Characterization of a Brain-specific Rho GTPase-Activating Protein, p200RhoGAP*

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The Rho GTPase-activating proteins (RhoGAPs) are a family of multifunctional molecules that transduce diverse intracellular signals by regulating Rho GTPase activities. A novel RhoGAP family member, p200RhoGAP, is cloned in human and mouse. The murine p200RhoGAP shares 86% sequence identity with the human homolog. In addition to a conserved RhoGAP domain at the N terminus, multiple proline-rich motifs are found in the C-terminal region of the molecules. Northern blot analysis revealed a brain-specific expression pattern of p200RhoGAP. The RhoGAP domain of p200RhoGAP stimulated the GTPase activities of Rac1 and RhoA in vitro and in vivo, and the conserved catalytic arginine residue (Arg-58) contributed to the GAP activity. Expression of the RhoGAP domain of p200RhoGAP in Swiss 3T3 fibroblasts inhibited actin stress fiber formation stimulated by lysophosphatidic acid and platelet-derived growth factor-induced membrane ruffling but not Bradykinin-induced filopodia formation. Endogenous p200RhoGAP was localized to cortical actin in native N1E-115 neuroblastoma cells and to the edges of extended neurites of differentiated N1E-115 cells. Transient expression of the RhoGAP domain and the full-length molecule, but not the catalytic arginine mutants, readily induced a differentiation phenotype in N1E-115 cells. Finally, p200RhoGAP was capable of binding to the Src homology 3 domains of Src, Crk, and phospholipase Cγ in vitro and became tyrosine-phosphorylated upon association with activated Src in cells. These results suggest that p200RhoGAP is involved in the regulation of neurite outgrowth by exerting its RhoGAP activity and that its cellular activity may be regulated through interaction with Src-like tyrosine kinases.

Rho family GTPases are key regulators of diverse cellular functions including actin-based morphological changes, gene induction, cell motility, and intracellular membrane trafficking (1, 2). They act as binary molecular switches that are turned on and turned off in response to a variety of extracellular stimuli. Rho proteins in the GTP-bound active state can interact with a number of effector targets that transduce signals leading to biological response (3). When the bound GTP is hydrolyzed to GDP, the Rho protein-mediated signaling is turned off. Three classes of regulatory proteins are involved in balancing Rho GTPases between the on- and off-states: the guanine nucleotide exchange factors that promote the release of bound GDP and facilitate GTP binding (4), the GTPase-activating proteins (GAPs)¹ that increase the intrinsic GTPase activity of Rho GTPases to accelerate the return of the proteins to the inactive state (5, 6), and the guanine nucleotide dissociation inhibitors that sequester the GDP-bound form of Rho GTPases and may also regulate their intracellular localization (7). One emerging theme from recent studies of Rho GTPase regulation is that a balanced act between the activation and the deactivation signals is required for effective signal flow through Rho GTPases, and this may involve concerted action of all classes of regulatory proteins (8). Understanding how such a balance is achieved in various physiological situations represents a major challenge in elucidating the regulatory mechanism of Rho GTPases.

Together with Rho family GTPases, RhoGAPs are found in eukaryotes ranging from yeast to human, suggesting an evolutionarily conserved role in eukaryotic cell regulation. So far over 30 RhoGAP family members have been reported, and more are found in mammalian genomes (9, 10). RhoGAPs may therefore far outnumber their cellular substrates, i.e., the Rho family GTPases that stand with 20 members. The overabundance of RhoGAPs evidently suggests that each RhoGAP may play a specialized role in regulating individual Rho GTPase activity and/or in mediating their specific functions. Moreover, the biochemical GAP activities of each RhoGAP must also be fine-tuned in cells by tight regulation in a spacial and temporal manner such that Rho GTPases would not be turned off all the time.

In neuronal systems, RhoGAPs and their substrate Rho GTPases have been implicated in regulating multiple processes of the morphological development of neurons, including axon growth, guidance, branch stabilization, and dendritic elaboration (6, 11). For example, the Rho-specific RhoGAP p190 has been shown to dissolve actin stress fibers when introduced into fibroblasts (12) and was found to be essential for exon stability in mushroom body neurons in Drosophila (13). Deletion of p190 in mice led to deregulation of Rho GTPase function and neuronal malfunction that is stemmed from Src kinase-regulated adhesion signaling defect (14). The discovery of a RhoGAP, oligophrenin-1, that is associated with X-linked mental retardation (15), further underlines the significance of RhoGAP in the nervous system. Thus, it is of particular interest to examine what role each individual RhoGAP might play in complex biological systems and how the RhoGAP activity is controlled in response to cellular stimulation.

