A δ-Catenin Signaling Pathway Leading to Dendritic Protrusions*

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δ-Catenin is a synaptic adherens junction protein pivotally positioned to serve as a signaling sensor and integrator. Expression of δ-catenin induces filopodia-like protrusions in neurons. Here we show that the small GTPases of the Rho family act coordinately as downstream effectors of δ-catenin. A dominant negative Rac prevented δ-catenin-induced protrusions, and Cdc42 activity was dramatically increased by δ-catenin expression. A kinase dead LIMK (LIM kinase) and a mutant Cofilin also prevented δ-catenin-induced protrusions. To link the effects of δ-catenin to a physiological pathway, we noted that (S)-3,5-dihydroxyphenylglycine (DHPG) activation of metabotropic glutamate receptor-induced dendritic protrusions that are very similar to those induced by δ-catenin. Furthermore, δ-catenin RNA-mediated interference can block the induction of dendritic protrusions by DHPG. Interestingly, DHPG dissociated PSD-95 and N-cadherin from the δ-catenin complex, increased the association of δ-catenin with Cortactin, and induced the phosphorylation of δ-catenin within the sites that bind to these protein partners.

δ-Catenin is a component of the synaptic adherens junction that is necessary for normal learning and memory (1). In the absence of δ-catenin, mice have severe deficits in several types of memory as well as synaptic plasticity. However, the functional basis for these deficits is not obvious, particularly because the morphological changes in δ-catenin null mice are minimal. δ-Catenin contains 10 Armadillo repeats (a 42-amino acid motif, originally described in the Drosophila segment polarity gene, armadillo) spaced in the characteristic arrangement of all members of this gene family which includes the prototypical member, p120ctn, as well as p0071, ARVCF (Armadillo Repeat gene deleted in Velo-Cardio-Facial syndrome) (2), and the plakophilins, both components of the desmosome (3–6). The core functions of this protein family are stabilization of cadherins by binding to a highly conserved sequence in the juxtamembrane region and regulatory coordination over Rho GTPases (7). δ-Catenin is localized to the post-synaptic adherens junction, collaborates with Rho GTPases to set a balance between neurite elongation and branching, and robustly induces dendritic protrusions (8). Among the cadherin binding family members, δ-catenin is the only one that is a neural-specific protein. However, δ-catenin null mice develop normally, whereas p120ctn can regulate synapse and spine development (9).

Because both p120ctn and δ-catenin are expressed in neurons, an important question is the added functionality provided by co-expression of these paralogs. In contrast to p120ctn, δ-catenin contains a short carboxyl-terminal motif that corresponds to a ligand sequence for PDZ (postsynaptic density-95 (PSD-95)/discs large/zona occludens-1) domain-containing proteins. Through the versatility of this domain, the multiple complex interactions of δ-catenin with the synapse arise. δ-Catenin binds to the synaptic scaffolding molecule (S-SCAM) (10) and to PSD-95 (11, 12), both of which contain multiple PDZ domains. δ-Catenin also binds via a PDZ interaction to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor-binding protein and the related glutamate receptor (GluR)-interacting protein (12). These scaffolding proteins interact with neurologin, a cell adhesion molecule, the N-methyl-D-aspartate receptor (13), and the GluR2 and GluR3 subunits of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (12). δ-Catenin also interacts with two members of the LAP (leucine-rich repeats and PSD-95/Dlg-A/ZO-1 (PDZ) domains) family of proteins. These are Erbin (14) and Densin-180 (15), which interacts with the metabotropic glutamate receptor scaffold protein Shank (16). δ-Catenin interacts with p190RhoGEF through AKT1-mediated phosphorylation, indicating an additional essential role for δ-catenin in dendritic morphogenesis (17). This complex set of interaction partners suggests that δ-catenin is pivotally positioned to coordinate synaptic activity with cell-cell adhesion properties.

* Support for this work was provided by the W. M. Keck Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4 and Tables S1 and SII.

