Rho-GTPase-activating Protein Interacting with Cdc-42-interacting Protein 4 Homolog 2 (Rich2)

A NEW Ras-RELATED C3 BOTULINUM TOXIN SUBSTRATE 1 (Rac1) GTPase-ACTIVATING PROTEIN THAT CONTROLS DENDRITIC SPINE MORPHOGENESIS*

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Background: Rich2 is a synaptic Rho-GAP (Rho-GTPase-activating protein) the target of which was unknown.

Results: We found that Rich2 controls dendritic spine morphogenesis by inhibiting Rac1 activity.

Conclusion: Rac1 is the target of Rich2 in spines.

Significance: We identified for the first time Rich2 as a Rac1-GAP protein that plays an important role in spine formation.

Development of dendritic spines is important for synaptic function, and alteration in spine morphogenesis is often associated with mental disorders. Rich2 was an uncharacterized Rho-GAP protein. Here we searched for a role of this protein in spine morphogenesis. We found that it is enriched in dendritic spines of cultured hippocampal pyramidal neurons during early stages of development. Rich2 specifically stimulated the Rac1 GTPase in these neurons. Inhibition of Rac1 by EHT 1864 increased the size and decreased the density of dendritic spines. Similarly, Rich2 overexpression increased the size and decreased the density of dendritic spines, whereas knock-down of the protein by specific si-RNA decreased both size and density of spines. The morphological changes were reflected by the increased amplitude and decreased frequency of miniature EPSCs induced by Rich2 overexpression, while si-RNA treatment decreased both amplitude and frequency of these events. Finally, treatment of neurons with EHT 1864 rescued the phenotype induced by Rich2 knock-down. These results suggested that Rich2 controls dendritic spine morphogenesis and function via inhibition of Rac1.

Dendritic spines are the sites of excitatory glutamatergic synapses in the principal neurons of mammalian brain structures. Their development is essential for establishing proper adult brain connectivity. Developmental alterations in density and morphology of spines have been found in many psychiatric and neurological disorders, such as mental retardation and epilepsy (1). Understanding the role of synaptic proteins in spine development is therefore an important issue, not only in term of physiological mechanisms, but also to identify potential therapeutic targets.

Development of dendritic spines implicates rearrangement of the actin network and trafficking of postsynaptic proteins (2–5). Rho-GTPases have been identified as key regulators of cytoskeleton structural changes in many cell types (6) and play major roles in spine development (5). Rho-GTPase activity is modulated by guanine nucleotide-dissociation inhibitors (GDIs), guanine nucleotide-exchange factors (GEFs), and GTPase-activating proteins (GAPs). GDIs sequester Rho proteins away from their targets, while GEFs and GAPs respectively up- and down-regulate the cycle between active GTP- and inactive GDP-bound states of Rho-GTPases. These modulatory proteins thus control dynamics of actin skeleton and spine remodeling (2, 4). Recent studies have reported implication of GEFs in dendritic spine formation. For instance, kalirin-7 is a GEF for Rac1, which plays important role in spine development. Its phosphorylation promotes its activity and leads to spine enlargement in hippocampal pyramidal neurons (7). Intersectin is a GEF for Cdc42 that functions as a multidomain adaptor for proteins involved in endocytosis and regulation of the cytoskeleton. Its activity is enhanced by the numb-binding protein in vivo and controls filopodia formation (8). GAP proteins have also been involved in dendritic spine morphogenesis during development. For instance, the Rho-GAP protein oligophrenin-1 has been shown to regulate the length of dendritic spines in vivo and in hippocampal cultures (9).

