RICS, a Novel GTPase-activating Protein for Cdc42 and Rac1, Is Involved in the β-Catenin-N-cadherin and N-Methyl-d-aspartate Receptor Signaling*

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Cadherin adhesion molecules are believed to be important for synaptic plasticity. β-Catenin, which links cadherins and the actin cytoskeleton, is a modulator of cadherin adhesion and regulates synaptic structure and function. Here we show that β-catenin interacts with a novel GTPase-activating protein, named RICS, that acts on Cdc42 and Rac1. The RICS-β-catenin complex was found to be associated with N-cadherin, N-methyl-d-aspartate receptors, and postsynaptic density-95, and localized to the postsynaptic density. Furthermore, the GTPase-activating protein activity of RICS was inhibited by phosphorylation by Ca2+/calmodulin-dependent protein kinase II. These results suggest that RICS is involved in the synaptic adhesion- and N-methyl-d-aspartate-mediated organization of cytoskeletal networks and signal transduction. Thus, RICS may regulate dendritic spine morphology and strength by modulating Rho GTPases.

Activity-induced changes in synaptic transmission efficacy, such as long term potentiation (LTP) and long term depression, have been postulated to be involved in information storage during learning. Many studies have revealed that synaptic remodeling and plastic changes in dendritic spine morphology play a role in synaptic plasticity (1–6). Furthermore, changes in synaptic strength have been shown to involve the structural and functional modifications of the molecules present in the postsynaptic density (PSD) (7–10), an electron-dense structure containing various structural and signaling molecules such as ion channels, scaffolding proteins, protein kinases, small G-proteins, cell adhesion proteins, and cytoskeletal proteins (11, 12).

The cadherins are a family of single-pass transmembrane proteins that mediate Ca2+-dependent, homophilic intercellular adhesion (13). Some members of the cadherin family of adhesion proteins are localized to synaptic junctions and have been implicated in synaptic plasticity (14, 15). For example, inhibitory antibodies to the first extracellular domain of N-cadherin, one of the classical cadherins enriched in neural cells, have been shown to attenuate the induction of LTP (16, 17). Also, antagonistic peptides containing the consensus sequence for cadherin dimer formation prevent the induction of LTP (16). Furthermore, inhibition of cadherin activity by a dominant-negative N-cadherin, as well as by mutation of αN-cadherin, has been shown to alter dendritic spine morphology (18), suggesting that cadherins function as regulators of synaptic plasticity by modulating spine morphology.

β-Catenin interacts with the cytoplasmic domain of classical cadherins and links cadherins and the actin cytoskeleton (19, 20). β-Catenin and N-cadherin are present in axons and dendrites prior to synapse formation and then cluster at developing synapses and form a symmetrical adhesion structure in synaptic junctions (21). Moreover, it has been reported recently (10) that neural activity induces redistribution of β-catenin from dendritic shafts into spines, where it interacts with cadherin to influence synaptic size and strength.

N-Methyl-d-aspartate (NMDA) receptors, which play a central role in synaptic plasticity, are heteromeric ion channels consisting of essential NR1 subunits and one or more of the modulatory NR2 subunits, NR2A-D (22–24). The NMDA receptors are associated with the PSD-95 family of proteins, the most abundant constituents of PSD, via its NR2 subunits (25–28). This interaction is important for specific localization of NMDA receptors in the PSD and its coupling to cytoskeletal networks and signaling molecules (29–33). β-Catenin is also known to be localized in PSD as a component of the NMDA receptor multiprotein complex (12). Furthermore, the β-catenin redistribution induced by depolarization is completely blocked by NMDA receptor antagonists, indicating that the redistribution is caused by synaptic activation of NMDA receptors (10). Also it has been reported that stimulation of NMDA receptors by the agonist NMDA induces molecular modification of N-cadherin, i.e. increased trypsin resistance, as well as pronounced dimerization of N-cadherin (7). Thus, β-catenin and N-cadherin are structurally and functionally linked to NMDA receptors.