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NOVEMBER 21, 2008 • VOLUME 283 • NUMBER 47

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 47, pp. 32781–32791, November 21, 2008

JOURNAL OF BIOLOGICAL CHEMISTRY 32781
Given the minimal morphological changes in the brains of δ-catenin gene-disrupted animals, the role of δ-catenin will likely emerge with more dynamic experiments related to synaptic activation. Neuronal culture provides a means to assess an intrinsic role for the protein independent of the developmental confounders in the reported gene disruption study (1). The signaling pathway downstream of metabotropic stimulation is particularly germane because the selective group 1 mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG) leads to calcium release from intracellular stores, local protein synthesis, and importantly, dendritic protrusive activity (18). Here we suggest that δ-catenin lies at a hub in the metabolotropic glutamatergic signaling network leading to actin cytoskeletal reorganization.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and DHPG Treatment**—Primary cultures were prepared from hippocampi of embryonic day 18 Sprague-Dawley rats as described previously (19). They were maintained in Neurobasal medium (Invitrogen) containing B27 supplement (Invitrogen) and 0.5 mM L-glutamine. HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in the same atmospheric conditions. For DHPG treatment, 21 days in vitro (DIV) neurons were washed once with prewarmed Neurobasal medium. Immediately afterward, DHPG (Sigma) was added at a final concentration of 100 μM, and cells were incubated for different time points at 37 °C. For the phosphoprotein analysis, cortical neurons were treated with DHPG for 30 min.

**Antibodies**—The affinity-purified antibody raised against a δ-catenin peptide corresponding to amino acids 434–530 in rabbit was described previously (20). A confirmatory δ-catenin antibody ( monoclonal) was purchased from BD Transduction Laboratories; synaptophysin and cortactin antibodies were from Sigma-Aldrich; PSD-95, phospho-Cofilin, GFP, and MAP2 antibodies were from Chemicon (Temecula, CA); HA antibody (monoclonal) was purchased from BD Transduction Laboratories; synaptophysin and cortactin antibodies were from Sigma-Aldrich; PSD-95, phospho-Cofilin, GFP, and MAP2 antibodies were from Chemicon (Temecula, CA); N-cadherin, β-actin, and cyclophilin B antibodies were from Abcam; αN-catenin (rat monoclonal, NCAT2) antibody was from the Hybridoma Bank of the University of Iowa. Phospho-LIMK1 antibody was from Cell Signaling Technology (Danvers, MA). Alexa 488, Alexa 568, Cy3, or Cy5-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were from Molecular Probes (Eugene, OR).

**DNA Preparation and cDNA Transfections**—A full-length δ-catenin cDNA was subcloned into pEGFP (Clontech, Palo Alto, CA) or into pCMVTag3B (Stratagene, La Jolla, CA) as described previously (20). pEGFP-δ-catenin* is a resistant full-length δ-catenin construct mutated at sites mapped to the region of Dpplx1 siRNA, prepared using site-directed mutagenesis. Expression plasmids for HA-tagged LIMK1 and their mutants or for S3A Cofilin were constructed as described (21, 22). The cDNA coding for Rac-dominant negative was a generous gift from Dr. Alan Hall (MRC, Laboratory for Molecular Cell Biology, London, UK). Three weeks after plating, hippocampal neurons were transfected with the Helios gene gun (Bio-Rad). To make the bullets, each cDNA was attached to gold particles. To achieve a uniform distribution of gold particles and reduce damage due to particle bombardment, mesh barriers (steel mesh plus 70-μm nylon mesh) were used as a diffusion screen for dissociated culture neurons. The expression of transfected cDNAs was detected 24–30 h later.

**siRNA Preparation and Transfection**—A set of siRNAs corresponding to mouse and rat δ-catenin mRNA was designed and synthesized by (Dharmacon) as follows: Duplex0 (Dpplx0)-(amino acid 581) 5'-GGCAGAGAUAAGGAGACUUU; Duplex1 (Dpplx1)-(amino acid 1216) 5'-GCAACAUUGUCGA-CUUCUAUU-3'; Duplex2 (Dpplx2)-(amino acid 749) 5'-GCA-GUGAGUCAUGCAAUU-3'; Duplex3 (Dpplx3)-(amino acid 1120) 5'-CCACGGAAUAGAGAGAAUU-3'; Duplex4 (Dpplx4)-(amino acid 1125) 5'-GAACACACCUCUCAGAAA-GUU-3'. Duplexes 1–4 were mixed to form the SMART pool (Dharmacon). Cyclophilin B (catalog #D-001136-01-20; Dharmacon) was used as a positive control, and siCONTROL siRNA (catalog # D-001210-01; Dharmacon) used as negative control. Duplex0 and Duplex1 were used for all of the immunostaining experiments. Transfections of siRNAs into neurons were performed using TransMessenger™ Transfection reagent (Qiagen) as described (23). Lipofectamine 2000 (Invitrogen) was used for co-transfections of reporter plasmid and siRNA sequences.

