group of GPs, which facilitate downstream signaling, effect the pharmacological properties of ligand binding, and terminate receptor activity after prolonged agonist stimulation (3, 4). Our focus is on a particularly complex GPCR, the α₁D-AR, which must bind syntrophins to become functional (5, 10). Previously, we discovered that syntrophins link α₁D-ARs to the DAPC (10) but had
yet to understand why this interaction is necessary for receptor function. In this study, we identified α-catulin as a unique member of this large GPCR signaling network. Biochemical and proteomic analysis indicates that α-catulin is directly recruited to the signalosome by a splice product of the α-DB gene, α-DB-1, and not the closely related splice variant α-DB2, whereas functional experiments reveal that α-catulin enhances α1D-AR functional coupling by binding and recruiting the Gaq11 signaling effector PLCl2 (Fig. 4G).

Why is the DAPC necessary for functional expression of α1D-ARs? One potential explanation is that the DAPC scaffolds α1D-ARs at discrete locations in post synaptic tissue, and loss of DAPC interactions results in altered α1D-AR spatial orientation in cells and/or loss of functional activity. Indeed, recent studies support the idea that α1D-ARs are expressed at the plasma membrane and intracellularly in native tissues (11, 30), but it remains unclear how the DAPC is regulated to alter α1D-AR localization. One study of particular interest revealed that α1D-ARs are clearly expressed both at the plasma membrane and intracellularly in freshly dissociated aortic smooth muscle cells but become completely intracellular and nonfunctional with culturing (31). Thus, it is likely that unidentified signals are required to maintain sequestration of α1D-ARs at their proper sites in vivo. Interestingly, the most prevalent α1D-AR interacting protein obtained in our TAP/MS analysis was another scaffolding protein, α-liprin, which is important for clustering a wide variety of signaling molecules in synapses and at the leading edge of focal adhesions, including the tyrosine phosphatase LAR, synaptotagmin, synaptobrevin, and glutamate receptors (32–36). Thus, it may be possible that α-catulin binding of α-liprin clusters α1D-ARs in vascular smooth muscle in close proximity to adjacent sympathetic neurons, where concentrations of norepinephrine are thought to be in high concentration and signals that disrupt liprin function result in loss of α1D-AR clustering and sequestration.

A major unanswered question in the DAPC field involves the functional significance of α-DB splice variants in vivo (20). The α-dystrobrevin gene produces five splice variants capable of interacting with the DAPC, and the most well studied of the splice variants, α-DB1 and α-DB2, differ only in the length of their C-terminal domain (α-DB2 is truncated at position 570, resulting in a loss of amino acids 570–766). Both isoforms are ubiquitously expressed and interact with the DAPC along with known binding partners syncoolin, dysbindin, and DAMAGE (20), yet a conclusive study addressing the necessity of α-DB splice variants remained elusive. It has been suggested that the α-DB1 C terminus acts as a regulatory domain that is modulated by tyrosine phosphorylation, or that the C terminus imparts specialized localization patterns in the neuromuscular junction (20). With this discovery of an α-DB1 splice variant-specific binding partner, it will be interesting in future studies to reveal how the α-catulin/DAPC complex regulates neuromuscular junction signaling (13).

Although few studies have examined the functional role of α-catulin, accumulating evidence strongly suggests it acts as a multipurpose scaffolding protein. For example, α-catulin has been shown to directly bind and modulate the enzymatic activity of Lbc-Rho guanine exchange factor (GEF), IkB kinase, and Ras (23–25). More recently, siRNA screens performed in Caenorhabditis elegans suggest that α-catulin facilitates proper localization and function of the BK channel SLO-1 (21, 37), and it is likely that α-DB1 recruits α-catulin to the BK channel, considering the high sequence homology in α-DB1 C-terminal domains across species (Fig. S2). A parallel study revealed that α-catulin bridges 5-hydroxytryptamine-2 receptor Gq/11 coupling to activation of the Lbc-RhoGEF/RhoA/ROCK pathway in pulmonary artery smooth muscle cells (22). Thus, these combined results implicate α-catulin as a unique regulator of membrane receptor function, and herein we provide a detailed molecular mechanism by which...
α-catulin links these membrane signaling proteins to the DAPC through α-DB1.

Our data suggest that α-catulin supersensitizes α₁D-AR functional responses by recruiting PLCζ2 and/or RhôGEFs. Interestingly, α₁-AR functional responses become supersensitized in vivo after sympathetic denervation (38, 39). Because the α₁D-AR is the only α₁-AR subtype that interacts with the DAPC α-catulin complex (5, 10), the findings of this study may provide a mechanistic explanation to the mystery of GPCR supersensitivity, a physiological process observed in hypertension and benign prostatic hypertrophy (12). Additionally, it is interesting to note that all identified α-catulin binding proteins are modulated by Guoq11 signaling (22, 29). Thus, we propose that α-catulin is a key component of Guoq11 signaling complexes, where it functions to recruit activator and effector proteins in close proximity to each other. Given the ubiquitous expression of α-catulin throughout the body, the large number of GPCRs containing PDZ domains in their C-terminal tails (40, 41), and the broad array of cellular functions controlled by Guoq11 signaling, it is likely that α-catulin plays an important role in numerous GPCR signaling networks.

