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p250GAP, a Novel Brain-enriched GTPase-activating Protein for Rho Family GTPases, Is Involved in the *N*-Methyl-D-aspartate Receptor Signaling

Takanobu Nakazawa,* Ayako M. Watabe,† Tohru Tezuka,* Yutaka Yoshida,* Kazumasa Yokoyama,* Hisashi Umemori,* Akihiro Inoue,‡ Shigeo Okabe,‡ Toshiya Manabe,†§ and Tadashi Yamamoto*‖

*Division of Oncology, Department of Cancer Biology and †Division of Neuronal Network, Department of Basic Medical Sciences, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; ‡Department of Anatomy and Cell Biology, School of Medicine, Tokyo Medical and Dental University, Tokyo, 113-8519, Japan; and §Division of Cell Biology and Neurophysiology, Department of Neuroscience, Faculty of Medicine, Kobe University, Kobe 650-0017, Japan

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N-Methyl-D-aspartate (NMDA) receptors regulate structural plasticity by modulating actin organization within dendritic spines. Herein, we report identification and characterization of p250GAP, a novel GTPase-activating protein for Rho family proteins that interacts with the GluRε2 (NR2B) subunit of NMDA receptors in vivo. The p250GAP mRNA was enriched in brain, with high expression in cortex, corpus striatum, hippocampus, and thalamus. Within neurons, p250GAP was highly concentrated in the postsynaptic density and colocalized with the GluRε2 (NR2B) subunit of NMDA receptors and with postsynaptic density-95. p250GAP promoted GTP hydrolysis of Cdc42 and RhoA in vitro and in vivo. When overexpressed in neuroblastoma cells, p250GAP suppressed the activities of Rho family proteins, which resulted in alteration of neurite outgrowth. Finally, NMDA receptor stimulation led to dephosphorylation and redistribution of p250GAP in hippocampal slices. Together, p250GAP is likely to be involved in NMDA receptor activity-dependent actin reorganization in dendritic spines.

INTRODUCTION

The adaptive properties of brain circuitry require dynamism of neuronal connectivity that includes conversion of transient events into long-lasting changes in synaptic transmission. *N*-methyl-D-aspartate (NMDA) receptors are involved in the experience-dependent plasticity (Feldman and Knudsen, 1998; Fischer *et al*., 2000; Lendvai *et al*., 2000). NMDA receptors in postnatal and adult brains modulate synaptic plasticity by regulating their localization, voltage-controlled Ca^{2+} influx, downstream signaling events, and morphological changes of dendritic spines (Kennedy, 2000; Scannevin and Huganir, 2000; Sheng and Pak, 2000; Hering and Sheng, 2001; Yuste and Bonhoeffer, 2001). These functions are likely to reside in complex formations of NMDA receptors with various proteins, including scaffolding proteins, kinases, phosphatases, cytoskeletal proteins, and other signaling molecules (Husi *et al*., 2000). Through cooperation of these molecules, NMDA receptors mediate long-lasting changes in synaptic strength (Kennedy, 2000; Scannevin and Huganir, 2000; Sheng and Pak, 2000; Hering and Sheng, 2001; Yuste and Bonhoeffer, 2001).

Members of the Rho family of small GTPases, including RhoA, Rac1, and Cdc42, are critical regulators of actin cytoskeleton organization (Ridley and Hall, 1992; Nobes and Hall, 1995; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). As such, these proteins regulate a variety of cellular

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‖ Corresponding author. E-mail address: tyamamot@ims.u-tokyo.ac.jp.

Abbreviations used: BAP, bacterial alkaline phosphatase; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GDI, guanine nucleotide dissociation inhibitor; GluR, glutamate receptor; CRIB, Cdc42/Rac-interactive binding domain GST, glutathione *S*-transferase mAb, monoclonal antibody NMDA, *N*-methyl-D-aspartate PSD, postsynaptic density RBD, Rho-binding domain SH3, *src* homology 3.

processes, including migration, adhesion, and morphological change. RhoA regulates formation of focal adhesions and subsequent assembly of stress fibers. Rac1 regulates formation of membrane lamellae, and Cdc42 triggers outgrowth of peripheral spike-like protrusions called filopodia (Ridley and Hall, 1992; Nobes and Hall, 1995; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). Like Ras GTPases, Rho family GTPases cycle in a tightly regulated manner between a GDP-bound inactive state and a GTP-bound active state. This cycling is regulated by the action of three major classes of proteins: guanine nucleotide exchange factors (GEFs), guanine nucleotide dissociation inhibitors (GDIs), and GTPase-activating proteins (GAPs). GEFs stimulate the replacement of GDP by GTP, which results in activation of the substrate GTPases. In contrast, GAPs stimulate the relatively weak intrinsic GTP-hydrolyzing activity of their substrate GTPases, thereby inactivating them. GDIs block dissociation of GDP from the GTPases (Lamarche and Hall, 1994; Whitehead *et al*., 1997; Sasaki and Takai, 1998).

Dendritic spines are actin rich, and their shape and motility are influenced by the actin cytoskeleton (Matus *et al*., 1982; Kaech *et al*., 1997; Fiala *et al*., 1998; Hering and Sheng, 2001). Rho family GTPases are thought to regulate turnover of dendritic spines (Luo *et al*., 1996; Threadgill *et al*., 1997; Ruchhoeft *et al*., 1999; Lee *et al*., 2000; Wong *et al*., 2000). For example, constitutively active Rac1 disrupts the morphology of dendritic spines in pyramidal neurons (Nakayama *et al*., 2000; Tashiro *et al*., 2000). Dominant negative Rac1 and constitutively active RhoA cause a progressive reduction in spine number (Nakayama *et al*., 2000; Tashiro *et al*., 2000). Stimuli that induce long-term potentiation result in alteration of spine size, increase of synaptic surface area, and perforation of postsynaptic density (Sorra and Harris, 1998; Engert and Bonhoeffer, 1999; Maletic-Savatic *et al*., 1999; Toni *et al*., 1999). Application of NMDA receptor antagonist blocks these changes, indicating that dendritic spine density and morphology are influenced by NMDA receptor activity (Engert and Bonhoeffer, 1999; Maletic-Savatic *et al*., 1999; Toni *et al*., 1999). Conversely, actin cytoskeleton is involved in NMDA receptor channel properties, NMDA receptor-mediated long-term potentiation, and NMDA receptor localization (Rosenmund and Westbrook, 1993; Allison *et al*., 1998; Kim and Lisman, 1999). Intriguingly, some actin binding proteins and regulators of the actin cytoskeleton, such as α-Actinin, Cortactin, Vinculin, Spectrin, and Kalirin-7, interact directly or indirectly with NMDA receptors, providing a potential link between the NMDA receptor and actin filaments (Wyszynski *et al*., 1998; Husi *et al*., 2000; Penzes *et al*., 2001). Thus, the signaling pathways that link NMDA receptors to the postsynaptic actin cytoskeleton are apparently important for synaptic plasticity; however, the underlying mechanism is poorly understood.

Herein, we report identification and characterization of p250GAP, a novel brain-enriched GAP for Rho family GTPases. p250GAP is the first GAP for Rho family GTPases shown to be enriched in the NMDA receptor complex. We provide evidence suggesting that p250GAP links NMDA receptor activity to actin reorganization.

MATERIALS AND METHODS

Plasmids

The human p250GAP cDNA (KIAA 0712, GenBank/EMBL/DDBJ accession no. AB018255) clone was obtained from the Kazusa DNA Research Institute (Chiba, Japan). A GAP inactive mutant (R58I) of p250GAP was generated by polymerase chain reaction. The FLAG-tagged and Myc-tagged p250GAP expression plasmids were constructed in the pME18S vector (Takeuchi et al., 1993). For preparation of glutathione *S*-transferase (GST)-fusion proteins in *Escherichia coli* BL21 (Nakazawa *et al*., 2001), p250GAP cDNA fragments encoding, respectively, amino acids 1055–1738, 1055–1371, 1371–1738, 1552–1738, and 1371–1518 were cloned in-frame into pGEX plasmids (Amersham Biosciences, Piscataway, NJ). For production of the GAP domain of p250GAP fused to GST (GST-GAP), the wild-type and R58I mutant of p250GAP cDNA fragments encoding amino acids 1–263 were cloned in-frame into pGEX plasmids. The expression plasmid pME-GluRε2 (NR2B) was described previously (Nakazawa *et al*., 2001). The expression plasmids pEFBOSMycWT-RhoA, pEFBOSMycWT-Cdc42, pEFBOSMycWT-Rac1, pEFBOSMycN19RhoA, and pEFBOSMycN17Cdc42 were kindly provided by Y. Takai (Osaka University, Osaka, Japan). The plasmid for GST-Cdc42/Rac-interactive binding domain of Pak (GST-CRIB) was kindly provided by C. Sasakawa (University of Tokyo, Tokyo, Japan). The plasmid for GST-Rho binding domain of Rhotekin (GST-RBD) (Reid *et al*., 1996) was constructed by reverse transcription-polymerase chain reaction. The constructs were verified by dideoxynucleotide sequencing.