To further elucidate the role of β-catenin in synaptic function, we attempted to identify novel binding partners of β-catenin. In the present study, we show that β-catenin interacts with a novel GTPase-activating protein for Cdc42 and Rac1,
termed RICS (RhoGAP involved in the β-catenin-N-cadherin and NMDA receptor signaling). RICS was found to localize to the PSD and to be associated with N-cadherin, PSD-95, and NMDA-R, in addition to β-catenin. Furthermore, we demonstrate that its GAP activity is downregulated through phosphorylation by Ca2+-calmodulin-dependent protein kinase II (CaMKII), a kinase critical for synaptic plasticity that is activated by NMDA receptor-mediated influx of Ca2+ (34).

EXPERIMENTAL PROCEDURES

Plasmid Construction—The human RICS cDNA clone KIAA0712 was provided by T. Nagase (Kazusa DNA Research Institute) and subcloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen). Mutant RICs were generated by PCR. The authenticity of all mutants was verified by DNA sequencing. For retrovirus-mediated expression of RICS, the RICS cDNA was inserted into pMX-puro, provided by T. Kitamura.

Two-hybrid Screening—Two-hybrid screening was performed as described previously (43).

In Vitro Binding Assay—[35S]Methionine-labeled RICS was synthesized by in vitro transcription-translation using the TNT coupled reticulocyte lysate system (Promega). Proteins fused to glutathione S-transferase (GST) were synthesized in E. coli Escherichia coli and purified by absorption to GSH-Sepharose (Amersham Biosciences). GST fusion proteins immobilized to beads were incubated in in vitro translation products in Buffer A (0.1% Triton X-100, 100 mM Tris-HCl, 140 mM NaCl, 1 mM EDTA, 1 mM sodium vanadate, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM p-aminophenylmethanesulfonyl fluoride hydrochloride, pH 8.0) and then washed extensively with Buffer A. Proteins adhering to the beads were analyzed by SDS-PAGE followed by autoradiography.

Preparation of PSDs from Mouse Brain—The PSD fractions were isolated from mouse brain following the protocols described previously (78, 77). Briefly, adult mouse brains were homogenized in an ice-cold solution containing 0.32 M sucrose, 1 mM HEPES-KOH, pH 7.4, 1 mM NaHCO3, 1 mM MgCl2, 0.1 mM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, 0.5 mg/ml leupeptin, 10 mM Tris-HCl, pH 8.0, and then centrifuged at 1,000 × g for 10 min at 4 °C. The resulting pellet was further fractionated by discontinuous sucrose density gradient centrifugation at 82,500 × g for 2 h to obtain the synaptoosome fraction. The synaptosome fraction was solubilized with 0.5% Triton X-100, and the PSD (One Triton) pellet was collected by centrifugation at 82,500 × g for 30 min. The PSD (One Triton) pellet was re-extracted in 0.5% Triton X-100 and centrifuged at 201,800 × g for 1 h to obtain the PSD (Two Triton) pellet. Alternatively, the PSD (One Triton) pellet was treated with 3% N-lauroyl sarcosinate and then centrifuged at 201,800 × g for 1 h to obtain the PSD (One Triton − Sarcosyl) pellet. All pellets were resuspended in 40 mM Tris-HCl, pH 8.0. To dissolve the PSD (Two Triton) pellet, 0.3% SDS was added. Isolated PSD fractions were checked for the absence of synaptophysin, a presynaptic protein, and concentration of PSD-95 by immunoblotting analysis prior to use in other experiments.

Antibodies—Antibodies to RICS were prepared by immunizing rabbits with peptides containing amino acids 670–735, 933–1009, 1518–1577 and 1675–1738, respectively. Antibodies were purified by affinity chromatography using columns to which the antigens used for immunization had been linked. Antibodies to N-cadherin, PSD-95 (used for immunoprecipitation and immunoblotting), NR2B (for immunocytochemistry) and RhoA were from Transduction Laboratories. Antibodies to RhoA and Cdc42 were from Santa Cruz Biotechnology, Inc.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were performed as described elsewhere (49, 50). Briefly, the PSD (One Triton) (50 μg) was first solubilized in 2% SDS in IP buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, 5 mM EDTA, 1 mM sodium vanadate, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and then diluted with 5 volumes of IP buffer containing 2% Triton X-100. The lysates were incubated with 10 μg of anti-RICS antibody and 30 μl of protein G-Sepharose (1:1 slurry) overnight at 4 °C. Immuno complexes were washed once with IP buffer containing 1% Triton X-100, once with IP buffer containing 1% Triton X-100 plus 500 mM NaCl, and finally three times with IP buffer. Immunoprecipitates were subjected to immunoblotting analysis. The blots were probed with the indicated antibodies and then visualized with alkaline phosphatase-conjugated secondary antibodies (Promega).