¹ The abbreviations used are: GAP, GTPase-activating protein; GST, glutathione S-transferase; PAK, p21-activated kinase; PBD, p21-binding domain; SH, Src homology; HA, hemagglutinin; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; TRITC, tetramethylrhodamine isothiocyanate; PLCy, phospholipase Cγ; LPA, lysophosphatidic acid.

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In the present work, we describe the identification and initial characterization of a novel member of the RhoGAP family, p200RhoGAP. p200RhoGAP is brain-specific and acts as a GAP to down-regulate RhoA and Rac1 activities in vitro and in vivo. In N1E-115 neuroblastoma cells it is localized at the cortical actin site in the undifferentiated state and at the ends of the neurite extensions in the differentiated state. Overexpression of the RhoGAP domain or full-length p200RhoGAP in N1E-115 cells induced a differentiation phenotype. Moreover we found that the p200RhoGAP binds to the SH3 domain of Src and is tyrosine-phosphorylated upon association with active Src in cells. These results suggest that this novel RhoGAP may have a role in neuronal differentiation, and its cellular function may be subject to the regulation by Src or Src-like kinases.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Rabbit antiserum against p200RhoGAP were raised using an N-terminal polypeptide derived from p200RhoGAP (residues 11–25; KRQGILKERVFGCDL) that was conjugated to KLH as an immunogen. Immunization and enzyme-linked immunosorbent assay analysis for the verification of the specificity of the antibody were performed by Sigma-GeneSys. Antiserum was purified by protein A affinity chromatography according to the manufacturer’s protocol (Pierce). The anti-GST monoclonal antibody was purchased from Sigma, and the anti-HA antibody was purchased from Roche Molecular Biochemicals. Fluorescein isothiocyanate-conjugated antibodies and rhodamine-conjugate phallolidin were purchased from Molecular Probes. Monoclonal anti-Rac1 antibody was obtained from Frome Cruz Biotechnology, Inc., anti-Rac1 was from Upstate Biotechnology, Inc., and anti-Cdc42 was from BD Biosciences. Anti-phospho-tyrosine and anti-Src antibodies were obtained from Upstate Biotechnology, Inc.

**DNA Constructs**—A human cDNA clone containing a RhoGAP homology domain, KIAA0712, was obtained from Dr. Takahiro Nagase at the Research Institute of Medical Sciences (Chiba, Japan). The cDNA sequence was sequenced by using the BLAST program in NCBI genomic data base for the human p200RhoGAP and in the Celera genomic data base for the murine p200RhoGAP. The exon sequences were identified manually by the GT-AG rule (16). Recombinant RhoGAP domain of p200RhoGAP (residues 1–251) was generated by polymerase chain reaction of the coding cDNA and subsequent subcloning of the PCR fragment into the BamHI and EcoRI sites of pGEX-2T vector (Amersham Biosciences). GAP/R58K) that bears a mutation of arginine to lysine at the predicted catalytic arginine (residue 58) was generated by introducing a point mutation using PCR-based site-directed mutagenesis (17) and was cloned into pGEX-2T vector similarly. GST fusion constructs of Cdc42, Rac1, and RhOa were described before (17, 18). GST fusion constructs of SH3 domains of Src, PLCγ, p85α, Crk, Abl, spectrin, and Grb2 (C) were kindly gifted from Dr. Steve Taylor (University of California). Production and purification of the GST fusion proteins were as described (17, 18). The purity of the recombinant proteins was estimated at >90% by Coomassie Blue staining of the polyacrylamide gel electrophoresis of the purified proteins.