**Immunoblot Analysis and Quantification**—Proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad), and ECL (Amersham Biosciences) was used for detection. Immunoblots of δ-catenin knockdown and DHPG treatments were quantified using Meta Morph (Universal Imaging Corp., West Chester, PA) and ImageQuant software (Amersham Biosciences). β-Actin was used for loading normalization. Percent of inhibition was calculated relative to the mock-transfected samples. Statistical significance was calculated using Student’s t test or analysis of variance.

**Immunocytochemistry**—Neurons were fixed with 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline (PBS) for 15 min at room temperature, washed 3 times in PBS, and permeabilized for 5 min in PBS containing 0.25% Triton X-100. After washing and blocking, the cells were incubated with primary antibody for 90 min at room temperature or overnight at 4°C. Secondary antibodies were applied for 1 h at room temperature. Secondary antibodies conjugated to Alexa 488 and Alexa 568 were used for double labeling and Cy3- and Cy5- were used in the presence of GFP-expressing cells.

**Immunoprecipitation**—After each incubation, the medium was aspirated, cells were washed in ice-cold phosphate-buffered saline to stop the reaction and lysed with lysis buffer (15 mM Tris, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 120 mM NaCl, 25 mM KCl, 1 mM Na3VO4, 20 mM NaF, and protease inhibitor mixture, Roche Applied Science), and protein G beads were added for pre-clearing for 30 min. After centrifugation, the supernatant was immunoprecipitated with the specific antibody overnight at 4°C, and the immunoprecipitates were captured by protein G beads or A beads (Pierce). Normal mouse or rabbit IgG were used as negative controls (Santa Cruz Biotechnology).

Phosphoprotein Sequence Analysis by Liquid Chromatography-Tandem Mass Spectrometry—Two-week-old cortical cultures were treated with vehicle control or 100-μm DHPG or
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then reattached to the HPLC system. A gradient was formed, and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid).

As each peptide was eluted they were subjected to electrospray ionization, and then they entered into an LTQ linear ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). Eluting peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences were determined by matching protein or translated nucleotide databases with the acquired fragmentation pattern by the software program, Sequest (ThermoFinnigan) (26). The modification of 80 mass units to serine, threonine, and tyrosine was included in the data base searches to determine phosphopeptides. Each phosphopeptide that was determined by the Sequest program was also manually inspected in ensure confidence.

\textbf{Image Acquisition and Quantification}—Confocal images were obtained with an Olympus Fluoview1000 confocal laser scanning microscope. Olympus images were obtained using oil 60× and 40× objectives with sequential acquisition settings at a resolution of 1024 × 1024 and 512 × 512 pixels, respectively. Each image was a 0.5-μm z-series of 7–13 images averaged 2–4 times. Confocal scanning settings of pinhole, brightness, and contrast were kept the same for all images when intensity was compared. Additional digital zoom factor of \( \times 3 \)–5 was used as well. MetaMorph software was used for morphometric analysis. Maximum projection of the z-stack images was flattened into a single image and was used for all quantitative analysis, in parallel with the z-stack image. The density of dendritic protrusions and their lengths were measured blinded in neurons transfected with GFP or GFP-δ-catenin constructs. Representative counts were confirmed by an independent observer.

\textbf{DHPG/RNAi Quantification}—After DHPG treatment the neurons were stained with δ-catenin and PSD-95 antibodies. GFP was used to visualize the shape of the cell and the protrusions. 7–10 neurons of three experiments were analyzed for each condition. 2–3 segments of 50–100-μm secondary branched dendrites were counted from each neuron. All the

250 μm of the antagonist (S)-\( \alpha \)-methyl-4-carboxyphenylglycine for 30 min. Cells were lysed, and δ-catenin was immunoprecipitated using 50–70 μg of the δ-catenin polyclonal antibody, separated on SDS-PAGE, and stained with Coomassie Blue. δ-Catenin bands were cut from the gels and analyzed by the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA) as follows. Gel pieces were subjected to a modified in-gel trypsin digestion procedure (24). On the day of analysis the samples were reconstituted in 5 μl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nanoscale reverse-phase HPLC capillary column was created by packing 5-μm C18 spherical silica beads into a fused silica capillary (75-μm inner diameter × 12-cm length) with a flame-drawn tip (25). After equilibrating the column, each sample was pressure-loaded off-line onto the column. The column was 12

\textbf{FIGURE 1. Overexpression of δ-catenin increases protrusions in mature neurons.} A, representative images of 21-DIV neurons that were transfected with either GFP or GFP-δ-catenin at 3 DIV. GFP or GFP-δ-catenin (green) were used to visualize the neuron morphology. Neurons were fixed and stained with δ-catenin (red) and PSD-95 (blue). The merge images show the overlap of GFP or GFP-δ-catenin expression and δ-catenin and PSD-95 staining. Images (e–h) are high magnification of the box in a–d and m–p of i–l. Arrows indicate axon. Scale bars, 50 and 5 μm for low and high magnification, respectively. B, quantification and statistical significance of five experiments. Histograms show the mean ± S.E. (n = 30 neurons per condition by Student’s t test). ***, \( p < 0.001 \).