Primary Neuron Culture and Immunostaining—Hippocampal primary neuronal cultures prepared from embryonic day 18–19 rat embryos were plated on coverslips coated with poly-L-lysine (50 μg/ml) (5 × 104 cells/well). Cultures were grown in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen) and 0.5 mM glutamine. After 3 weeks, cultured hippocampal neurons were fixed with 2% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature and then ice-cold methanol for 10 min at −20 °C. Cells were double-stained with antibodies to RICS and PSD-95 antibodies to RICS and β-catenin, or antibodies to RICS and MAP2. The staining patterns obtained with antibody to RICS were visualized with fluorescein isothiocyanate-labeled anti-rabbit antibodies (ICN Biomedicals); those obtained with antibodies to MAP2, PSD-95, NR2B, and β-catenin were visualized with RITC-labeled anti-mouse antibody (ICN Biomedicals). The cells were photographed with a Carl Zeiss LSM510 laser scanning microscope.

GAP Assay—The GAP domains of RICS (amino acids 1–263) and p50RhoGAP (amino acids 189–439) fused to GST were expressed in E. coli. His-tagged Rho GTPhases (0.1 μg each of protein) generated by the baculovirus system were preincubated with 1 μM [32P]GTP (γ-32P/GTP (γ-32P, 6000 Ci/mmol) in a mixture (20 μl) containing 20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 5 mM EDTA, 0.1 mM dithiothreitol for 10 min at 30 °C. After the addition of MgCl2 (final concentration 20 mM), 10 mM GST, GST-RICS-GAP, or GST-p50RhoGAP in GAP buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, 1 mM GTP, 0.86 mg/ml bovine serum albumin) was added to the mixture and incubated at room temperature, and 10 μl samples were removed at 0, 2, 4, and 6 min, diluted in 1 ml of ice-cold Buffer B (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2), and filtered through nitrocellulose membranes prefilled with Buffer B. After washing twice with 10 ml of ice-cold Buffer B, the radioactivity remaining on the filter was determined.

Phosphatase Assay—Lysate were prepared from mouse brain, and immunoprecipitation experiment was performed as described above. The immunoprecipitates were washed three times with BAP buffer (20 mM Tris-HCl, pH 8.0, 1 mM MgCl2) and incubated at 30 °C for 5 min and then bacterial alkaline phosphatase (TAKARA) was added to the reaction mixtures and incubated for 5 min before termination by SDS-PAGE sample buffer.

RBD and PSD Assay—The Rho-binding domain (RBD; amino acids 2–89) of mouse rhokin and p21-binding domain (PSD; amino acids 65–136) of mouse PAK-3 were prepared as GST fusion proteins. NIH3T3 cells infected with Ret-RICS were lysed with lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml protein, 0.86 mg/ml bovine serum albumin) and then bacterial alkaline phosphatase (TAKARA) was added to the reaction mixtures and incubated for 5 min before termination by SDS-PAGE sample buffer.

RESULTS

Molecular Cloning of the RICS cDNA—We screened a mouse 17-day-embryo cDNA library by the yeast two-hybrid system using the armadillo repeats 10–12 of mouse β-catenin as bait and obtained a cDNA fragment of a novel gene, which we have designated RICS (Fig. 1). Addition of the nucleotide sequence of
the upstream portion of the clone obtained by the 5′-rapid amplification of cDNA ends system revealed a composite sequence containing a 5,220-bp-long open reading frame, encoding a predicted protein of 1740 amino acids. Its human homolog corresponds to KIAA0712 and contains a 5214-bp-long open reading frame, encoding a predicted protein of 1738 amino acids (Fig. 1A). The predicted amino acid sequence of RICS reveals that its amino-terminal region possesses striking amino acid homology to several GAPs that are modulators of the Rho family of small G-proteins (Fig. 1, B and C). In particular, the GAP domain of RICS shares 67.9% identity with that of CdGAP (35) (Fig. 1, B and C). In addition, the central region of RICS contains three Pro-rich sequences, each of which conforms to the Src homology 3-binding motif (Fig. 1A).