3 The abbreviations used are: GEF, guanine nucleotide-exchange factor; Cdc42, cell division cycle 42 protein; DIV, days in vitro; EPSC, excitatory postsynaptic current; GFAP, glial fibrillary acidic protein; GluA1, glutamate receptor subunit A1; NeuN, neuronal specific nuclear protein; RhoA, Ras homolog family member A protein; GAP, GTPase-activating protein; PMA, phorbol 12-myristate 13-acetate.
Rich2 is a Rho-GAP domain containing protein that belongs to a complex implicated in the organization of the sub-apical actin network of epithelial cells (10). This protein has been cloned several years ago (11) and we have recently shown that it is a Rho-GAP protein involved in endosomal recycling and AMPA receptor GluA1 subunit exocytosis during synaptic long term potentiation (12). However, the Rho-GTPase regulated by Rich2 in neurons remained unknown. Here we found that Rich2 specifically controls spine morphogenesis of hippocampal neurons via regulation of the Rho-GTPase Rac1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Neuronal hippocampal cultures were prepared from 17.5 day embryonic mice and grown in Neurobasal medium supplemented with B27 and 10% fetal bovine serum (FBS). Hippocampal neurons cells were transfected at DIV-9 with Lipofectamine 2000 (Invitrogen, Cergy-Pontoise, France) according to the manufacturer’s standard protocol. COS-7 cells were plated in DMEM (Invitrogen/Life Technology, Invitrogen) supplemented with 4 mM Glutamax, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% FBS.

**Antibodies and DNA Constructs**—Rabbit polyclonal anti-Rich2 antibody was generated by targeting the specific Rich2 sequence, SPDMDPADRRQPEQC. Cysteine was linked to hemocyanin by sulfolink (Pierce, Thermo Fisher Scientific, Brebières, France) and the complex injected into rabbits using a previously described protocol (13). The antibody was purified by affinity chromatography using the related peptide coupled to activated-CH Sepharose (Amersham Biosciences, GE Healthcare Europe, Saclay, France) and characterized elsewhere (12). The mouse anti-Rac1 and mouse anti-Cdc42 antibodies were purchased from BD Biosciences (Le Pont-de-Claix, France, Cat. 67B9).

Rich2 constructs were generated from IMAGE clone of mouse Rich2 (clone 6825221) from RZPD German Resource Center for Genome Research. The wild-type Rich2 cDNA were cloned in pCMV-flag2B-Rich2 plasmid (12). The Sh-Rac1-GFP, Sh-Unr-GFP (unrelated/control) were generated in pGHSuper-GFP vector using the following sequences. Sh-Rac1: CCAATGAACCAGTCAGTAA, sh-Unr: ATTCTATCAGTCCACG.

**GTPase Assay**—For GTPase measurements, 0.1 µg of recombinant wild-type Rac, Rho, or Cdc42 was preloaded with 10 µCi of [γ-32P]GTP in 20 µl of 20 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5 mM EDTA, and 0.1 mM DTT. The mixture was incubated for 10 min at 30 °C, and the reaction terminated by adding 5 µl of 0.1 M MgCl2. The resulting [γ-32P]GTP-loaded GTPase solutions were stored on ice. For the GAP assays, we used equimolar amounts of GTPases and GST-Rich2. The P50 Rho-GAP protein was purchased from Cytoskeleton (Tebu-bio, France, Cat. GS501-B). Three µl of the [γ-32P]GTP-loaded GTPase were added to a 30-µl mixture of 20 mM Tris-Cl, pH 7.5, 1 mM non-radioactive GTP, 0.87 mg/ml bovine serum albumin, and 0.1 mM DTT with the GST-Rich2. The mixture was incubated at 30 °C, 5-µl aliquots were removed after 0, 3, 6, 9, and 12 min, and the reaction was stopped by addition of 1 ml of ice-cold buffer A (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, and 5 mM MgCl2). The samples were collected on nitrocellulose filters and washed with 10 ml of ice-cold buffer A, and the portion of [γ-32P]GTP remaining bound to the GTPases was determined by scintillation counting.