\textbf{Number of Protrusions/10 μm}
FIGURE 2. Downstream effectors of δ-catenin protrusive activity. A, levels of total and active GTP-bound Rac1 and Cdc42 were measured in Chinese hamster ovary cells transfected with either GFP or GFP-δ-catenin. Equal amounts of protein were extracted and subjected to Rac1 activity assay or Cdc42 activity assay. a, representative immunoblot showing the total and GTP-bound Rac1. b, quantification and statistical significance of five experiments. c, representative immunoblot showing the total and GTP-bound Cdc42. d, quantification and statistical significance of six experiments. Histograms show the mean ± S.E. (Student t test). **, p < 0.01; ***, p < 0.001. δ, a and b, a neuron transfected with GFP-δ-catenin (green) and HA-tagged LIMK1-wild type (red/merge). e and d, image showing a neuron co-transfected with δ-catenin (green) and HA-tagged LIMK1-kinase dead (red/merge). Note the absence of protrusive expansions in the co-transfected neuron. e, high power micrograph of b and f of a, g, and h, images showing a neuron transfected with GFP-δ-catenin (green) plus HA-tagged LIMK1-wild type (not shown); neurons were fixed 18 h after transfection and counterstained for MAP2. Note the exuberant protrusions. i, high power micrograph showing a dendritic segment from a neuron transfected with GFP-δ-catenin (green) and Rac dominant negative (red); note the absence of protrusive activity. j, high power micrograph showing a dendritic segment for a neuron transfected with GFP-δ-catenin (green) and constitutively active Cofilin (S3A-Cofilin; red); note the absence of protrusive activity. C, DIV representative neurons that were transfected with either GFP-δ-catenin (a–c) or co-transfected with Cdc42 dominant negative (Cdc42-DN) and GFP-δ-catenin (d–f and g–i) and immunostained as indicated. c is a high magnification of the rectangle in a, f, of d and i of g. Scale bars, 10 and 5 μm for low and high magnification, respectively.

RESULTS

δ-Catenin Recruits Small Rho GTPases to Induce Protrusive Activity—To understand the mechanistic basis for the observation that δ-catenin can induce dendritic protrusions, we overexpressed δ-catenin in mature primary cultured neurons and observed the expected increase in dendritic protrusions (Fig. 1). As shown previously (8, 17, 33), δ-catenin dramatically induced protrusions. Neurons were transfected with either GFP alone or full-length GFP-δ-catenin and analyzed for the number of protrusions at 20 DIV based on GFP visualization, δ-catenin, and PSD95 immunolocalization (Fig. 1). The elaboration of protrusions was restricted to the dendritic tree despite GFP-
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The effects of DHPG stimulation. Ad21-DIV neurons were induced with DHPG for the indicated times and subjected to Rac1-GTP and Cdc42-GTP activity assays. Representative immunoblots show the total and GTP-bound Rac1 and total and GTP-bound Cdc42. Also shown are quantification of five experiments of Rac1 activity assays (b) and of Cdc42 activity assays (c). The relative activity of each GTPase was determined by quantifying each band of GTP-bound GTPase and the total amount of GTPase using the Scion software program. Histograms show the mean ± S.E. **p < 0.01. B, DHPG induced morphological changes in hippocampal neurons. DHPG treatments were performed in 21-DIV cultured neurons for 30 min. For visualization, GFP was transfected using a gene gun and double immunolabeled with PSD-95 and synaptophysin. Compare images a and b without treatment to images c and d, high power images of rectangles in a and c, respectively. The merged images show co-localized PSD-95 and synaptophysin along the dendritic shaft and extending into some spine-like structures as white puncta. Fewer co-localized puncta are visible after DHPG. C, quantification of the average length of protrusions of vehicle control and DHPG-treated neurons. DHPG resulted in a significant increase in the average length of protrusions related to the vehicle control (***, p < 0.001). Histograms show the mean ± S.E. (n = 20 neurons in each experiment by Student's t test). Scale bars, 50 μm (a and c) and 10 μm (b and d).