Antibodies

Polyclonal antibodies against p250GAP and postsynaptic density (PSD)-95 were raised by immunizing rabbits with a glutathione *S*-transferase (GST) protein fused with human p250GAP (amino acids 1401–1738) and human PSD-95 (amino acids 1–45), respectively. Anti-GluRε2 (NR2B) monoclonal antibody (mAb), anti-pY mAb (RC20AP), anti-RhoA mAb, and anti-Rac1 mAb were purchased from Transduction Laboratories (Lexington, KY). Anti-Synaptophysin mAb, anti-FLAG mAb (M2), and anti-FLAG antibodies were from Sigma-Aldrich (St. Louis, MO). Anti-MAP2 mAb was from Leinco Technology (Ballwin, MO). Anti-extracellular signal-regulated kinase (ERK) antibodies, anti-Myc mAb (9E10), and anti-Cdc42 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK antibodies were from Cell Signaling Technology (Beverly, MA). Anti-GluRε2 (NR2B) antibodies were from Chemicon International (Temecula, CA).

Yeast Two-Hybrid Screening

Yeast two-hybrid screening was conducted with a human adult brain cDNA library (BD Biosciences Clontech, Palo Alto, CA) as described previously (James *et al*., 1996). The cytoplasmic region of GluRε2 (NR2B) (amino acids 900-1482) was used as a bait.

Cell Culture and DNA Transfection

Neuro-2A cells (IFO 50081) were purchased from Health Science Research Resources Bank (Osaka, Japan). Human embryonic kidney (HEK)293T cells and Neuro-2A cells were cultured as described previously (Brouns *et al*., 2001; Nakazawa *et al*., 2001). HEK293T cells were transfected using calcium phosphate precipitation (Nakazawa *et al*., 2001). Two days later, the cells were harvested for protein preparation. Neuro-2A cells were plated on glass coverslips in six-well plates and transfected with plasmid DNAs (1 μg/well) by using FuGene 6 (Roche Diagnostics, Indianapolis, IN).

Preparation of Lysates, Immunoprecipitation, and Immunoblotting

Lysates of HEK293T cells, whole telencephalons, and hippocampal slices were prepared as described previously (Nakazawa *et al*., 2001). In brief, HEK293T cells expressing GluRε2 (NR2B) were lysed in TNE buffer [1% (wt/vol) Nonidet P-40, 50 mM Tris-Cl, pH 8.0, 120 mM NaCl, 5 mM EDTA, 0.2 mM Na_3VO_4 with aprotinin at 50 U/ml] and the cleared lysates were incubated at 4°C for 1 h GST-fusion proteins immobilized on glutathione-Sepharose 4B. Bound proteins were separated by centrifugation and washed with TNE buffer. Immunoprecipitation and immunoblotting were performed as described previously (Nakazawa *et al*., 2001).

Northern Blot Analysis and In Situ Hybridization

Northern blot analysis was carried out as described previously (Yoshida *et al*., 2000). In situ hybridization was performed with α-^{35}S-UTP–labeled cRNA probe (Yoshida *et al*., 2000). A partial mouse cDNA fragment (corresponding to nucleotides 5130–5569 in human p250GAP cDNA) was obtained by reverse-transcription-polymerase chain reaction and cloned into pBluescript II KS+ (Stratagene, La Jolla, CA). After verification of the sequence, the cRNA for GAP was prepared by in vitro transcription and used as a probe.

Preparation of PSD Fraction

Synaptosome and PSD (one Triton X-100 extraction) of adult mouse telencephalon were prepared as described previously (Carlin *et al*., 1980).

Immunocytochemistry

Neuro-2A cells and hippocampal neurons were fixed with methanol for 10 min at −20°C, blocked with 5% normal goat serum and then incubated with appropriate antibodies. The primary antibodies were visualized using goat anti-mouse or anti-rabbit IgG conjugated to fluorescein isothiocyanate (Sigma-Aldrich), Cy3 (KPL, Gaithersburg, MD; and Jackson Immunoresearch Laboratories, West Grove, PA), or Alexa 488 (Molecular Probes, Eugene, OR).

RhoGAP Assay

In vitro RhoGAP assay was carried out as described previously (Lamarche-Vane and Hall, 1998). In brief, recombinant RhoA, Rac1, and Cdc42 (100 ng) were preloaded with [γ-^{32}P]GTP (10 μCi, 6000 Ci/mmol) in 30 μl of 20 mM Tris-Cl, pH 7.5, 25 mM NaCl, 0.1 mM dithiothreitol, and 5 mM EDTA for 10 min at 30°C. After the addition of $MgCl_2$ (20 mM at final concentration), the preloaded GTPases (20 nM at final concentration) were diluted with 20 mM Tris-Cl, pH 7.5, 0.1 mM dithiothreitol, 1 mM GTP, 0.86 mg/ml bovine serum albumin, and 10 nM GST or GST-GAP domain. The mixture (30 μl) was incubated at room temperature, and 10-μl samples were removed at 0, 3, and 6 min, diluted in ice-cold wash buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$), and filtered through nitrocellulose filters. Filters were washed with the wash buffer, dried, and counted. The RhoGAP activity of p250GAP in vivo was analyzed as described previously (Yamaguchi *et al*., 2001). Briefly, HEK293T cells were transfected with the expression plasmids for Myc-tagged RhoA, Cdc42, or Rac1 together with or without FLAG-tagged wild-type or R58I mutant of p250GAP. The cells were lysed for 5 min with the ice-cold lysis buffer (50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 1% Nonidet P-40, 10% glycerol for Cdc42 and Rac1; 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 30 mM $MgCl_2$, 0.1% Triton X-100, 10% glycerol for RhoA), and then the lysates were centrifuged for 5 min at 10,000 × *g*. The supernatants were incubated with 20 μg of GST-CRIB for Cdc42 and Rac1 for 30 min, or 40 μg of GST-RBD for RhoA for 60 min. After washing the beads with the lysis buffer, the bound proteins were resolved on SDS-PAGE, and subjected to immunoblotting with anti-Myc mAb. To analyze the GAP activity of endogenous p250GAP in brain, the p250GAP immunoprecipitates from brain lysates were incubated with extracts of HEK293T cells transiently transfected with plasmids encoding Myc-tagged RhoA, Cdc42, or Rac1. GTP-loaded RhoA, Cdc42, or Rac1 was then collected from the lysates by using GST-CRIB or GST-RBD immobilized on glutathione-Sepharose. After washing the beads with the lysis buffer, the bound proteins were resolved on SDS-PAGE, and subjected to immunoblotting with anti-Myc mAb.

Pharmacological Treatment of Hippocampal Slices

Mouse hippocampal slices were prepared as described previously (Nakazawa *et al*., 2001). Slices were submerged beneath continuously perfusing artificial cerebrospinal fluid (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 11 mM glucose) that had been saturated with 95% O_2 and 5% CO_2. Slices were then treated with or without 50 μM NMDA (Sigma-Aldrich) for 5 min in the presence or absence of a selective NMDA receptor antagonist DL-APV (Tocris Cookson, Ballwin, MO). Then, the slices were frozen in liquid N_2. The TNE buffer-soluble slice lysates were prepared as described above. The whole slice lysates were prepared by boiling in SDS-PAGE sample buffer (65 mM Tris-Cl, pH 6.8, 5% 2-mercaptoethanol, 3% SDS, 10% glycerol, 1 mg/ml bromophenol blue). The lysates were subjected to immunoblotting with antibodies against p250GAP, PSD-95, ERK, and phospho-ERK. For quantification, immunoreacted protein bands were analyzed with NIH Image software. The intensity of the band of p250GAP was indicated relative to that of PSD-95.

Phosphatase Treatment

Slice lysates (40 μg of protein) and p250GAP immunoprecipitated from slice lysates were incubated with bacterial alkaline phosphatase (Takara, Kyoto, Japan) at 100 U/ml for 3 h (Nakazawa *et al*., 2001).