The cDNAs encoding RhoGAP domain (residues 1–251), the domain containing the arginine mutation (GAP/R58K), full-length p200RhoGAP, the full-length p200RhoGAP containing the arginine mutation (p200R58K), and the C-terminal region of p200RhoGAP (p200-C; residues 224–1738) were cloned into the mammalian expression vector pCEFL-GST to be expressed as GST fusions and into the pKH3 vector to be expressed as (HA)3 fusions. The cDNAs were also cloned into a retroviral vector pMX-GFP that bicistronically expresses green fluorescent protein (GFP) to be expressed by retroviral infection (19). The pKH3 constructs of the HA-tagged Rho GTPases were described before (19), and the pCDNA3 constructs expressing kinase-deficient Src (Src[K295R/Y527F]), wild type c-Src, and constitutively active Src (Src[Y527F]) were obtained from Dr. Steve Taylor (University of California).

**Northern and Western Blotting**—For Northern blot analysis, repressible cisplatin-resistant cells were harvested from transfected cells. Cells were briefly washed with phosphate-buffered saline (PBS), pH 7.4, and were resuspended in RNAzol B solution (Tel-Test). The tissues were homogenized by using a Teflon homogenizer in gentle strokes, and the RNA extraction was performed by adding chloroform to 1/3 of the volume of the homogenate. After the extraction, the aqueous phase was taken for alcohol precipitation of RNA. Equal amount of RNA (30 μg) was loaded on each lane for gel electrophoresis and subsequent transfer to a nitrocellulose membrane. The membrane was probed with 32P radiolabeled cDNA probes prepared by the random priming method (Amersham Biosciences). A 668-bp PCR fragment corresponding to the residue’s C terminus of the RhoGAP domain of p200RhoGAP (residues 758–1002) was used as a template for the random priming labeling. The hybridization conditions were as recommended by Amersham Biosciences. A human multiple tissue RNA blot was obtained from Clontech, Inc. for the Northern blot analysis following similar protocols.

For Western blot analysis, cell lysates or co-precipitates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 5% skim milk in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl for 1 h and probed with primary antibodies followed by horseradish peroxidase-coupled secondary antibody for enhanced chemiluminescence analysis (Amersham Biosciences).

**In Vitro GTPase Activity Assay**—The intrinsic and GAP-stimulated GTPase activities of Cdc42, Rac1, and RhOa were measured as described by the nitrocellulose filter-binding method (17, 18). Briefly, recombinant G-proteins were preloaded with [γ-32P]GTP (1 μCi, 6000 Ci/mmol, PerkinElmer Life Sciences) in a 100-μl buffer containing 50 mM HEPES, pH 7.6, 0.2 mg/ml bovine serum albumin, and 0.5 mM EDTA for 10 min at ambient temperature before the addition of MgCl2 to a final concentration of 5 mM. An aliquot of the [γ-32P]GTP-loaded proteins was mixed with GAP buffer containing 50 mM HEPES, pH 7.6, 100 mM NaCl, 0.2 mg/ml bovine serum albumin, and 5 mM MgCl2 in the presence or absence of GAP. At different time points the reaction was terminated by filtering the reaction mixture through nitrocellulose filters followed by washing with 10 ml of ice-cold buffer with 50 mM HEPES, pH 7.6, and 10 mM MgCl2. The radioactivities retained on the filters were then subjected to quantification by scintillation counting.

**Cell Culture and Transfection**—Swiss 3T3 cells and COS-7 cells were cultured and maintained as described previously (19). N1E-115 neuroblastoma cells were obtained from ATCC. The cells were cultured in dishes coated with 20 μg/ml laminin and in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in 10% CO2 at 37 °C. For transient expression, COS-7 cells were plated in 6-well plates at a density of 5 × 105 cells/dish. The next day, plasmid constructs were transfected into the cells by using LipofectAMINE Plus (Invitrogen) according to the manufacturer’s protocol. For transient expression in N1E-115 cells, the cells were seeded in 6-cm dishes at a density of 6 × 105, and the transfections were carried out by using Cytofectin (BioRad) following the manufacturer’s protocol. 48 h after the transfections, cells were either fixed for immunofluorescence analysis or washed with ice-cold PBS once, and the whole cell extracts were prepared in a cell lysis buffer containing 50 mM Tris, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 5 mM MgCl2, 0.5 mM Na3VO4, 0.5% Nonidet P-40, 50 mM NaF, 10% glycerol, 10 mM β-mercaptoethanol, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Sigma) for 30 min at 4 °C.