TABLE 1
Affect of regulators downstream of δ-catenin on dendritic protrusions

| Constructs transfected (1 μg/construct) | Number of protrusions/25 μm p Value mean ± S.E. |
|----------------------------------------|--------------------------------------------------|
| GFP                                    | 6.2 ± 0.8 NS                                     |
| GFP (2 μg)                             | 6.6 ± 0.4 NS                                     |
| GFP-δ-catenin                          | 11.5 ± 0.5 <0.01                                |
| GFP-δ-catenin + LIMK1 wt               | 19.5 ± 1.2 <0.001                               |
| GFP-δ-catenin + LIMK1 kd               | 2.7 ± 0.4 <0.01                                 |
| GFP-δ-catenin + S3A Cofilin            | 2.9 ± 0.6 <0.01                                 |
| GFP-δ-catenin + Rac-DN                 | 2.3 ± 0.8 <0.01                                 |
| GFP-δ-catenin + Y27632                 | 22.7 ± 0.9 <0.001                               |
| GFP + LIMK1 wt                         | 9.2 ± 0.4 <0.01                                 |
| GFP + LIMK1 kd                         | 1.8 ± 0.6 <0.01                                 |
| GFP + Y27632                           | 14.4 ± 0.6 <0.01                                |

δ-catenin signal throughout the neuron including the axon (Fig. 1A).

We had previously linked δ-catenin-induced protrusions and branching to RhoA inhibition (8), a finding supported by studies of δ-catenin paralogs (34). Rac activation by δ-catenin was demonstrated by increased Rac activity in Chinese hamster ovary cells which overexpressed δ-catenin (Fig. 2A, a and b). A dominant negative Rac prevented δ-catenin protractive activity (Fig. 2Bi). Cdc42 activity also dramatically increased in the presence of δ-catenin expression (Fig. 2A, c and d), and a dominant negative Cdc42 prevented δ-catenin protractive activity in 21 DIV neurons (Fig. 2C). Thus, activation of Rac and Cdc42 occurred simultaneously with Rho inhibition and collectively appeared to operate downstream of the δ-catenin ability to induce protrusions. Our data are not consistent with Kim et al. (17), who detected no effect on Cdc42 and Rac1 activity in the presence of δ-catenin overexpression. However, our data are consistent with many reports showing that the Rho family of small GTPases (particularly RhoA, Rac1, Cdc42) are well known regulators of the actin cytoskeleton that have profound influence on spine morphogenesis (35–37).

An active form of Rac can increase the activity of LIMK1 (38). Activation of LIMK phenocopied the increased protractive activity associated with δ-catenin overexpression (Fig. 2B, g and h). Neurons co-transfected with δ-catenin and an HA-tagged LIMK1-kinase dead mutant did not elaborate protrusions (Fig. 2B, c, d, and f) and Table 1). If LIMK acts downstream of δ-catenin, one would expect to see a similar blockade of protractive activity with mutant actin depolymerizing factor/Cofilin. Actin depolymerizing factor/Cofilin is the only substrate identified for LIMK (39) which phosphorylates Cofilin (at serine 3) when in its active phosphorylated state (phospho-LIMK1). The phosphorylation of Cofilin is an inactivation step that prevents actin binding and thereby promotes actin protractive activity by reducing actin dynamics. S3A Cofilin is a mutant that cannot be phosphorylated by LIMK1 and, therefore, is constitutively active. S3A is, therefore, functionally equivalent to the LIMK kinase dead mutant, and it too blocked δ-catenin-induced protrusions (Fig. 2Bi).
δ-Catenin Is an Intermediary in Group I mGluR Activation—

Interestingly, we found that Group I mGluR activation also operates through these small Rho GTPases. 5 min after DHPG stimulation, 21 DIV hippocampal neurons increased Rac and Cdc42 activity significantly (Fig. 3A). Furthermore, delivery of DHPG to neurons induced spine elongation and filopodial elaboration (18). We replicated these morphological effects of DHPG (Fig. 3, B and C). We next sought to determine whether reduced levels of δ-catenin would interfere with the elaboration of DHPG-induced protrusions.