RESULTS

p250GAP Is Isolated as a GluRε2 (NR2B) Subunit-interacting Protein

To clarify the glutamate receptor-mediated signaling pathways, we screened for molecules that interact with the GluRε2 (NR2B) subunit of the NMDA receptor. We carried out yeast two-hybrid screening by using the cytoplasmic region of GluRε2 (NR2B) as a bait. We obtained 23 candidate clones from a cDNA library of 5.0×10^6 individual clones prepared from human adult brain. Database searches showed that one of the clones was a partial cDNA of the KIAA0712 clone identified by the Kazusa DNA Research Institute (Figure 1A). KIAA0712 contains a predicted open reading frame of 5217 base pairs that encodes a polypeptide of 1738 amino acids. Sequence analysis of KIAA0712 revealed that the KIAA0712-encoded protein contained a RhoGAP-like domain at its amino terminus (Figure 1A). Because an apparent molecular size of the protein, as was determined by its electrophoretic mobility, was 250 kDa, the protein was termed p250GAP. As shown in Figure 1B, the RhoGAP homology domain encompassed ~160 amino acids. The predicted amino acid sequence of p250GAP was 27 and 26% identical with that of p190RhoGAP and p50RhoGAP, respectively (Figure 1B). p250GAP contained several proline-rich sequences that may serve as SH3 binding sites (Figure 1A).

Figure 1. Sequence of human p250GAP. (A) Protein sequence and domain structure of p250GAP. The RhoGAP domain is boxed, proline-rich sequences are double underlined, and the cDNA sequence obtained by two-hybrid screening (amino acids 1056–1738) is underlined. (B) Homology between RhoGAP domains of p250GAP, p190RhoGAP, and p50RhoGAP. Identical residues are highlighted in black, and the predicted critical residues for GAP activity are marked with asterisks.

To demonstrate that p250GAP interacts with GluRε2 (NR2B) in mammalian cells as well, HEK293T cells were transfected with an expression plasmid encoding FLAG-tagged p250GAP together with or without a GluRε2 (NR2B) plasmid. Then the lysates of the transfectants were subjected to a coimmunoprecipitation experiment. As shown in Figure 2A, GluRε2 (NR2B) was readily detectable in anti-FLAG immunoprecipitates. Coimmunoprecipitation of GluRε2 (NR2B) with anti-FLAG antibody was dependent on expression of p250GAP (Figure 2A). This finding indicates that p250GAP and GluRε2 (NR2B) do indeed interact with each other in mammalian cells, when they are coexpressed. To identify the regions of p250GAP that interact with GluRε2 (NR2B), various regions of p250GAP were produced as bacterial fusion proteins linked to GST. The GST fusion proteins immobilized on glutathione-Sepharose were incubated with lysates of HEK293T cells expressing GluRε2 (NR2B), and then GluRε2 (NR2B) bound to the fusion protein were examined (Figure 2B). As shown in Figure 2B, the GST fusion proteins, GST-1, -3, and -5, but not GST-2 and -4, precipitated GluRε2 (NR2B). Therefore, the sequence of amino acid residues 1371–1518 in p250GAP contained the GluRε2 (NR2B) association site. We further performed coimmunoprecipitation experiments by using mouse brain extracts. Incubation of brain lysates with anti-p250GAP antibodies resulted in coprecipitation of GluRε2 (NR2B) with p250GAP (Figure 2C). p250GAP and GluRε2 (NR2B) were not coimmunoprecipitated with preimmune serum or antibodies preabsorbed with immunogen (Figure 2C). The data suggested that p250GAP interacted with GluRε2 (NR2B) in brain.

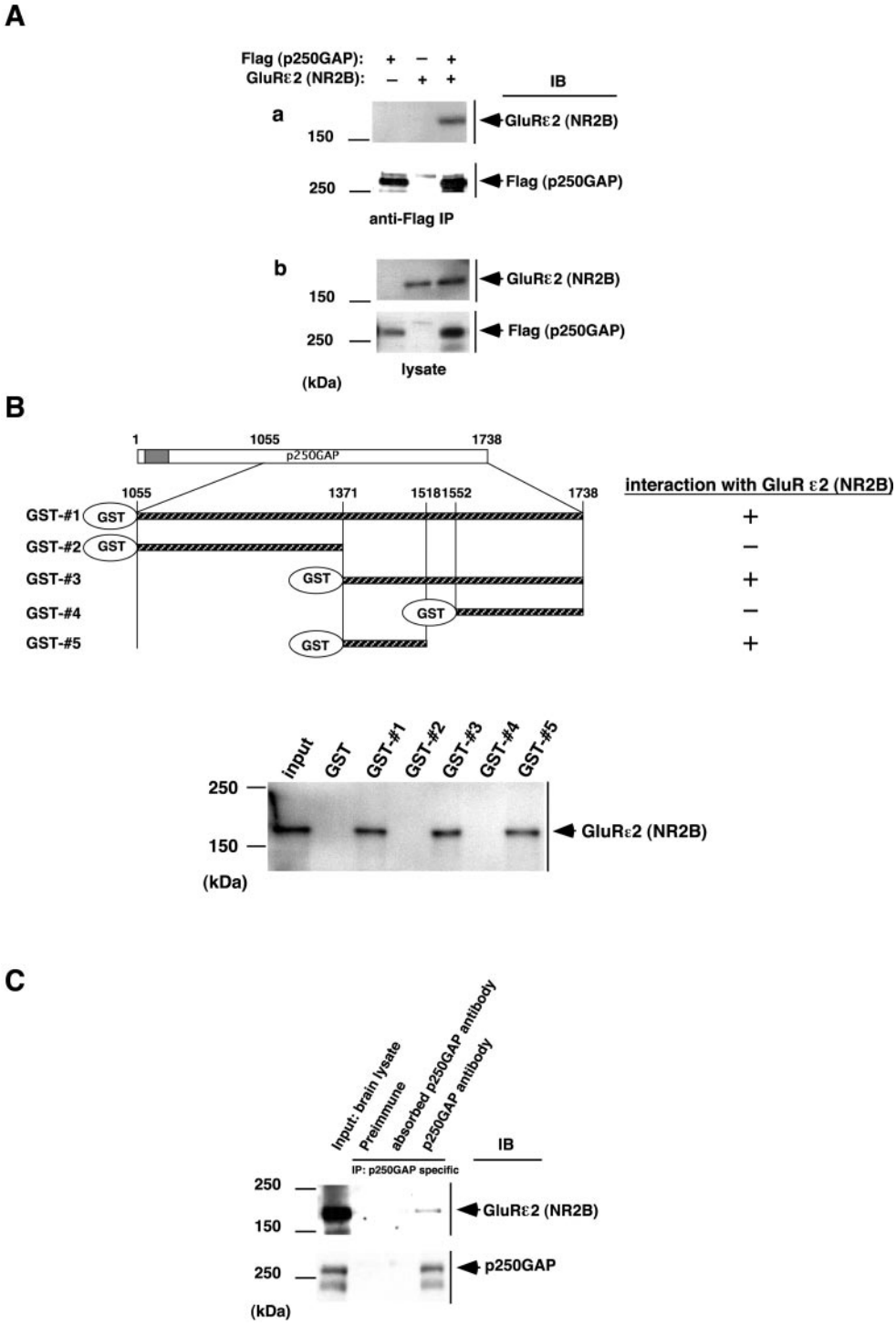

Figure 2. Interaction between p250GAP and GluRε2 (NR2B). (A) Interaction between p250GAP and GluRε2 (NR2B) in HEK293T cells. HEK293T cells were transfected with combinations of expression plasmids for FLAG-tagged p250GAP and GluRε2 (NR2B). Two days later, the cells were lysed and anti-FLAG immunoprecipitates (IP, a) and cell lysates (b) were subjected to immunoblotting (IB) with anti-GluRε2 (NR2B) mAb (top) and anti-FLAG mAb (bottom). (B) Mapping of the regions in p250GAP required for interaction with GluRε2 (NR2B). HEK293T cells were transfected with the expression plasmid for GluRε2 (NR2B). The lysates were incubated with 1 μg of the GST or GST-fusion proteins (GST1–5) immobilized on glutathione-Sepharose. After centrifugation, the beads were washed and subjected to immunoblotting with anti-GluRε2 (NR2B) mAb. (C) Interaction between p250GAP and GluRε2 (NR2B) in mouse brain. Brain lysates were immunoprecipitated with preimmune sera, anti-p250GAP antibodies, or anti-p250GAP antibodies preabsorbed with immunogen. The immunoprecipitates and input were immunoblotted (IB) with anti-GluRε2 (NR2B) (top) mAb and anti-p250GAP antibodies (bottom).