The recombinant retroviruses expressing various p200RhoGAP constructs were generated by using the retroviral packaging Phoenix cell system (20) with a retroviral construct that bicistronically expresses green fluorescent protein as described (19). Swiss 3T3 and N1E-115 cells were infected with the respective retroviruses according to an established protocol (20). To observe ligand-induced actin structural changes, Swiss 3T3 cells were serum-starved overnight and challenged for 10 min with 20 ng/ml lysophosphatidyl ethanolamine (Sigma), 10 ng/ml PDGF (Upstate Biotechnology, Inc.) or 100 ng/ml Bradykinin (Sigma) before fixation.

**Immunocytochemistry**—For immunofluorescence staining, Swiss 3T3 cells were fixed with 3.8% formaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. The cells were then incubated with 1% bovine serum albumin in PBS for 30 min followed by incubation with the TRITC-conjugated phalloidin (Molecular Probes) to visualize the actin filaments. In N1E-115 cells, endogenous p200RhoGAP was detected by staining the cells with a polyclonal antibody followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit antibody. The actin filaments were stained with TRITC-conjugated phalloidin (Molecular Probes). The fluorescence images were obtained by using a Leica fluorescence microscope.

**GTPase Effector Pull-Down Assay**—The p21-binding domain (PBD) pull-down assay was performed using the HOXOS system (20) that contained native Rho family GTPases were performed as described (19). Briefly, COS-7 cells were cotransfected with HA-tagged Rho GTPases expressing plasmid and a vector expressing various GST-tagged p200RhoGAP cDNAs. 48 h post-transfection, the cell lysates prepared in a lysis buffer containing
FIG. 1. A, the predicted amino acid sequences of human and mouse p200RhoGAP. Human p200RhoGAP was identified from a human cDNA library (KIAA0712). The mouse p200RhoGAP sequences were identified from the Celera mouse genome data base. The mouse homolog shares 86% identity with human p200RhoGAP. The RhoGAP domain is in bold, and the proline-rich regions are in bold italic.

B, alignment of RhoGAP domains of p200RhoGAP (human and mouse), mouse CdGAP, human n-chimaerin, rat p190 RhoGAP, and human Cdc42GAP (p60RhoGAP). Identical residues are indicated by stars, and conserved residues are indicated by asterisks. C, sequence alignment of the proline-rich regions in p200RhoGAP (human and mouse), Cdc42GAP, BCR, 3BP-1, p85a, and CdGAP. X, non-conserved residues; P, proline-preferred; α, hydrophobic residue; P, conserved proline.
50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 1% Triton X-100, protease inhibitors, 1 mM phenylmethylsulfonyl fluoride were incubated with GST fusion of the PBDs of PAK1, WASP, or Rhotekin that were bound on glutathione beads. The expression of each protein was confirmed on Western blots using either anti-HA or anti-GST antibodies. The amount of RhoA-GTP, Rac1-GTP, or Cdc42-GTP that was pulled down by the bead-associated PBD of PAK1, WASP, or Rhotekin was detected by immunoblotting using anti-HA antibody. Quantification of Western blots was carried out using a Fujifilm LAS-1000 digital imaging system.

RESULTS

Identification of p200RhoGAP—We have been interested in identifying novel RhoGAPs that display distinctive tissue distribution patterns. Data base searches using the conserved RhoGAP domain as a probe led us to a few previously uncharacterized protein sequences that show different extent of homology to the known RhoGAPs. Among these proteins, we identified a cDNA clone predicted to encode 1738 amino acids containing a RhoGAP domain at the N terminus (GenBank™ accession numbers AB018255 and KIAA0712; see Fig. 1A). Comparison of this cDNA clone with the corresponding human genomic sequences in the NCBI data base (accession number AP000751) revealed that a guanine base at nucleotide position 414 was missing in the KIAA clone, resulting in a loss of 17 amino acids at the N terminus and a reading frameshift. This gene is located at chromosome 11q. We named this novel putative RhoGAP p200RhoGAP based on its predicted molecular mass. Using the human cDNA sequences, we further identified the mouse homolog of p200RhoGAP by searching the Celera mouse genome data base (GA_x5J8B7W6RL5; see Fig. 1A). Both the human and the mouse cDNA sequences are composed of 12 exons, and mouse p200RhoGAP shows 86% sequence identity to human p200RhoGAP.