**Rho-GAP Function with RAICHU Probes**—The pRAICHU probes for Cdc42, RhoA, and Rac1 were generously provided by M. Matsuda (15). COS-7 cells plated in 96 wells were transfected with the different probes and stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA) over 10 min to activate Rho-GTPases. Fluorescence intensity at 480 nm (CFP) and 530 nm (YFP) were measured using a Safire II fluorimeter (TECAN, Lyon, France) after CFP stimulation at 430 nm. FRET efficiency was then determined by measuring the sensitized emission of the acceptor as previously described (16). Data were expressed as the mean ± S.E. and statistical tests performed using the Student’s t test.

**FRET Intensity Measurement and FRET Image Acquisition**—FRET image acquisition in neurons transfected with pRAICHU probes was performed using a LSM-Meta 510 Zeiss confocal microscope (Carl Zeiss S.A.S., Le Pecq, France). CFP excitation was performed at 405 nm, and image fluorescence emission signals were collected from 415 nm to 580 nm. A fluorescence spectrum was thus generated from images acquired every 10 nm (see Fig. 3B). Ratio of images obtained at 525 nm and 480 nm provided FRET images. Calculation of FRET efficiency was obtained by dividing spectral values of individual dendritic spines obtained at 525 nm and 480 nm (Fig. 2, D and E).

**Electrophysiology**—Mouse hippocampal cultures were continuously perfused with the following external medium (mM): NaCl (140), MgCl2 (2), CaCl2 (2), KCl (3), HEPES (10), d-glucose (10), tetrodotoxin (0.0003), bicucullin (0.01), pH 7.4 and osmolarity of 330 mOsm. The transfected neurons were selected on the basis of their fluorescence (YFP) and recorded at room temperature using the whole-cell configuration of the
patch-clamp technique. The recording pipettes had a resistance of 3–5 MOhm when filled with the following medium (mM) KCl (140), HEPES (10), d-glucose (10), pH 7.2 and osmolarity of 300 mOsm.

Miniature EPSCs (mEPCs) were recorded at −65 mV membrane potential through an Axopatch 200B amplifier (Axon Instruments, Union City, CA), filtered at 1 kHz and then digitized at 3 kHz using Axotape software (Axon Instruments). Currents were analyzed using the pClamp 9 software (Axon Instruments). All the detected events were re-examined and accepted or rejected on the basis of visual examination. Once more than 100 events had been collected from a neuron, the average frequency and amplitude of the events were measured on the total duration of the sample. Data obtained from the indicated number (n) of cells were expressed as the mean ± S.E. and analyzed using the statistical Student’s t test.

**Immunofluorescence Staining and Analysis**—Cells were fixed for 20 min in 4% paraformaldehyde buffered in phosphate-buffered saline (PBS) followed by three washes in PBS. They were then permeabilized in PBS supplemented with 2% BSA and 0.1% Triton X-100 and incubated for 1 h at room temperature with appropriate primary antibodies in the same buffer. Mouse

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**FIGURE 1. Rich2 is localized in dendritic spines.** A, immunostaining of E18.5 rat hippocampal CA1, CA2, and CA3 regions with anti-NeuN (green) and anti-Rich2 (red) antibodies (upper panel). In this and the following panels, right images represent merged red and green images. Middle panel: similar to the upper panel, but in an enlarged region of CA1 of a different sample. Lower panel: immunostaining of E18.5 rat hippocampal with an anti-GFAP antibody. Please note localization of Rich2 in neurons (NeuN labeling, arrows), but not glial cells (GFAP labeling; Scale bars: 100 and 40 μm, respectively). B, DIV-21 hippocampal neurons treated with phalloidin (green) and anti-Rich2 antibody (red) to visualize F-actin and endogenous Rich2, respectively. Arrows indicate co-localization of Rich2 and actin in dendritic spines (scale bars: 10 and 1 μm, respectively). C, dendritic portion of a DIV-15 cultured hippocampal neuron immunolabeled with an anti-bassoon antibody showing the synaptic localization of Rich2, (arrows, scale bar: 1 μm).
or rabbit secondary antibodies coupled to Alexa Fluor 488 (Molecular Probes, Invitrogen, Cergy-Pontoise, France) or cyanine 3 (Jackson ImmunoResearch Laboratories, Interchim, Montluçon, France) were incubated for 1 hour at room temperature. Cells were observed under an Axio-Imager Z1 Zeiss microscope equipped with appropriate epifluorescence and filters (Carl Zeiss S.A.S., Le Pecq, France; GFP: 475 ± 25 and 530 ± 70 nm for excitation and emission respectively, cyanine 3: 545 ± 25 and 605 ± 70 nm for excitation and emission respectively) to discriminate between green and red fluorescence. Image quantification was performed using ImageJ software (National Institutes of Health). In morphological studies, spines were defined as dendritic protrusions with neck and head, whereas filopodia were defined as thin dendritic protrusions devoid of head.