Expression of p250GAP mRNA Is Enriched in Brain

To gain insights into possible function of p250GAP, we examined tissue distribution of the p250GAP mRNA expression. Northern blot analysis of RNAs from adult mouse tissues showed that the p250GAP mRNA was expressed at high levels in telencephalon and at low levels in cerebellum, colon, small intestine, and kidney (Figure 3A). Furthermore, in situ hybridization analysis showed that the level of p250GAP mRNA was high in cortex, corpus striatum, hippocampus, and thalamus of adult mouse brain (Figure 3, B and C). The expression pattern was very similar to that of GluRε2 (NR2B) (Watanabe *et al*., 1992). p250GAP mRNA

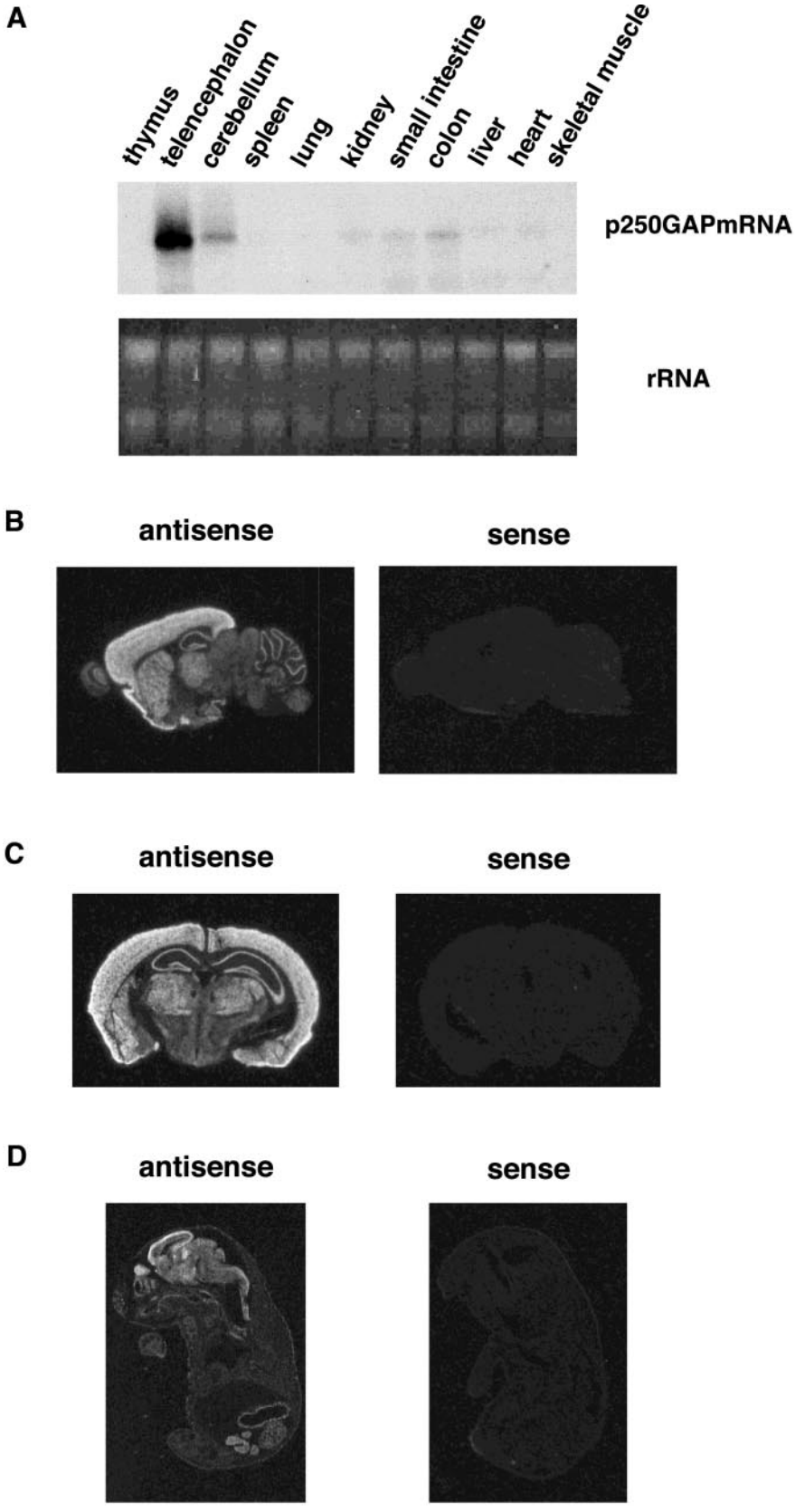

Figure 3. Expression pattern of p250GAP. (A) Northern blot analysis. A blot with RNAs from adult mouse tissues was probed with a fragment of mouse p250GAP cDNA. The amounts and quality of RNAs were verified by EtBr staining. (B–D) In situ hybridization of p250GAP mRNA in mouse tissue sections. A parasagittal section of adult brain (B), a coronal section of adult brain (C), or a sagittal section of E18.5 embryo (D) was hybridized with an α-^{35}S-UTP–labeled mouse p250GAP cRNA probe.

was also expressed at a high level in developing brain and at low levels in small intestine and kidney (Figure 3D).

p250GAP Is Concentrated in the Postsynaptic Density

We visualized p250GAP in 20-d-old cultured primary hippocampal neurons with antibodies against MAP2, p250GAP, and GluRε2 (NR2B). The cultured primary neurons have mature synapses with fully differentiated PSDs. p250GAP was strikingly abundant in punctate structures arrayed along dendrites (Figure 4A). As p250GAP associates with GluRε2 (NR2B) in vivo (Figure 2C), p250GAP was colocalized with GluRε2 (NR2B) at the spine (Figure 4B). In addition, exogenous p250GAP expressed using Sindbis virus-mediated expression system colocalized with PSD-95 (our unpublished data). These results provided further evidence that p250GAP was localized at the dendritic spines and was associated with GluRε2 (NR2B) in vivo. Consistently, Western blot of subcellular fractions from mouse forebrain with antibodies against p250GAP, GluRε2 (NR2B), PSD-95, RhoA, Cdc42, Rac1, and Synaptophysin revealed that p250GAP, GluRε2 (NR2B), and PSD-95 were all enriched in the isolated PSD fraction (Figure 4C). Furthermore, the data revealed that RhoA and Rac1 were present in the PSD fraction. In contrast, Cdc42 was hardly detectable in the PSD fraction (Figure 4C).

p250GAP Is a GAP for Cdc42 and RhoA

To determine whether p250GAP encodes a functional GAP activity toward Rho GTPases, amino acids 1–263 of wild-type and R58I mutant of p250GAP were bacterially expressed in Arg-58 of p250GAP corresponds to the conserved amino acid of RhoGAPs, which is known to be required for GAP activity (Barrett *et al*., 1997; Li *et al*., 1997). GAP assay with [γ-^{32}P]GTP-loaded Rho family proteins revealed that the RhoGAP-like domain of p250GAP was active on RhoA and Cdc42, but hardly on Rac1 (Figure 5A)

Next, the ability of p250GAP to regulate the GTP-loaded states of RhoA, Cdc42, and Rac1 in cells was tested. Because Rho GTPases bind downstream effectors when in the GTP-bound state, a GST fusion protein containing the CRIB of Pak and the RBD of Rhotekin can be used to assay the levels of active Cdc42, active Rac1, and active RhoA, respectively. HEK293T cells were transiently transfected with plasmids encoding FLAG-tagged wild-type and R58I mutant of p250GAP together with Myc-tagged RhoA, Cdc42, or Rac1. GTP-loaded RhoA, Cdc42, or Rac1 was then collected from the cell lysates by GST-CRIB or GST-RBD. Simultaneous expression of wild-type p250GAP clearly reduced the amount of GTP-loaded Cdc42 and GTP-loaded RhoA, whereas expression of p250GAP had little effect on the GTP-loaded status of Rac1 (Figure 5B). Apparently, GAP activity of p250GAP R58I mutant toward RhoA and Cdc42 was less than that of wild-type p250GAP. We then tested the ability of endogenous p250GAP to regulate the GTP-loaded states of Rho family GTPases. The anti-p250GAP immunoprecipitates from mouse brain lysates were incubated with extracts of HEK293T cells transiently transfected with plasmids encoding Myc-tagged RhoA, Cdc42, or Rac1. GTP-loaded RhoA, Cdc42, or Rac1 was then collected from the reaction mixtures with GST-CRIB or GST-RBD. Incubation of wild-type p250GAP immunoprecipitates with extracts from RhoA, Cdc42, or Rac1 expressing HEK293T cells clearly reduced GTP-Cdc42 and GTP-RhoA but not GTP-Rac1 (Figure 5C). These results suggest that p250GAP promotes GTP hydrolysis on RhoA and Cdc42 in vivo.