A sequence comparison analysis indicated the existence of an N-terminal RhoGAP domain, as well as five C-terminal proline-rich domains in human p200RhoGAP (four proline-rich domains in the mouse homolog). In the N-terminal RhoGAP domain, the critical residues that are required for the GAP activity, including a highly conserved arginine (Arg-58), are well represented, suggesting that the GAP domain might be involved in the down-regulation of Rho GTPases (Fig. 1B). The RhoGAP domain of p200RhoGAP is most similar to that of Cdc42GAP with 70% sequence identity (21) and less to that of other RhoGAPs (36% identity to n-chimaerin, 27% to Cdc42GAP, and 28% to p190). However, outside the RhoGAP region the sequences of p200RhoGAP diverge from other RhoGAPs. Moreover, the proline-rich sequences present in all five proline-rich regions of p200RhoGAP appear to confer to the class II SH3 domain binding consensus (21) that are found in other signaling RhoGAPs including p190, p85α, Cdc42GAP, Bcr, and 3BP-1 (Fig. 1C).

To examine the tissue expression pattern of p200RhoGAP, we prepared a multiple tissue Northern blot using various mouse organs harvested from a 6-week-old male C57/BL6 mouse. To ensure the specificity of the signal, a radiolabeled probe was designed from the sequences outside of the conserved N-terminal RhoGAP domain. The Northern blot analysis showed that p200RhoGAP is specifically expressed in the brain but not in other tissues examined (Fig. 2). Similar Northern blot pattern was seen in a human multi-tissue mRNA blot (data not shown). Because a single hybridization signal was observed in a number of repeated hybridization experiments, it seems that p200RhoGAP does not have closely related family members that cross-react with the probe.

In Vitro and in Vivo GAP Activities of p200RhoGAP—To determine the function of the N-terminal RhoGAP domain of p200RhoGAP, we performed an in vitro GTPase activity test using the RhoGAP domain, as well as a mutant RhoGAP domain carrying a point mutation (R58K) at the predicted catalytic arginine site. When incubated with RhoA, Rac1, or Cdc42,
the RhoGAP domain could significantly stimulate the GTPase activity of all three GTPases whereas the R58K mutant failed to do so (Fig. 3). The RhoGAP domain showed higher GAP efficiency toward RhoA compared with Rac1 or Cdc42. In addition, the presence of increasing concentrations of the RhoGAP domain further enhanced the stimulatory effect (Fig. 3). These results indicate that the RhoGAP domain of p200RhoGAP can function as a GAP for Rho GTPases in vitro.

A number of effector proteins are known to be able to form a tight complex exclusively with the active form of RhoGTPases through their PBD (3). We next examined the in vivo substrate specificity of p200RhoGAP toward Rho GTPases using the immobilized GST fusion PBDs of Rhotekin, WASP, and PAK1 as bait for selective binding of RhoA-GTP, Cdc42-GTP, and Rac1-GTP, respectively. In COS-7 cells, wild type RhoA, constitutively active mutant of RhoA (V14RhoA), or dominant negative mutant of RhoA (N19RhoA) was cotransfected with a plasmid expressing the RhoGAP domain of p200RhoGAP, the catalytic arginine mutant of the RhoGAP domain (GAP(R58K)), the full-length, or the full-length mutant (p200(R58K)) of p200RhoGAP. GTP-bound Cdc42, Rac1, and RhoA were detected by the effector domain pull-down assay using the GBDs of His6-tagged WASP, PAK1, and Rhotekin immobilized on beads, respectively. The representative Western blots of the pull-down assays are shown on the left in each panel, and the statistical results from three independent experiments are plotted on the right.