Expression of RICS mRNA and Protein—Northern blot analysis detected a doublet 9.8- and 10-kb mRNA. Both bands were detected at high levels in mouse kidney, brain, testis, and heart and at low levels in skeletal muscle, liver, lung, and spleen (Fig. 2A).

To identify the gene product, we generated antibodies to the carboxyl-terminal portion (amino acids 1518–1578) of RICS and confirmed that the antibody reacts specifically with RICS generated by in vitro translation (Fig. 2B). The antibody also recognized Myc-tagged RICS that was exogenously expressed in 293 cells and immunoprecipitated with anti-Myc antibody (Fig. 2B). Immunoblotting analysis of a lysate from mouse brain and human colorectal tumor DLD-1 cells with anti-RICS antibody but not total IgG from nonimmunized rabbit detected a doublet of 250 and 210 kDa (see Fig. 2B and Fig. 3C). Also, immunoprecipitation-immunoblotting experiments with anti-RICS antibody but not total IgG of nonimmunized rabbit detected both 250- and 210-kDa proteins (Fig. 2B), and precipitation of these proteins was inhibited by preincubation of the antibody with the antigen used for immunization (Fig. 3C). Furthermore, three other antibodies raised against different epitopes also detected the 250- and 210-kDa proteins (data not shown). These results suggest that the 250- and 210-kDa proteins identified by the anti-RICS antibodies are the RICS gene products. The nature of the doublet is currently under investigation. In addition, the migration of the 250- and 210-kDa RICS proteins was accelerated by phosphatase treatment of RICS immunoprecipitates (Fig. 2B).
these proteins was not increased by phosphatase treatment in the presence of a phosphatase inhibitor, β-glycerophosphate. These results suggest that RICS is phosphorylated in living cells.

**RICS Is Present in the PSD Fraction**—We next examined the subcellular localization of RICS by subcellular fractionation of mouse brain (Fig. 2C). Similar to PSD-95, RICS was found to be concentrated in the synaptosomes and in the Triton X-100 insoluble fraction (One-Triton and Two-Triton PSD; see “Experimental Procedures”). Most of RICS was present in the PSD fraction even after extraction with N-lauroylsarcosinate, suggesting that RICS is tightly bound to the core structure of the PSD.

**RICS Binds to β-Catenin in Vitro**—The minimal fragment of RICS obtained in the two-hybrid screen contained the carboxy-terminal 557 amino acids. Two-hybrid assays using deletion fragments of RICS further confirmed that a fragment containing amino acids 1182–1371 was positive for interaction with β-catenin, whereas a fragment lacking this region was not (Fig. 3A). A fragment containing amino acids 1182–1303 was weakly positive. Consistent with these results, RICS generated by in vitro translation was found to specifically interact with β-catenin fused to GST (GST-β-catenin), whereas RICS lacking amino acids 1182–1371 did not interact with GST-β-catenin (Fig. 3B).

**RICS Interacts with β-Catenin in Vivo**—To examine whether RICS is associated with β-catenin in vivo, we subjected a lysate from mouse brain to immunoprecipitation with anti-RICS antibody and then immunoblotted with anti-β-catenin antibody. As shown in Fig. 3C, RICS was found to coprecipitate with β-catenin, and this coprecipitation was inhibited by preincubation of anti-RICS antibody with the antigen used for immunization. Also, immunoprecipitation of the lysate with anti-β-catenin and subsequent immunoblotting with anti-RICS antibody revealed an association between RICS and β-catenin (Fig. 3C). Preincubation of the anti-β-catenin antibody with the antigen prevented coprecipitation of β-catenin and RICS. These results suggest that RICS interacts with β-catenin in vivo.