p250GAP Regulates Neurite Outgrowth in Neuroblastoma Cells

Rho family GTPases regulate neurite outgrowth in Neuro-2A neuroblastoma cells (Brouns *et al*., 2001). To establish the biological significance of p250GAP in neuronal cells, we examined the effect of p250GAP expression on neurite outgrowth of Neuro-2A cells. Neuro-2A cells were transiently transfected with plasmid encoding wild-type p250GAP, R58I mutant of p250GAP, mutationally inactive Cdc42 (N17Cdc42), or mutationally inactive RhoA (N19RhoA). The mock-transfected cells undergo neuronal differentiation associated with extensive neurite outgrowth after serum withdrawal (Figure 6Aa, Ab) (Brouns *et al*., 2001). Without serum, expression of N17Cdc42 suppressed neurite outgrowth in Neuro-2A cells (Figure 6Ac, Ad). Expression of wild-type p250GAP also suppressed neurite outgrowth under this condition (Figure 6Ae, Af). The ratio of total neurite length of cells transfected with N17Cdc42 and wild-type p250GAP to that of mock-transfected cells were 9.5 ± 5.4 and 13.4 ± 2.9% (mean ± SEM) ($p < 0.01$), respectively (Figure 6C). Neuro-2A cells also undergo neuronal differentiation associated with extensive neurite outgrowth upon Rho inactivation by C3 transferase (Brouns *et al*., 2001). In agreement with this, expression of N19RhoA induced extensive neurite outgrowth in the presence of serum (Figure 6Bk, Bl). Expression of wild-type p250GAP also induced extensive neurite outgrowth under this condition (Figure 6Bm, Bn). The ratio of total neurite length of cells transfected with N19RhoA and wild-type p250GAP to that of mock-transfected cells were 975 ± 70.0 and 942 ± 37.5% (mean ± SEM) ($p < 0.01$), respectively (Figure 6D). Expression of R58I mutant of p250GAP had no effect on these morphological changes (Fig 6Ag, Ah, Bo, Bp), suggesting that these effects were completely dependent on the GAP function of p250GAP. These results suggest that RhoA and Cdc42 serve as physiological substrates for p250GAP in neuronal cells.

NMDA Receptor Stimulation Leads to Redistribution of p250GAP in Hippocampal Slices

Association of p250GAP with NMDA receptor led us to investigate whether NMDA receptor activity modulates p250GAP behavior. Hippocampal slices were stimulated with NMDA, and TNE buffer-soluble or whole slice lysates (see MATERIALS AND METHODS) were subjected to immunoblot analysis with antibodies against p250GAP, PSD-95, phospho-ERK, and ERK. As shown in Figure 7, A and B, NMDA stimulation led to a significant decrease in the level of p250GAP in TNE buffer-soluble fraction. Levels of PSD-95 and ERK were little affected by the NMDA treatment (Figure 7, A and B). Phosphorylation of ERK was increased upon NMDA receptor stimulation (Figure 7, A and C) as described previously (English and Sweatt, 1996). The ratio of the level of p250GAP in the TNE buffer-soluble fraction of NMDA stimulated slices to that of mock-stimulated slices was 0.54 ± 0.006 (mean ± SEM) ($p < 0.0001$) (Figure 7B). The

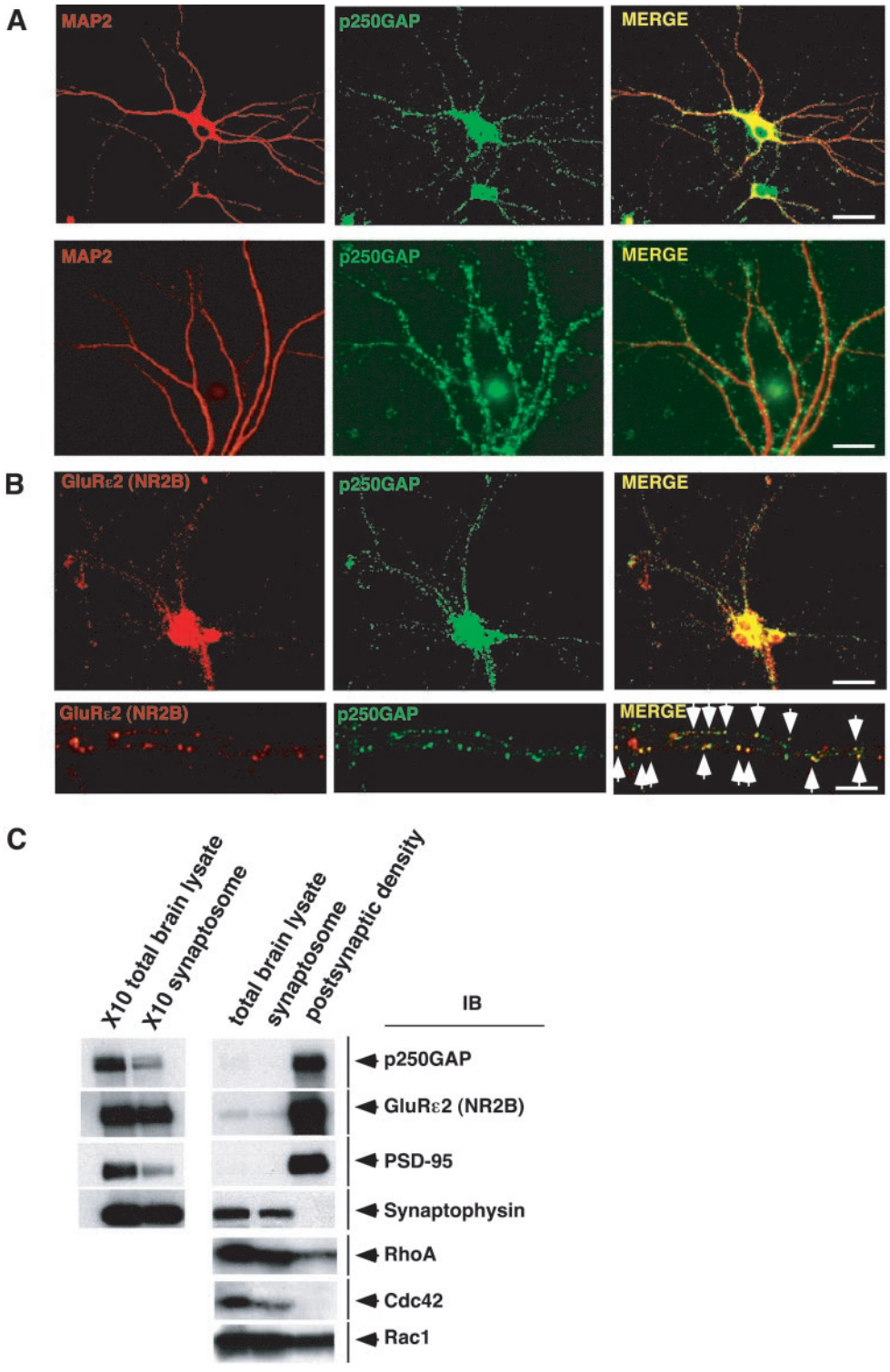

Figure 4. Enrichment of p250GAP in postsynaptic density. (A) Localization of p250GAP at punctate structures arrayed along dendrites. Hippocampal neurons were dissociated at embryonic day 17.5, cultured for 20 d, and stained with anti-p250GAP antibodies (green) and anti-MAP2 mAb (red). Bars, 25 μm (top). Lower panels show higher magnification images. Bar, 10 μm (bottom). (B) Colocalization of p250GAP with GluRϵ2 (NR2B). The hippocampal neurons were stained with antibodies against p250GAP (green) and GluRϵ2 (NR2B) (red). Bars, 25 μm (top). Lower panels show higher magnification images. Arrows indicate colocalization of p250GAP with GluRϵ2 (NR2B). Bars, 5 μm. (C) p250GAP in isolated PSD fraction. A sample of 50 μg (left) or 5 μg (right) of mouse forebrain lysates and synaptosomes, and the PSD extracted once with Triton X-100 (5 μg), were subjected to immunoblotting (IB) with antibodies against p250GAP, GluRϵ2 (NR2B), PSD-95, Synaptophysin (a presynaptic marker), RhoA, Cdc42, and Rac1.

decrease in p250GAP was completely blocked by application of a selective NMDA receptor antagonist DL-2-amino-5-phosphonovalerate (DL-APV) (Figure 7, A and B). On the other hand, the levels of p250GAP in whole slice lysates of NMDA-stimulated slices and in mock-stimulated slices were unchanged (Figure 7, C and D). These results suggest that NMDA receptor stimulation leads to redistribution of p250GAP in hippocampal neurons.