A variety of siRNAs were tested for their ability to knockdown δ-catenin in heterologous cells by co-transfection of the siRNAs, GFP, and GFP-δ-catenin into HEK293 cells. Transfected proteins were immunoprecipitated with GFP antibody and analyzed by Western blot with GFP and δ-catenin antibodies (Fig. 4A). Although all of the sequences showed some degree of knockdown of GFP-δ-catenin in HEK293 cells, sequences Dplx0 and Dplx1 were optimal (Fig. 4A). A developmental blot showed that δ-catenin started expressing 1 day after plating (Fig. 5B). δ-Catenin RNAi induced a small reduction in MAP2 (data not shown) that just reached significance, perhaps due to the attenuation of the dendritic tree. The level of PSD-95 did not change by Western blot (data not shown).

To rescue the phenotypic effect of δ-catenin inhibition on dendritic protrusions, we transfected a mutated form of GFP-δ-catenin* (resistant to Dplx1 siRNA) together with δ-catenin siRNA Dplx1 (Fig. 5, C and D). The expression of the resistant GFP-δ-catenin* construct reversed the phenotype, and ~80% of the protrusions were restored using immunohistochemistry compared with the neurons co-transfected with GFP and non-targeting siRNA (Fig. 5D).

Neurons treated with δ-catenin siRNAa were unresponsive to DHPG (Fig. 6 and supplemental Fig. S4). Neurons transfected with non-targeting siRNA showed an increase in the total number and lengths of protrusions as expected after DHPG treatment (Fig. 3, B and C). In the control non-targeting siRNA sample, the mean total number of protrusions was 7.48 ± 1/10 μm, a value that decreased by 20% to 4.96 ±

### Figure 4. RNAi knock-down of δ-catenin.

A suppression of δ-catenin expression in HEK293 cells. pEGFP-δ-catenin was co-transfected with pEGFP and Dplx0 or Dplx1 or Dplx2 or Dplx3 or Dplx4 or SMART pool or non-targeting siRNA sequences. GFP antibodies were used for immunoprecipitation, and blots were hybridized with GFP and δ-catenin antibodies. All siRNA sequences inhibited the expression of δ-catenin in heterologous cells. B, efficiency and specificity of δ-catenin siRNAs in cultured hippocampal neurons. Immunoblot of 14 DIV hippocampal neurons transfected with the indicated duplex siRNAs, δ-catenin antibodies were used to detect δ-catenin protein, and β-actin antibodies were used as a loading control. Dplx0 and Dplx1 suppressed δ-catenin efficiently; all other the siRNA sequences inhibited δ-catenin knockdown neurons. Representative images of 20 DIV that were co-transfected with GFP and δ-catenin siRNA sequences (Dplx1 or Dplx0) and visualized by co-transfection with GFP. Spines and filopodia were counted and plotted as described under "Experimental Procedures." The immunoblot shows δ-catenin siRNA-dependent silencing. The histogram shows the mean ± S.E. (n = 13 neurons per condition from four experiments by Student’s t test).***, p < 0.001; *, p < 0.05.

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We replicated these morphological effects of δ-catenin inhibition on dendritic protrusions. Neurons transfected with GFP-δ-catenin* (resistant to Dplx1 siRNA) together with δ-catenin siRNA Dplx1 (Fig. 5, C and D). The expression of the resistant GFP-δ-catenin* construct reversed the phenotype, and ~80% of the protrusions were restored using immunohistochemistry compared with the neurons co-transfected with GFP and non-targeting siRNA (Fig. 5D).

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1.54/10 μm (Fig. 6B, p < 0.001) after δ-catenin RNAi. DHPG stimulation of the control non-targeting siRNA sample significantly increased total protrusions to 8.49 ± 0.77/10 μm (Fig. 6B, p < 0.05), but in the presence of δ-catenin RNAi no DHPG effect occurred; the mean total number of protrusions remained similar to siRNA treatment alone (5.07 ± 0.88/10 μm).

A more detailed analysis of the dendritic protrusion categories based on length and shape (see “Experimental Procedures”) revealed more specific effects. Protrusions of 0–3 μm represent mostly mature dendritic spines, and they account for the majority (94%) of the total protrusions under control conditions (Fig. 6B). δ-Catenin silencing affected this population dramatically; the number of protrusions in this category decreased by 46% (from 7.05 ± 0.63 to 2.8 ± 1.31/10 μm, p < 0.001, Fig. 6B). Mushroom-type spines were measured as part of the 0–3-μm category, and they showed a significant loss in the δ-catenin inhibited neurons (from 3.58 ± 0.47 to 1.75 ± 0.6/10 μm, p < 0.001, Fig. 6B).