Immunohistology—The brain was dissected and fixed by immersion with 4% paraformaldehyde in PBS for 24 h at 4 °C. It was then cut frontally with a vibratome into 50-μm thick sections. After careful rinsing in PBS, sections were incubated overnight at 4 °C with the rabbit anti-Rich2 antibody (1:100 dilution) in PBS, Triton X-100 0.1, and 2% BSA.

Immunoprecipitation—Immunoprecipitation experiments (IP) were performed using rabbit anti-Rich2 antibodies, and immunoprecipitated proteins were revealed by Western blot with mouse anti-Rac1 antibody. In IP, 5 μg of either rabbit antibody (control condition) or Rich2 antibody (IP) were added. Protein-A Sepharose (Amersham Biosciences) was used to immunoprecipitate IgGs.

Statistical Analyses—Values were expressed as mean ± S.E. of at least three independent experiments. In bar graphs, they were compared with control using Student’s t test. For GTPase measurements, curves were compared with control using two-way ANOVA test. Values of p < 0.05 were considered as significantly different.

RESULTS

Rich2 Neuronal Localization—We found Rich2 expression in the pyramidal layers of mouse hippocampus at embryonic age 18.5 (E18.5). Double-immunolabeling with neuronal (NeuN) and glial (GFAP) markers revealed that Rich2 was highly expressed in neurons and undetectable in glial cells (Fig. 1A). In 21 days in vitro (DIV 21) cultured mouse hippocampal neurons, F-actin and Rich2 immunostaining colocalized in dendritic spines (Fig. 1B). Furthermore, Rich2 immunostaining colocalized with the synaptic marker Bassoon (Fig. 1C). Altogether, these results suggested a post-synaptic localization of Rich2 in cultured hippocampal neurons.

Rich2 Is a Rho-GAP for Rac1 in Neurons—Rich2 displays a Rho-GAP domain (11). We therefore addressed the issue of the Rho-GAP function of this protein. We found that Rich2 was able to stimulate GTP hydrolysis of [γ-32P]GTP-loaded Rac1 and Cdc42, and to a lesser extent RhoA, in vitro (Fig. 2, A–C). We then examined if this applied to in cellulo conditions using the RAS and Interacting protein Chimeric Unit (RAICHU) probe system, (15). A RAICHU probe is a genetically encoded FRET-based probe consisting of the Rho GTPase (RhoA, Rac1, or Cdc42) conjugated to the donor fluorochrome CFP, and the GTPase-binding domain of its binding partner conjugated to the acceptor fluorochrome YFP (Fig. 3A). Interaction between the GTP-bound GTPase and the GTPase-binding domain brings CFP into close proximity of YFP, thereby generating a FRET signal upon stimulation of the donor CFP. The pRAI-
CHU-Rac1, pRAICHU-RhoA, or pRAICHU-Cdc42 constructs were transfected separately with or without Rich2 in COS-7 cells, and their respective GTP-bound forms measured by FRET. We found that co-expression of Rich2 decreased FRET signals of all three pRAICHU probes, thus indicating that Rich2-inactivated RhoA, Rac1, and Cdc42 (Fig. 2D). These results showed that the GAP domain of Rich2 was functional and equally effective on all members of the Rho-GTPase family in COS-7 cells.