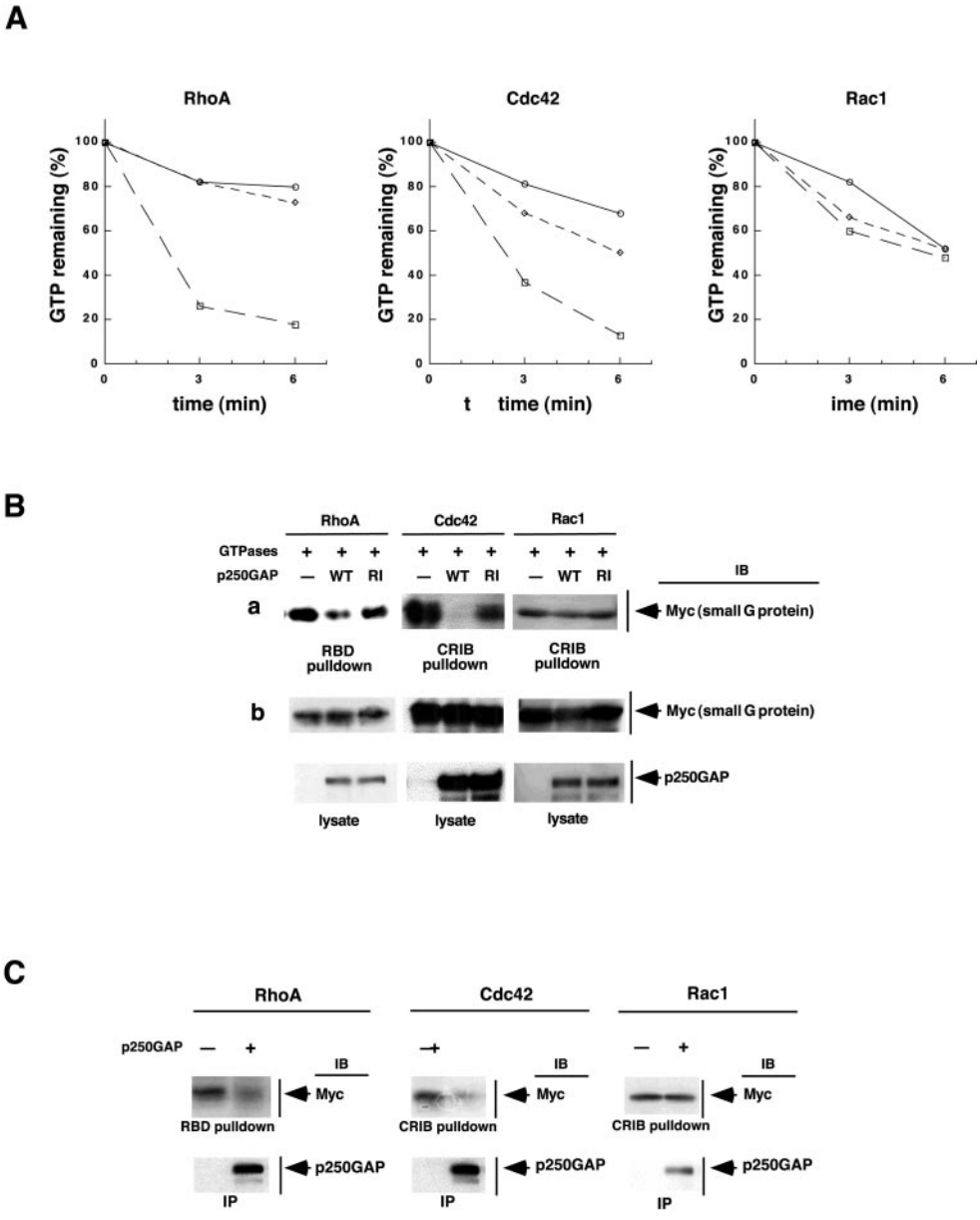

Figure 5. Characterization of GAP activity of p250GAP. (A) GAP activity of recombinant GAP domain of p250GAP. Equal concentrations (10 nM) of recombinant GST (circle), GST-GAP domain of wild-type p250GAP (square), and R58I mutant of p250GAP (diamond) were added to in vitro GAP assay with 20 nM [γ-^{32}P]GTP-loaded RhoA, Cdc42, or Rac1. (B) GAP activity of p250GAP in HEK293T cells was analyzed by Rho GTPase effector pulldown assays. HEK293T cells were transiently transfected with expression plasmids for Myc-tagged RhoA, Cdc42, or Rac1 together with or without (−) FLAG-tagged wild-type (WT) or R58I mutant (RI) of p250GAP. Lysates of the cells were pulled down by GST-CRIB (for Cdc42 and Rac1) or GST-RBD (for RhoA) immobilized on glutathione-Sepharose. Levels of GTP-loaded RhoA, Cdc42, and Rac1 were analyzed by immunoblotting with anti-Myc mAb (a). Whole-cell lysates were verified by immunoblotting with anti-Myc mAb (b, top) and anti-p250GAP antibodies (b, bottom). (C) GAP activity of endogenous p250GAP in mouse brain. Lysates of HEK293T cells expressing Myc-tagged RhoA, Cdc42, or Rac1 were incubated with (+) or without (−) p250GAP immunoprecipitates from brain lysates (IP). GTP-loaded RhoA, Cdc42, or Rac1 in the lysates was then collected by GST-CRIB or GST-RBD and detected by anti-Myc mAb (top). p250GAP immunoprecipitates were verified by anti-p250GAP antibodies (bottom). All experiments were repeated more than three times.

NMDA Receptor Stimulation Leads to Dephosphorylation of p250GAP in Hippocampal Slices

As shown in Figure 7A, NMDA stimulation led to not only a significant decrease in the level of p250GAP in TNE buffer-soluble fraction but also an increase in its migration on SDS-PAGE. When the proteins in the lysate of unstimulated slices were treated with bacterial alkaline phosphatase (BAP), migration of p250GAP on SDS-PAGE increased to resemble that of p250GAP derived from NMDA-stimulated slices (Figure 7A). The migrations of PSD-95 and ERK were little changed by BAP treatment. These suggest that p250GAP is a phospho-protein and is subjected to dephosphorylation after NMDA stimulation. It was possible that p250GAP is tyrosine-phosphorylated in hippocampal slices, because p250GAP expressed together with kinase active Fyn in HEK293T cells becomes tyrosine-phosphorylated (our unpublished data). To examine whether p250GAP is tyrosine-phosphorylated in brain and tyrosine-dephosphorylated after NMDA stimulation, equal amounts of p250GAP were immunoprecipitated from mock- and NMDA-stimulated hippocampal slices and subjected to immunoblotting with an anti-pY antibody. The anti-pY immunoreactivity clearly decreased upon NMDA-stimulation (Figures 7E), suggesting that p250GAP is tyrosine-dephosphorylated after the NMDA receptor stimulation. The ratio of the tyrosine-phosphorylation level of p250GAP in the NMDA-stimulated slices to that of mock-stimulated slices was 0.39 ± 0.05