Interestingly, the numbers of the protrusions in the 3–4- and 4–10-μm categories increased after δ-catenin inhibition, probably due to loss of N-cadherin stability in the absence of δ-catenin (40, 41). Protrusions in these categories, especially the 4–10-μm category, resemble immature spines seen in developing neuronal cultures.

Under control conditions, DHPG mainly increased the numbers of protrusions in the length categories of 3–4 μm (from 0.31 ± 0.28 to 1.01 ± 0.46, p < 0.001) and the 4–10 μm (from 0.07 ± 0.16 to 0.59 ± 0.25, p < 0.001, Fig. 6B). This increase did not occur under δ-catenin silencing conditions (Fig. 6B). Taken together, DHPG failed to induce its expected effects on dendritic protrusions in the presence of δ-catenin knockdown (Fig. 6).

mGluR Activation Reconfigures δ-Catenin Binding Partners and Induces δ-Catenin Phosphorylation—To search for the effects of mGluR stimulation on δ-catenin, 21 DIV hippocampal neurons were treated with DHPG, and changes in δ-catenin binding partners were probed. After DHPG incubation, δ-catenin was immunoprecipitated, and the precipitated pellets were blotted with antibodies against the δ-catenin interactor proteins. Cortactin, PSD-95, and N-cadherin. DHPG induced a reconfiguration of the δ-catenin complex over a similar time frame as the elaboration of increased dendritic protrusions. After treatment with DHPG for 20 min, co-immunoprecipitated Cortactin increased, whereas co-immunoprecipitated N-cadherin and PSD-95 decreased (Fig. 7).

One mechanism by which mGluR activation may act upon δ-catenin is by inducing its phosphorylation. δ-Catenin was immunoprecipitated from cultured rat cortical neurons either with or without DHPG stimulation, and mass spectroscopy identified phosphorylation sites with a DHPG-induced on-off state (Fig. 8 and supplemental Table SII). Most of these sites lie in interaction domains with δ-catenin binding partners. These
phosphorylation sites include Ser-693 in the fourth Arm repeat, which interacts with classical cadherins (42), Ser-1242 at the pemutaneous serine related to PDZ interactions, and Ser-1063/Thr-1064 and Ser-1098 two sites between the Arm repeats and RhoA, Rac1, Cdc42) are well known regulators of the actin downstream. The Rho family of small GTPases (particularly mGluR activation upstream and to the Rho family GTPases, with decreased Rac1 and increased phosphorylation may operate via p120ctn (43–45) in its role in organizing actin into filaments. Here we report that δ-catenin inhibits RhoA (8) by decreasing the expression of δ-catenin with Cortactin and Cortactin were significantly increased after treatments for 20 min. Histograms show mean intensity of normalized co-immunoprecipitated bands ± S.E. (PSD-95, n = 14; N-cadherin, n = 5; Cortactin, n = 12 in each experiments, *, p < 0.05, **, p < 0.01 compared with no treatment by Student’s t test).

**DISCUSSION**

The results described here link δ-catenin to group 1 mGlurR activation upstream and to the Rho family GTPases downstream. The Rho family of small GTPases (particularly RhoA, Rac1, Cdc42) are well known regulators of the actin cytoskeleton that have profound influence on spine morphogenesis (35). RhoA inhibits, whereas Rac and Cdc42 promote, the growth and/or stability of dendritic spines. Consistent with the cellular phenotype induced by δ-catenin, expression of δ-catenin inhibits RhoA (8) by decreasing the binding between p190RhoGEF and RhoA, resulting in lower levels of GTP-RhoA (17). A similar mechanism of RhoA inhibition may operate via p120ctn (43–45) in its role in organizing actin into filaments. Here we report that δ-catenin also activates Rac and Cdc42, a finding that follows from the decreased Rho activity. A further parallel with p120ctn is the observation that p120ctn loss resulted in mis-regulation of Rho-family GTPases, with decreased Rac and increased RhoA activity (9). Farther downstream, δ-catenin expression
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is associated with phosphorylation of LIMK and Cofilin. Consistent with these findings are other observations that LIMK knock-out mice have short and smaller dendritic spines and, like δ-catenin knock-out mice, have enhanced hippocampal long-term potentiation (46). After exploratory activity in vehicle-treated rats compared with rats treated with the N-methyl-D-aspartate receptor antagonist (+/−)-3-(2-carboxyypiperazin-4-yl)propyl-1-phosphonic acid (CPP) dense phosphorylated Cofilin immuno-reactivity appeared in enlarged spines of hippocampal field CA1 (47).