Having established the suitability of the pRAICHU probe for Rich2 GAP activity measurement, pRAICHU-Cdc42, pRAICHU-Rac1, or pRAICHU-RhoA constructs were co-transfected with Rich2 in cultured hippocampal neurons for FRET imaging experiments. We first determined the FRET efficiency by confocal microscopy (LSM-Meta 510 Zeiss microscope) in neurons. Images were taken every 10 nm from 415 to 580 nm using the LSM-Meta 510 Zeiss confocal microscope. This resulted in a fluorescence spectrum for every pixel of the images. Scale bar in B: 1 μm. D, hippocampal neurons were co-transfected with Rich2 (DIV-9) or si-Rich2 (DIV-11) and pRAICHU-Rac1, pRAICHU-RhoA, or pRAICHU-Cdc42 constructs, and then tested at DIV-12. Images obtained from portions of dendrites of cultured hippocampal neurons transfected with either the Rac1, RhoA, or Cdc42 pRAICHU probe, in the absence (control) and presence of co-transfected Rich2 or si-Rich2. The right panels show 525/480 ratio images corresponding to FRET signals (scale bar: 2 μm) and enlarged views of the squared areas. E, histogram shows FRET efficiency obtained with Rac1, RhoA, and Cdc42 pRAICHU probes. FRET efficiency in dendritic spines was calculated from fluorescence spectrum images generated by the confocal microscope similar to those shown in D (n = 50 for Rac1, n = 65 for RhoA, n = 54 for Cdc42, n = 52 for Rac1 + Rich2, n = 55 for RhoA + Rich2, n = 59 for Cdc42 + Rich2, n = 87 for Rac1 + si-Rich2, n = 74 for RhoA + si-Rich2, n = 48 spines for Cdc42 + si-Rich2, *, p < 0.05).
efficiency by photobleaching the YFP acceptor module of the pRAICHU-Cdc42 probe. Photobleaching of YFP decreased the peak of fluorescence emission at 525 nm and increased the fluorescence emission at 480 nm, indicating that genuine FRET responses were recorded with the probe (Fig. 3, B and C). FRET imaging was then carried out in dendrites of DIV 21 cultured
Rich2 is a Rho-GAP for Rac1 in neurons. Altogether, these experiments showed that Rich2 is a Rho-GAP for Rac1 in neurons.

Rich2 Controls Dendritic Spine Morphogenesis and Function via Rac1—We then investigated whether Rich2 controlled spine morphogenesis in developing neurons and if this involved its interaction with Rac1. DIV 9 hippocampal neurons were transfected with pEGFP and either Rich2 or si-Rich2. At this stage of development, the dendrites do not yet display spines. The effects of Rich2 and si-Rich2 on dendritic spine growth were then analyzed at DIV12. Rich2 increased the size of spine head and decreased spine density, while si-Rich2 had opposite effects on spine size. Like Rich2, si-Rich2 decreased spine density (Fig. 5, A–C). The si-RNA resistant mutant of Rich2 (Rich2-mt) rescued the phenotype on spine size and density (Fig. 5, A–C). Neither Rich2 nor si-Rich2 significantly modified the density of filopodia (control 1.25 ± 0.25, Rich2 1.2 ± 0.20 and si-Rich2 1.4 ± 0.30 filopodia per 10 μm).

Patch-clamp recording performed in the transfected cultured hippocampal neurons showed that Rich2 increased the amplitude and decreased frequency of miniature ESPCs (mEPSCs).
SCs; Fig. 5, D–F), whereas si-Rich2 decreased both amplitude and frequency of mEPSCs (Fig. 5, G–I). These functional synaptic modifications were consistent with the observed spine morphological changes. Taken together these results suggested that Rich2 controls the formation of functional dendritic spines.