The RICS-β-Catenin Complex Is Associated with N-Cadherin, NMDA Receptors, and PSD-95 in Vivo—Because β-catenin is known to be associated with the cadherin family of proteins, we examined whether the RICS-β-catenin complex is associated with E-cadherin and N-cadherin. Immunoblot analysis of the RICS immunoprecipitates with anti-E-cadherin and anti-N-cadherin antibodies revealed that E-cadherin (data not shown) and N-cadherin (Fig. 3D) both coprecipitate with RICS. Immunoblot analysis of the E-cadherin and N-cadherin immunoprecipitates with anti-RICS antibody further confirmed that E-cadherin and N-cadherin coprecipitate with RICS (data not shown). Coprecipitation of these proteins was not observed when anti-RICS antibody preincubated with the antigen was used for immunoprecipitation (Fig. 3D). These results suggest that RICS, β-catenin, and E-cadherin/N-cadherin are contained in the same complex in vivo.

The β-catenin-cadherin complex has been reported to be linked to the NMDA-R-PSD-95 complex (12). Therefore, we next examined whether RICS is contained in the NMDA receptor-PSD-95 complex. When the RICS immunoprecipitate was subjected to immunoblot analysis with anti-NR2A/2B and anti-PSD-95 antibodies, respectively, both NR2A/2B and PSD-95 were found to coimmunoprecipitate with RICS (Fig. 3D). Immunoblot analysis of the NR2A/2B and PSD-95 immunoprecipitates with anti-RICS antibody also showed that NR2A/2B and PSD-95 coprecipitate with RICS (data not shown). However, the amounts of RICS detected in the NR2A/2B and PSD-95 immunoprecipitates were small, presumably because

**Fig. 2. RICS mRNA and protein.** A, expression of RICS mRNA in mouse adult tissues. Multiple tissue northern (MTN) blots of mouse adult tissues (Clontech) were probed with a cDNA encoding RICS. As a control, expression of actin mRNA was also examined. The positions of the RICS and actin mRNAs are indicated by arrowheads. B, identification and characterization of the RICS protein. Left panel, lanes 1 and 2, in vitro translated RICS was subjected to immunoblotting with total IgG from nonimmunized rabbit (lane 1) or anti-RICS antibody (lane 2). Lanes 3 and 4, Myc-tagged RICS exogenously expressed in 293 cells was immunoprecipitated with nonimmune IgG (lane 3) or anti-Myc antibody (lane 4) and then immunoblotted with anti-RICS antibody. Lanes 5 and 6, a lysate from DLD-1 cells was subjected to immunoblotting with anti-RICS antibody (lane 5) or nonimmune IgG (lane 6). Right panel, lane 1, a lysate from mouse brain was subjected to immunoblotting with anti-RICS antibody. Lanes 2–6, lysates from mouse brain were subjected to immunoprecipitation followed by immunoblotting with anti-RICS antibody. Lane 2, total IgG of nonimmune rabbit instead of anti-RICS antibody was used for immunoprecipitation. Lanes 4 and 5, the RICS immunoprecipitates were treated with bacterial alkaline phosphatase in the absence (lane 4) or presence of β-glycerophosphate (10 μM) (lane 5). C, RICS is enriched in the PSD in mouse brain. Mouse brain was fractionated as described under “Experimental Procedures.” Mouse brain homogenate (25 μg), the synaptosome fraction (25 μg), PSD extracted once (One Triton; 2.5 μg) or twice with Triton X-100 (Two Triton; 2.5 μg), and PSD extracted once with Triton X-100 followed by N-lauroylsarcosinate (One Triton + Sarcosyl; 2.5 μg) were subjected to immunoblotting analysis with antibodies to RICS, PSD-95, or synaptophysin.

NR2A/2B and PSD-95 are present in a great excess over RICS. On the other hand, focal adhesion kinase was not immunoprecipitated with RICS (Fig. 3D). Taken together, these results suggest that RICS, β-catenin, N-cadherin, NMDA recep-
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neuronal cultures was performed to examine the colocalization of RICS and PSD-95. When hippocampal cells were cultured for 3 weeks, RICS was expressed in MAP2-positive neurons and glial fibrillary acidic protein-positive glial cells. In neuronal cells, dendrites were brightly labeled by anti-RICS antibody and appeared as dots (Fig. 4, A–D). This punctate staining for RICS coincided with those observed for NR2B and PSD-95, although some fraction of NR2B and PSD-95 puncta did not contain for RICS (Fig. 4, H–M). Numerous RICS clusters colocalized with β-catenin clusters (Fig. 4, E–G). These staining patterns were not detected when antibody was preadsorbed with an excess amount of the antigens used for immunization.