The question remained as to what upstream stimuli trigger the pathway that links δ-catenin to the small Rho GTPases. The type I mGluR activation was a logical receptor to study because stimulation of this receptor by DHPG induced dendritic protrusions that resembled those of δ-catenin. Furthermore, DHPG stimulation, like δ-catenin expression, also increased Rac and Cdc42 activity in 21 DIV hippocampal neurons (Fig. 3A). We found that activation of the type I mGluR receptors by DHPG dissociated δ-catenin from PSD-95 and from N-cadherin while increasing its association with Cortactin (Fig. 7). mGluR activation also induced δ-catenin phosphorylation at the protein-protein interaction sites where PSD-95, N-cadherin, and Cortactin bind. Thus, dissociation of δ-catenin from the post-synaptic scaffold releases a latent tendency to elaborate filopodia by multiple pathways related to both small Rho GTPases and Cortactin (Fig. 9). Previous studies have shown that Rac is locally activated in dendritic spines (48), and Cdc42 is a key module in dendritic spine formation (49). In older neurons with established synapses, Cortactin knockdown results in depletion of dendritic spines, and in response to synaptic stimulation and N-methyl-D-aspartate receptor activation, Cortactin redistributes rapidly from spines to the dendritic shaft (50). Thus, reconfiguration of

FIGURE 8. Sites of DHPG-induced phosphorylation on δ-catenin as determined by mass spectrometry. 14-DIV cortical neurons were treated for 30 min with DHPG or (S)-α-methyl-4-carboxyphenylglycine (MCPG) or no treatment. Endogenous phosphorylated δ-catenin was analyzed by phospho-mass spectrometry. A, list of the trypsin phosphotryptic fragments found to be DHPG-inducible. + indicates specific phosphorylation of Ser/Thr site(s), whereas − indicates that the Ser/Thr site(s) was not phosphorylated under the indicated stimulation conditions. B, Ser-693, Ser-1063 or Thr-1064, Ser-1064, and Ser-1242 sites (blue) were phosphorylated, whereas Ser-264 and Ser-273 sites (red) were dephosphorylated. The δ-catenin binding domains and interacting proteins are indicated. Amino acids numbers are based on the rat δ-catenin protein sequence.

FIGURE 9. The δ-catenin pathway. The model indicates the series of δ-catenin-related events that occur after group I mGluR activation. The reconfiguration of the δ-catenin complex is indicated by green arrows, and a networked pathway of activation and inhibitory signals that lead to actin polymerization and increased dendritic protrusions are shown. DHPG-induced release of δ-catenin from its submembranous partners N-cadherin and PSD-95 may set up a collaboration with Cortactin, Rac, Rho, Cdc42, LIMK, and Cofilin to reorganize actin. ROCK, Rho kinase; PAK, p21-activated kinase.
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δ-catenin binding partners, and a shift in its functional localization may induce filopodia (Fig. 9).

Finally, RNAi knockdown of δ-catenin prevented DHPG from inducing filopodia. Interestingly, reducing δ-catenin had a unique phenotype that had some superficial parallels with δ-catenin overexpression. In both cases, filopodial elaboration occurred. However, the magnitude of the response and, more importantly the qualitative nature, differed greatly. Reduced δ-catenin resulted in fewer filopodia than overexpression, and the filopodia that did emerge were restricted to a size category consistent with immature protrusions (Fig. 6). We suggest that in contrast to δ-catenin overexpression, which likely operates through small Rho GTPases and Cortactin, δ-catenin suppression likely operates by destabilization of cadherin and the initiation of filopodia emergence reported under these conditions (40, 41, 51). In fact, we did observe reduced N-cadherin in the presence of δ-catenin RNAi (Fig. 5).

Once dissociated from its submembranous partners, δ-catenin collaborates with Rac, LIMK, Cofilin, and Cortactin in convergent pathways that assemble actin filaments (Fig. 9). Together these findings reveal a signaling pathway that captures a broad network of proteins to coordinate the precise effects on actin dynamics needed for plasticity. These coordinated effects may establish the antagonistic relationship between Rac activation and Rho inhibition that leads to protrusive activity (52). However, the picture is by no means complete. Additional proteins such as Cordon-Bleu (53) are likely to serve between Rac activation and Rho inhibition that leads to protrusive activity that caps off with p21-activated kinase (PAK) (65).

Acknowledgments—We thank Andrea S. Witt and Michael Majewski for preparing some of the δ-catenin constructs and Steve Gygi for the mass spectroscopy.

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