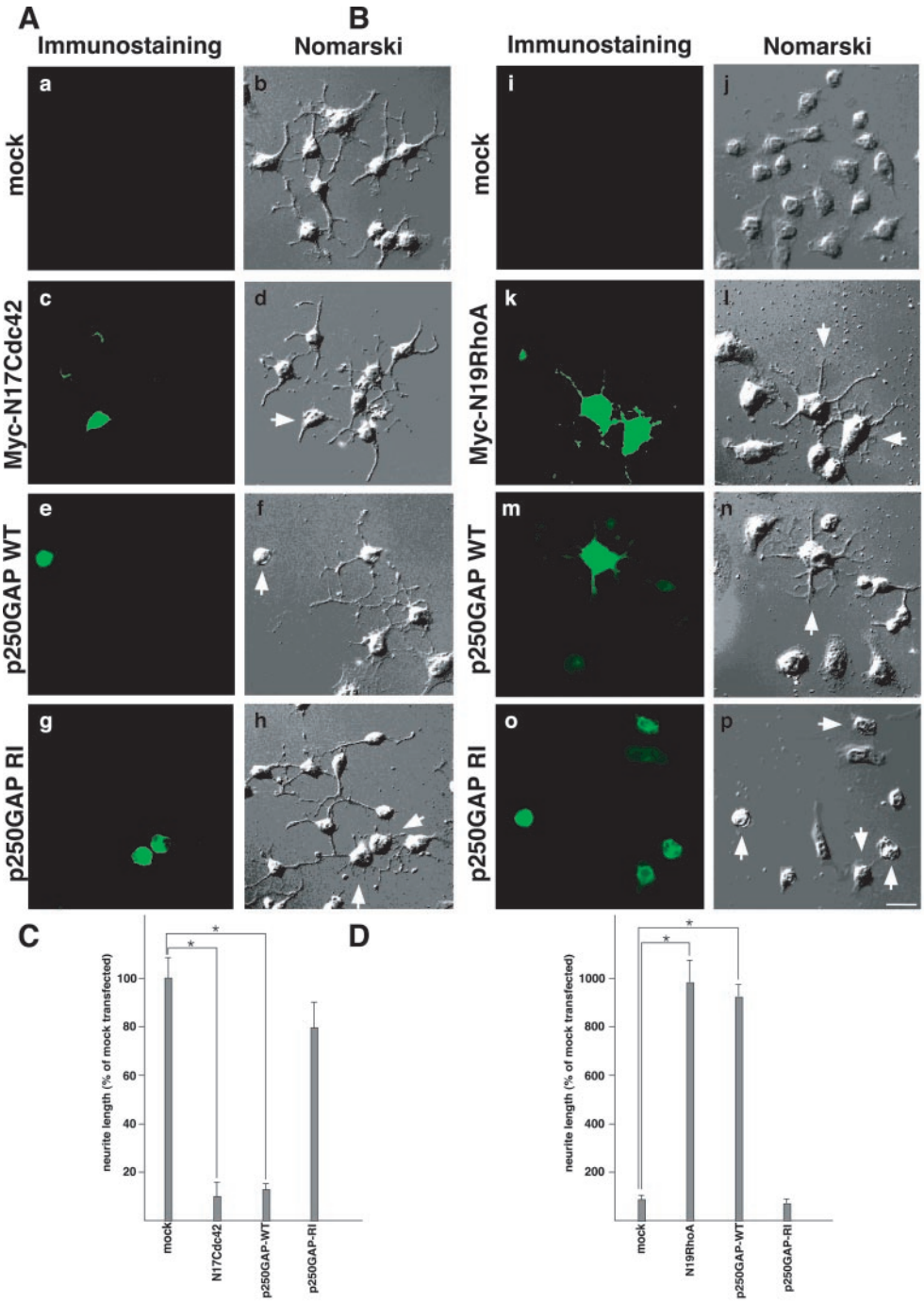

Figure 6. Regulation of neuritogenesis in Neuro-2A cells by overexpression of p250GAP. (A) Suppression of neuritogenesis after serum withdrawal by overexpression of wild-type p250GAP. Neuro-2A cells were transiently transfected with expression plasmids for mock (Aa and antibody), Myc-tagged N17Cdc42 inactive mutant (Ac and Ad), FLAG-tagged wild-type (WT) p250GAP (Ae and Af), or R58I mutant (RI) of p250GAP (Ag and Ah). (B) Induction of neuritogenesis by overexpression of wild-type p250GAP in the presence of serum. Neuro-2A cells were transiently transfected with an expression plasmid for mock (Bi and Bj), Myc-tagged N19RhoA inactive mutant (Bk and Bl), FLAG-tagged wild-type (WT) p250GAP (Bm and Bn), and R58I mutant (RI) of p250GAP (Bo and Bp). (A and B) The cells were fixed and viewed using a Nomarski microscope (antibody, Ad, Af, Ah, Bj, Bl, Bn, and Bp). Exogenous expression of the proteins was confirmed by anti-Myc mAb or anti-FLAG antibodies staining (Aa, Ac, Ae, Ag, Bi, Bk, Bm, and Bo). Bar, 20 μm. (C and D) The neurite length of 20 cells was calculated using TI workbench software (kindly provided by T. Inoue, University of Tokyo). Results were shown as the mean from three independent experiments. Values are mean ± SEM, *$p < 0.01$, mock transfected versus N17Cdc42, N19RhoA, wild-type (WT) p250GAP, or RI mutant of p250GAP (Student's *t* test).

(mean ± SEM) ($p < 0.01$) (Figure 7F). Unlike the results shown in Figure 7A, immunoblotting of the anti-p250GAP immunoprecipitates from the TNE-solubilized lysates with anti-p250GAP detected similar amounts of p250GAP regardless of NMDA stimulation (Figure 7E). This was most likely due to low efficiency of immunoprecipitation with the anti-p250GAP antibodies that were used in the experiments. NMDA-induced dephosphorylation of p250GAP on residues other that tyrosine is under investigation.

DISCUSSION

Recent data suggest that NMDA receptors regulate actin reorganization in dendritic spines (Engert and Bonhoeffer, 1999; Maletic-Savatic *et al*., 1999; Toni *et al*., 1999). However, the signaling pathways that link NMDA receptor activity to the postsynaptic actin cytoskeleton are poorly understood. We identified a novel GAP for Rho family GTPases, termed p250GAP, and showed that it interacts