RICS Possesses GAP Activity for Rho GTPases in Vitro—Because the amino acid sequence of RICS has homology to the GAPs for Rho GTPases, we examined whether RICS also functions as a GAP for Rho, Rac1, and/or Cdc42. When the GAP domain of RICS fused to GST was incubated with RhoA, Rac1, or Cdc42 bound to [γ-32P]GTP, it was found to stimulate hydrolysis of GTP to GDP (Fig. 5A). Arg-58 and Lys-98 of the GAP domain of RICS correspond to the conserved amino acid residues of RhoGAPs, which are known to be required for GAP activity per se and for interaction with Rho GTPases, respectively (36, 37). We therefore generated mutated versions of RICS, designated RICS-R58A and -K98A, in which Arg-58 and Lys-98, respectively, were replaced with Ala. Unexpectedly, RICS-R58A showed GAP activity against RhoA, Rac1, and Cdc42, although RICS-K98A was inactive (Fig. 5A). After further considering the three-dimensional structure of the Rho GTPases-GAP complexes (38–40), we then replaced Arg-58 with Ile and Met, respectively, and generated RICS-R58I and -R58M. As expected these mutants were found to possess no GAP activity with respect to RhoA, Rac1, or Cdc42 (Fig. 5B; see below). These results indicate that RICS possesses GAP activity toward RhoA, Rac1, and Cdc42.

RICS Possesses GAP Activity for Cdc42 and Rac1 in Vivo—We next examined whether RICS exhibits GAP activity toward Rho GTPases in vivo. NIH3T3 cells were infected with a retrovirus encoding Myc-tagged RICS (Ret-RICS), and GAP activity of RICS was examined by RBD or PBD assay (41, 42). We found that expression of RICS resulted in decreases in the levels of the active forms of Cdc42 and Rac1, whereas expression of the inactive mutants RICS-R58I or -R58M did not (Fig. 5B). On the other hand, RICS induced a slight reduction in the level of the active form of RhoA. Under these experimental conditions, the total amount of Cdc42, Rac1, and RhoA remained constant. These results suggest that RICS possesses GAP activity for Cdc42 and Rac1 in vivo.

RICS Is Phosphorylated by CaMKII—RICS possesses 24 consensus sites (RXX(S/T)) for phosphorylation by CaMKII. We therefore examined whether RICS is phosphorylated by purified CaMKII in the presence of Ca2+ and/or calmodulin. As shown in Fig. 6A, RICS immunoprecipitated from the PSD fraction of mouse brain lysate was phosphorylated by purified CaMKII in a Ca2+- and calmodulin-dependent manner (Fig. 6A). Phosphorylation of RICS was inhibited by the addition of the CaMKII inhibitor KN-93 to the kinase reaction mixture (Fig. 6A). These results suggest that RICS is phosphorylated by CaMKII.

The GAP Activity of RICS Is Inhibited by Phosphorylation by CaMKII—We next investigated whether phosphorylation by CaMKII affects the GAP activity of RICS against Cdc42. RICS immunoprecipitated from the PSD fraction of mouse brain lysate was phosphorylated by purified CaMKII, and its GAP activity against Cdc42 was measured. The GAP activity of RICS was inhibited following phosphorylation in the presence of Ca2+ and calmodulin (Fig. 6B). Inhibition of GAP activity

RICS Is Localized to the Synapse in Cultured Hippocampal Neurons—Immunofluorescent staining of rat hippocampal

tor, and PSD-95 may be contained in the same complex in vivo.