As we found that Rich2 is a Rho-GAP for Rac1, we reasoned that Rac1-V12 should mimic the effect of si-Rich2. As expected, transfection of Rac1-V12 decreased spine size and density (Fig. 6, A–C), as it was the case for si-Rich2 (Fig. 5, A–C). In the same line, if the effect of Rich2 involved inhibition of Rac1 activity, overexpression of Rac1-V12 should rescue the phenotype induced by Rich2. Both Rac1-V12 and Rich2 decreased spine number. It is therefore difficult to conclude on the implication of the Rich2-Rac1 pathway on spine density regulation. However, Rac1-V12 decreased, while Rich2 increased spine size. Interestingly, in the presence of Rac1-V12, Rich2 was unable to increase spine size, thus suggesting that Rich2 and Rac1-V12 acted via a common pathway to control spine size (Fig. 6, A and B).

The Rac1 inhibitor, ETH 1864, mimicked the effect of Rich2 transfection as it increased the size and decreased the density of dendritic spines, and this effect was partially rescued by transfection of si-Rich2 (Fig. 6, A, D, E). In addition, sh-RNAs against Rac1 but not Cdc42 (sh-Rac1 and sh-Cdc42, respectively characterized in Fig. 7A) inhibited the effect of Rich2 on spine size. (Fig. 7B). We observed a decrease in spine density in all the conditions tested (Fig. 7C). Taken together these results suggested that Rich2 controls spine morphogenesis via inhibition of Rac1 activity, but not Cdc42.

DISCUSSION
Our results show that Rich2 is a neuronal protein that is present in dendritic spines of developing hippocampal pyra-
midal neurons. In these neurons, Rich2 displays a GAP activity that we found to be specific for Rac1. This role had been previously suggested by knock-down of Rich2 in Caco-2 epithelial cells, which increased Rac1 activity (10). However according to previous findings (11), in non-neuronal cells as well as in vitro, Rich2 displayed a Rho-GAP activity also for Rho-A and Cdc42. We found that Rich2 controls dendritic spine morphogenesis and function via specific inhibition of Rac1. Indeed, si-Rich2, which is expected to potentiate Rac1 activity, reduced spine size and mEPSC amplitude, whereas Rich2 overexpression, which was expected to down-regulate Rac1 activity, induced spine enlargement and increased mEPSC amplitude. Consistent with these results, we observed that the constitutively active form of Rac1, Rac1-V12, reduced spine size, as it is the case in developing Purkinje neurons but not in mature hippocampal neurons where it induces opposite effect (17, 20).

Our results suggest that an optimal balance of Rac1 activity is required for adequate control of spine morphogenesis during development. As expected, Rich2 and Rac1-V12 induced opposite effects on spine size. Furthermore pharmacological inhibition of Rac1 activity mimicked Rich2 effect (spine enlargement). However Rac1 knock-down also reduced spine size. This apparent discrepancy may simply result from the fact that a sufficient amount of Rac1 is required to control spine morphogenesis.

We also found that the Rho-GAP Rich2 influenced spine number. A decrease in spine density was observed with all treatments tested, including Rich2 and Rac1-V2, as well as both knock-down and over-expression of Rich2. Therefore no firm conclusion could be drawn for the control of Rac1 activity by Rich2 and spine number. Previous studies showing that both inhibition and over-activation of Rho-GTPases similarly decrease synaptic density in developing cultured hippocampal neurons (17, 20–22) support our results. For instance both overexpression and knock-down of α1-chimaerin, a GAP for Rac1, was shown to decrease the number of spines, increase the number of filopodia, and induced formation of spines with multiple protrusions (23, 24). This suggests more complex tuning of Rac1 activity to control spine density. An alternative hypothesis could be that Rich2 displays other GAP activity on different GTPase family.

Other studies have shown that oligophrenin, a GAP for RhoA, controls spine morphogenesis (9, 25). This protein is mutated in patients affected by mental retardation (26). The GAP p190 also regulates spine density by modulating of RhoA activity via PAR-6 (partition defective 6)/αPKC complex (27). The present study further extends the spectrum of Rho-GAP proteins and their regulations that control spine morphogenesis, thus providing new insights in the mechanisms responsible of synaptogenesis.
Rich2 Controls Dendritic Spine Morphogenesis

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