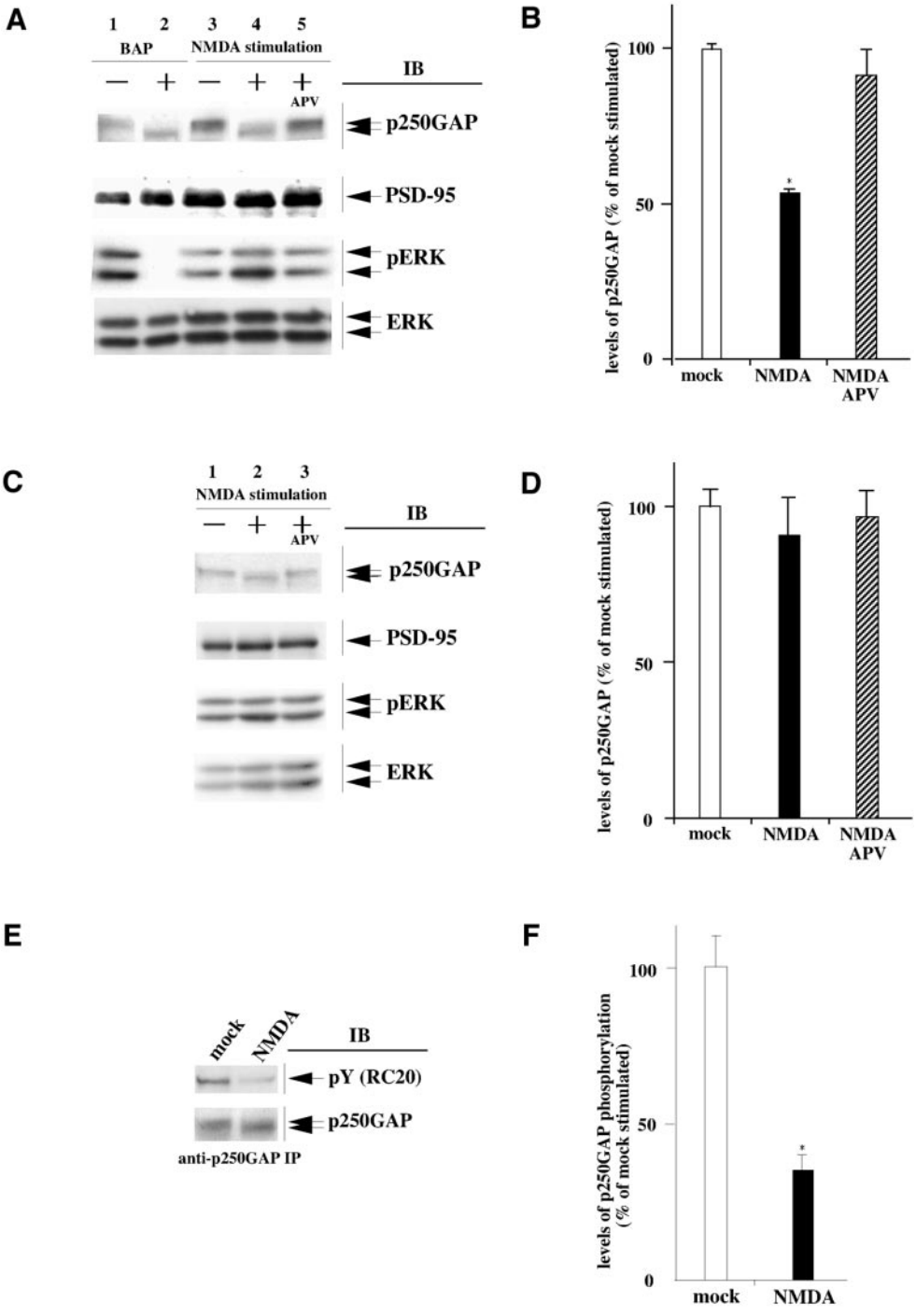

Figure 7. Redistribution and dephosphorylation of p250GAP after NMDA receptor stimulation in hippocampal slices. (A and C) Redistribution of p250GAP after NMDA receptor stimulation. Shown are representative blots of TNE buffer-soluble (A) or whole (C) lysates with antibodies against p250GAP, PSD-95, phospho-ERK, and ERK. The samples were prepared from mouse hippocampal slices that were mock stimulated (lane 3 in A and lane 1 in C), or stimulated with 50 μM NMDA for 5 min, in the absence (lane 4 in A and lane2 in C) or presence (lane 5 in A and lane 3 in C) of 100 μM DL-APV. In parallel experiments, the lysates of mock-stimulated slices were treated with (lane 2 in A) or without (lane 1 in A) BAP and were subjected to immunoblotting with antibodies against p250GAP, PSD-95, phospho-ERK, and ERK. (B and D) Quantification of the p250GAP level. Results were shown as the mean from three independent experiments. Values are mean ± SEM, *$p < 0.0001$, mock stimulated versus NMDA stimulation (Student's *t* test). (E) Dephosphorylation of p250GAP after NMDA receptor stimulation. The anti-p250GAP immunoprecipitates from mock-stimulated or NMDA stimulated slices were blotted with anti-pY mAb (RC20) (top) and then with anti-p250GAP antibodies (bottom). (F) Quantification of p250GAP phosphorylation level. The Student's *t* test value (0.39 ± 0.05: mean ± SEM, *$p < 0.01$) was from three independent experiments.

with the GluRε2 (NR2B) subunit of the NMDA receptor. p250GAP was targeted to dendritic spines and highly concentrated in PSD (Figures 2 and 4). Because the PSD-95/NMDA receptor complex is tightly associated with the PSD (Allison *et al*., 1998), this complex can provide rigid structural support for the recruitment of other PSD proteins. It is therefore possible that the recruitment of p250GAP to dendritic spines is mediated by a direct interaction with NMDA receptor complex. We also showed that the NMDA receptor activity could regulate p250GAP behavior (Figure 7, further discussed below). To our knowledge, p250GAP is the first GAP for the Rho family GTPases whose behavior is modulated by NMDA receptor activity.

p250GAP promoted GTP hydrolysis on both Cdc42 and RhoA in vitro and in vivo (Figures 5 and 6), suggesting that activities of Cdc42 and RhoA are regulated by p250GAP. Nevertheless, we assume that p250GAP targets RhoA but not Cdc42 in dendritic spines, because the amount of Cdc42 in PSD was under detectable level (Figure 4C). Rho family GTPases play a central role in dendritic spine plasticity (Luo *et al*., 1996; Threadgill *et al*., 1997; Ruchhoeft *et al*., 1999; Lee *et al*., 2000; Nakayama *et al*., 2000; Tashiro *et al*., 2000; Wong *et al*., 2000; Hering and Sheng, 2001). We assume that p250GAP might be involved in morphological rearrangements of dendritic spines through regulation of the RhoA activity.

NMDA receptor-mediated synaptic activity modulates spine morphology, spine turnover, and synaptic transmission (Fischer *et al*., 1998; Halpain *et al*., 1998; Sorra and Harris, 1998; Engert and Bonhoeffer, 1999; Maletic-Savatic *et al*., 1999; Okabe *et al*., 1999; Parnass *et al*., 1999; Toni *et al*., 1999; Yuste and Bonhoeffer, 2001). In these processes, NMDA receptors must transduce highly localized signals to the actin cytoskeleton specifically in the region of the dendritic spines where they are activated. Because p250GAP interacts directly with NMDA receptors (Figure 2), NMDA receptors would be able to modulate p250GAP behavior to elicit spatially restricted activation of RhoA only at the site where they are activated. In fact, we showed that NMDA stimulation induces changes in the extractability of p250GAP (Figure 7, A–D). Moreover, we preliminarily observed changes in the immunostaining pattern of virally expressed GFP-tagged p250GAP in response to NMDA receptor stimulation (our unpublished data). Redistribution of p250GAP upon NMDA receptor stimulation may be one of the mechanisms of functional modulation of p250GAP. Intriguingly, p250GAP is a phospho-protein and can be phosphorylated by Fyn and CaMKII (Figure 7E; our unpublished data). When NMDA receptors were strongly stimulated, p250GAP was tyrosine-dephosphorylated (Figure 7E). Dephosphorylation of p250GAP may affect its interaction with proteins in the PSD, such as NMDA receptors, scaffold proteins, and cytoskeletal proteins.

Reduction of the level of NMDA receptor-associated p250GAP upon NMDA stimulation would result in activation of RhoA to alter local cytoskeletal arrangements in stimulated dendritic spines. Stimulation of cultured hippocampal neurons with 50 μM NMDA for 5 min results in rapid and extensive loss of spines (Halpain *et al*., 1998). The NMDA receptor-mediated regulation of p250GAP may contribute to a neuroprotective mechanism by which the number of dendritic spines is reduced (Halpain *et al*., 1998). The idea is consistent with the result that expression of active RhoA dramatically reduces the spine number (Tashiro *et al*., 2000). Stimulation of cultured hippocampal neurons with 50 μM NMDA for 5 min also results in disruption of AKAP–MAGUK complexes by remodeling the dendritic actin (Gomez *et al*., 2002). This triggers dephosphorylation of AMPA receptors, which subsequently induces homosynaptic long-term depression (Tavalin *et al*., 2002). Thus, NMDA receptor regulation of p250GAP may contribute to an induction of long-term depression as well by remodeling the dendritic actin. Further studies are needed to establish the mechanism by which NMDA receptors regulate p250GAP function as well as function of other regulators for Rho family proteins in the receptor complex, and thereby the localized actin remodeling.

Rho family GTPases in neural tissues regulate neuronal migration, axon growth, axon guidance, dendrite elaboration, and synapse formation (Jalink *et al*., 1994; Luo *et al*., 1996; Zipkin *et al*., 1997; Ruchhoeft *et al*., 1999; Bateman *et al*., 2000; Bito *et al*., 2000; Li *et al*., 2000; Luo, 2000). Because exogenous expression of p250GAP regulates neurite outgrowth in Neuro-2A cells (Figure 6), p250GAP may regulate dendrite outgrowth. Although, it is not clear whether the interaction between p250GAP and NMDA receptors is important in this regulation, it is worthy to note that NMDA receptors regulate growth of the dendritic arbor in *Xenopus* central neurons (Li *et al*., 2000). In immature neurons, p250GAP may also be involved in regulation of axon growth and guidance similar to Ephexin, a GEF for Rho GTPases that is involved in the Eph tyrosine kinase signaling (Shamah *et al*., 2001). Because p250GAP mRNA is highly expressed not only in adult brain but also in developing brain, p250GAP as well as p190 RhoGAP might modulate the activities of Rho family GTPases to direct several actin-dependent morphogenetic processes required for normal neural development (Brouns *et al*., 2000).

In summary, we suggest that p250GAP is an important link between NMDA receptors and Rho family GTPases, and thus the actin cytoskeleton. Further studies on p250GAP will unravel the importance of spine plasticity regulated by NMDA receptor activity.

During revision of this manuscript, others have reported cloning of Grit/p200RhoGAP/Rics (Nakamura *et al*., 2002; Moon *et al*., 2003; Okabe *et al*., 2003) that is identical to p250GAP. Although Okabe *et al.* (2003) have demonstrated that Rics is associated with NMDA receptors and localized to the PSD, we would like to emphasize that our study is the first to characterize the functional involvement of p250GAP in NMDA signaling.

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