RICS was subjected to immunoprecipitation with the indicated antibodies, fractionated by 6% SDS-PAGE, and immunoblotted with the antibodies indicated. Ag+, antibodies were preincubated with antigens before use in immunoprecipitation. In lanes labeled Lysate, 10% of lysates used for immunoprecipitation were applied. Control IgG, preimmune control serum was used for immunoprecipitation. WB, Western blot. D, the RICS-β-catenin complex is associated with N-cadherin, NMDA-R, and PSD-95 in vivo. Lysates prepared from the PSD fraction of mouse brain were subjected to immunoprecipitation with the indicated antibodies, fractionated by 6% SDS-PAGE, and immunoblotted with the indicated antibodies. Ag+, antibodies were preincubated with antigens before use in immunoprecipitation. In lanes labeled Lysate, 5% of lysates used for immunoprecipitation were applied.

Fig. 3. Association of RICS with β-catenin, N-cadherin, NMDA receptors, and PSD-95. A, mapping of the regions in RICS required for interaction with β-catenin. Deletion constructs of RICS were analyzed for their ability to interact with β-catenin in the two-hybrid system. Schematic representation of RICS deletion mutants and corresponding β-catenin-binding activities are shown. +, detectable activity; ±, very weak activity; −, no detectable activity. B, association of RICS with β-catenin in vitro. In vitro translated [35S]methionine-labeled full-length RICS or a RICS fragment lacking amino acids 1182–1371 were incubated with GST-β-catenin-Sepharose. The bound proteins were analyzed by SDS-PAGE followed by autoradiography. Ten % of in vitro translated proteins used for pull-down assays were applied to each Input lane. WT, wild-type. C, association of RICS with β-catenin in vivo. Lysates prepared from mouse brain were subjected to immunoprecipitation (IP) with the antibodies indicated, fractionated by SDS-PAGE, and immunoblotted with the antibodies indicated. Ag+, antibodies were preincubated with antigens before use in immunoprecipitation. In lanes labeled Lysate, 10% of lysates used for immunoprecipitation were applied. Control IgG, preimmune control serum was used for immunoprecipitation. WB, Western blot. D, the RICS-β-catenin complex is associated with N-cadherin, NMDA-R, and PSD-95 in vivo. Lysates prepared from the PSD fraction of mouse brain were subjected to immunoprecipitation with the indicated antibodies, fractionated by 6% SDS-PAGE, and immunoblotted with the indicated antibodies. Ag+, antibodies were preincubated with antigens before use in immunoprecipitation. In lanes labeled Lysate, 5% of lysates used for immunoprecipitation were applied.
was blocked when the phosphorylation reaction was performed in the presence of KN-93 or a nonhydrolyzable ATP analog, AMP-PNP. Furthermore, the GAP activity of RICS was not inhibited by phosphorylation in the absence of Ca\(^{2+}\) and calmodulin. These results imply that the GAP activity of RICS is inhibited by CaMKII-mediated phosphorylation.

**DISCUSSION**

In the present study, we found that \(\beta\)-catenin interacts with RICS via armadillo repeats 10–12. Furthermore, we showed that RICS is enriched in the PSD fraction and coimmunoprecipitates with N-cadherin, PSD-95, and NMDA receptors from the PSD of mouse brain. Consistent with this result, RICS, PSD-95, and NMDA receptors colocalized at the synapses in cultured hippocampal neurons. In addition, *in vitro* pull-down assays showed that *in vitro* translated RICS weakly interacts with NR2A/2B and PSD-95 but not with NR1 (data not shown). Although these weak interactions were reproducibly detected in our experimental conditions, we do not know whether these interactions are physiologically important. In any case, these results suggest that RICS, \(\beta\)-catenin, N-cadherin, PSD-95, and NMDA receptors are contained within the same complex *in vivo*. The PSD-95 family of proteins interacts with various proteins, including DAP (hDLG-associated protein)/SAPAP (synapse-associated protein 90-associated protein)/GKAP (guanylate kinase-associated protein) (43–46), SPAL/SPAR (47, 48), synGAP (49, 50), Kalirin-7 (51), CRIP3 (52), neurogin (53), and nNOS (54). Thus, RICS may be contained in a multicomponent complex consisting of adhesion proteins, NMDA receptors, and other various structural and signaling molecules.