Materials and Methods

Reagents. Antibodies generated against α-DB1, β-DB2, β-DB, pan-DB, utrophin, and syntrphin were described previously (42–44). Other antibodies were purchased from commercial sources: anti-dystrophin and anti-flag (Sigma), anti-HA (Cell Signaling), and anti-α-catulin (Abnova).

Constructs. pIRESpuro-GLUE (TAP vector) was kindly provided by Randal Moon (University of Washington, Seattle, WA). Mouse β₂-syntrophin was amplified by PCR to add 5′ EcoRI and 3′ NsiI restriction sites for subcloning into pIRESpuro-GLUE. Mouse β₂-syntrophin was amplified by PCR to add 5′ EcoRI and 3′ Pst restriction sites for subcloning into pIRESpuro-GLUE. The 5′ end of mouse α-dystrobrevin (shared by both α-DB1 and -2) was amplified using a primer containing an Asc I site in frame and an internal primer downstream of the endogenous BglII site. The PCR product and the C-terminal–specific regions of each isoform were cloned into pIRESpuro-GLUE. Human α-catulin-DDK/myc was purchased from OriGene. Human α-catulin cDNA was amplified by PCR to add 5′ BamHI and 3′ NotI for subcloning into pGEX-4T-2 (GE Healthcare) to generate GST-α-catulin. Human α-catulin cDNA was amplified by PCR to add 5′ NotI and 3′ BamHI for subcloning into pIRESpuro-GLUE to generate TAP-α-catulin. TAP-α-catulin truncations 1–4 were generated by using QuikChange Mutagenesis (Agilent Technologies) to introduce stop codons at base pairs 831, 1026, 1575, and 1731 of α-catulin. A1–277 TAP-α-catulin was generated by introducing an EcoRV restriction site at amino acid 831, followed by subcloning with EcoRV to remove base pairs 1–831 of α-catulin. TAP-α₁C-AR, TAP-α₁A-R, HIF-α₁C-AR, and HIF-α₁A-AR-6G-α-syntrophin have been described previously (10, 26).

Cell Culture and Transfection. HEK293 cells were propagated in DMEM containing 10% FBS, 10 mg/ml streptomycin, and 100 U/ml penicillin at 37 °C in 5% CO₂. The constructs were transfected using FuGENE HD transfection reagent (Roche Applied Science) when cells were 80% confluent.

Tandem Affinity Purification/Mass Spectrometry. TAP purification of all constructs has been described previously (10). MS on final eluates was done on a linear ion trap Fourier transform or linear trap quadrupole at the Fred Hutchinson Cancer Research Center (Seattle, WA).

Immunoprecipitations and Western Blotting. For TAP-α-DB1/2, TAP-α-catulin, and flag (DDK), immunoprecipitations were performed as previously described (10).

Blot Overlays. HEK293, TAP-α-DB1–expressing HEK293, and TAP-α–DB2–expressing HEK293 lysate was run on an 8% SDS/PAGE gel (Thermo Fisher Scientific) per the manufacturer’s instructions. Gels were transferred to nitrocellulose by using a wet transfer system per the manufacturer’s instructions. Membranes were blocked in 5% milk in Tris-buffered saline with Tween 20 (TBS) for 1 h at room temperature, followed by two brief washes in overlay buffer (150 mM NaCl, 2 mM MgCl₂, 20 mM Hepes (pH 7.5), 3% BSA, and 1 mM DTG). GST-α-catulin was diluted to 200 nM in overlay buffer and incubated with membranes overnight at 4 °C with rocking. GST-α-catulin was removed and membranes were washed 2× 10 min in overlay buffer, followed by two brief washes in TBS. Western blotting procedures described above were then used to detect proteins.

Immunohistochemistry. HEK293 cells were fixed in ice-cold 4% paraformaldehyde in PBS for 10 min at 25 °C. Cells were permeabilized in PBS containing 1% Triton X-100, 0.1% Tween 20 (TBST). After 10 min. Blocking, antibody incubation, and wash steps were done in cold PBS containing 1% BSA and 1% fish gelatin. Antibodies were incubated at 25 °C for 1 h and washed three times for 10 min. In vitro Alexa Fluor secondary antibodies were incubated overnight at 4 °C. Nuclei stain DRAQ5 (Axxora Platform) or actin stain phalloidin 635 (Invitrogen) were added for 20 min at 25 °C, and coverslips were then mounted on glass slides with In Vitrogen ProLong Gold. Cells were imaged on a Zeiss 510 META confocal microscope using a 100× oil immersion lens. Staining of wild-type mouse quadriceps was performed as previously described (42). Data analysis was performed with ImageJ software.

IP3 Assay. To knock down α-catulin expression, HEK293 cells were treated with 20 nM siRNA against α-catulin (Invitrogen Stealth RNAi siRNA). Twenty-four hours later both siRNA-treated and untreated cells were transfected with constructs as indicated. Twenty-four hours later siRNA cells were treated with 20 nM siRNA. Cells were then seeded into 24-well plates with 2 mM/ml ‘H-myoinositol (American Radiolabeled Chemicals). Twenty-four hours later cells were stimulated with 100 mM phenylephrine, and total inositol phosphates were purified as described previously (45). Total inositol phosphates purified were normalized to incorporated counts and analyzed in Prism software (GraphPad Software).

Radioligand Binding and ERK1/2 Assays. Radioligand binding assays and ERK1/2 phosphorylation assays have been previously described (10, 45).

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