We demonstrated that RICS possesses GAP activity for Cdc42 and Rac1. This result is consistent with the observation that the GAP domain of RICS is highly homologous to that of CdGAP, which also possesses GAP activity for Cdc42 and Rac1 (35). The Rho GTPases regulate the actin cytoskeleton (55, 56) and play an important role in the maintenance and reorganization of dendritic spines (57, 58). Rac1 is required for the maintenance of dendritic spines, whereas elevation of RhoA activity leads to pronounced simplification of dendritic branch patterns (59). Furthermore, Kalirin-7, a guanine nucleotide exchange factor for Rac1, has been reported to be a regulator of the postsynaptic actin cytoskeleton (51). On the other hand, it has been reported that overexpression of either an active or dominant-negative form of Cdc42 does not induce significant changes in the maintenance of spine density or morphology (60). Thus, RICS may be involved in the maintenance and reorganization of dendritic spines through its GAP activity toward Rac1.

It has been shown that NMDA receptor signaling influences dendritic branching, and this regulation involves Rho GTPases that are contained in the NMDA receptor complex (61). In addition, it has been reported that PSD-95 is involved in regulating spine structure and number; overexpression of PSD-95 in cultured hippocampus neurons leads to increases in the size and number of dendritic spines (62). Therefore, the fact that RICS is contained in the NMDA receptor-PSD-95 complex raises the possibility that activation of NMDA receptors leads
RICS is a novel GAP for Cdc42 and Rac1

RICS is a novel GAP for Cdc42 and Rac1. It has been postulated that the function of N-cadherin is to transiently change spine morphology. Indeed, inhibition of cadherin activity has been shown recently (18) to alter dendritic spine morphology, suggesting that cadherins function as regulators of synaptic plasticity by modulating this morphology. On the other hand, it has been reported that cadherin-mediated junction formation activates Rac1 (75) and that Rac1 is required for the maintenance of dendritic spines (59). These findings raise the possibility that the cadherin system regulates spine morphology by regulating Rho GTPases. Hence, it is possible that RICS contained in the N-cadherin β-catenin complex is involved in N-cadherin-mediated regulation of spine morphology.

It has been reported recently (10) that neural activity induces redistribution of β-catenin from dendritic shafts into spines and increases its association with cadherins, thereby promoting changes in synaptic structure and function (10). Thus, it would be interesting to determine whether RICS is also induced to redistribute together with β-catenin into spines or if RICS associates with redistributed β-catenin in the PSD. The redistribution of β-catenin is mimicked or prevented, respectively, by a tyrosine kinase or phosphatase inhibitor (10). Furthermore, Murase et al. (10) demonstrated that a point mutation in β-catenin Tyr-654 alters spine-shaft distribution; a mutant β-catenin, in which Tyr-654 is replaced with Phe, thereby abrogating phosphorylation, is concentrated in spines, whereas a mutant β-catenin, in which Tyr-654 is replaced with Glu to mimic phosphorylation, accumulates in dendritic shafts. Interestingly, RICS interacts with the armadillo repeats 10–12 of β-catenin and Tyr-654 resides in armadillo repeat 12. It would therefore be interesting to examine whether phosphorylation of β-catenin Tyr-654 regulates its interaction with RICS.

In conclusion, our results suggest that RICS is involved in the synaptic adhesion- and NMDA receptor-mediated organization of cytoskeletal networks and signal transduction. We speculate that RICS may regulate dendritic spine morphology and strength by regulating Cdc42 and Rac1.

During the revision of this paper, Nakamura et al. (79) and Moon et al. (80) reported the identification of Grit and p200RhoGAP, respectively, which are identical to RICS. Nakamura et al. (79) have shown that Grit may regulate neurite extension through association with the TrkA receptor and N-Shc and CrkL/Crk adapter molecules. Moon et al. (80) have demonstrated that p200RhoGAP may be involved in the regulation of neurite outgrowth, and its activity may be regulated through an interaction with Src-like tyrosine kinases. It has been shown that Grit prefers RhoA and Cdc42 to Rac1 whereas p200RhoGAP prefers RhoA and Rac1. Discrepancies between three papers (Refs. 79 and 80 and this article) are currently under investigation in our laboratory.

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RICS, a Novel GTPase-activating Protein for Cdc42 and Rac1, Is Involved in the β-Catenin-N-cadherin and N-Methyl-d-aspartate Receptor Signaling
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