Fig. 4. p200RhoGAP specifically activates the GTPase activities of RhoA and Rac1 in vivo. COS-7 cells were co-transfected with HA-tagged wild type, constitutively active or inactive mutant of Cdc42, Rac1 or RhoA, and the pCEFL vector, the vector containing the RhoGAP domain, the RhoGAP domain mutant (GAP(R58K)), the full-length, or the full-length mutant (p200(R58K)) of p200RhoGAP. GTP-bound Cdc42, Rac1, and RhoA were detected by the effector domain pull-down assay using the GBDs of His6-tagged WASP, PAK1, and Rhotekin immobilized on beads, respectively. The lysates of the transfectants were incubated with immobilized p21-binding domain of Rhotekin, and the co-precipitates were immunoblotted with anti-RhoA antibody. As shown in
A Novel Neuronal RhoGAP

Fig. 4A, constitutively active RhoA (V14RhoA) readily associated with GST-Rhotekin in the presence or absence of the RhoGAP domain, whereas dominant negative RhoA (N19RhoA) did not interact with this probe. The amount of active RhoA was significantly reduced (a 76% reduction) by the presence of the RhoGAP domain in the cells in comparison to the conditions in which the RhoGAP was absent. Furthermore, the catalytic arginine residue mutant of the RhoGAP domain, GAP(R58K), was only partially (24% reduction) effective in down-regulating RhoA activity (Fig. 4A). On the other hand, RhoA-GTP was slightly reduced by the full-length p200RhoGAP (a 30% reduction) and by the full-length molecule bearing the arginine mutation, p200(R58K) (a 22% reduction). These results suggest that the RhoGAP domain is an active GAP toward RhoA, and the full-length p200RhoGAP can weakly deactivate RhoA *in vivo*. Similar sets of experiments were also performed to examine the *in vivo* GTPase-activating efficacy of the RhoGAP domain and full-length p200RhoGAP toward Rac1 and Cdc42. As shown in Fig. 4B, neither the RhoGAP domain nor the full-length p200RhoGAP can inactivate Cdc42 effectively. In contrast, expression of the RhoGAP domain significantly reduced the Rac1-GTP species (a 67% reduction) whereas the full-length had only a minor effect (29% reduction) (Fig. 4C). Again, mutation of the catalytic arginine residue in the RhoGAP domain, Arg-58, to lysine reduced the negative regulatory effect of the GAP (a 23% reduction of Rac1-GTP). Taken together, these results indicate that p200RhoGAP preferentially regulates RhoA and Rac1 activity through the catalytic GAP domain in cells.

The Effects of the GAP Activity of p200RhoGAP on the Actin Structure of Fibroblasts—To investigate the cellular role of p200RhoGAP, the RhoGAP domain was expressed in Swiss 3T3 fibroblasts, and the effect on actin cytoskeleton was examined by staining actin filaments with TRITC-conjugated phalloidin. An expression vector that bicistronically expresses GFP as a marker was used to track the RhoGAP expressing cells in the culture dish. As shown in Fig. 5, the cells expressing RhoGAP domain became insensitive to lysophosphatidic acid stimulation under conditions that readily induced heavy stress fibers in the GFP expressing cells. When the RhoGAP domain expressing cells were stimulated with PDGF, minimal membrane ruffles were observed under conditions at which the GFP expressing cells displayed significant membrane ruffles on the cell surface (Fig. 5). Further, the RhoGAP domain of p200 did not affect Bradykinin-induced actin microspike formation that is mediated by Cdc42 activation (Fig. 5). These effects are similar to those observed in the case of CdGAP (22), another RhoGAP family member, and are consistent with a negative role of the RhoGAP domain in the regulation of Rac and Rho activities in these cells, because one of direct consequences of the inactivation of RhoA and Rac1 in fibroblasts is loss of stress fibers or cell rounding (1, 12). The effects of the RhoGAP domain, *i.e.* disruption of actin stress fibers and membrane ruffles stimulated by lysophosphatidic acid (LPA) or PDGF,
suggests that it favor RhoA and Rac1 as a substrate in this cellular context.

**Intracellular Localization and Neurite Induction in Neuroblastoma Cells**—Because the p200RhoGAP mRNA was found exclusively in the brain, we wished to determine the role of p200RhoGAP in a neuronal cell line. For this purpose, we raised a polyclonal antibody against p200RhoGAP by using a peptide fragment derived from a unique N-terminal region of the protein (residues 11–25; KQRGLKERVFGCDL) as an immunogen. As shown in Fig. 6A, Western blot analysis confirmed the expression of p200RhoGAP in a neuroblastoma cell line, N1E-115. The endogenous p200RhoGAP migrated on a 4–15% gradient SDS-PAGE as a band greater than 204 kDa, higher than the predicted 200-kDa size, suggesting potential post-translational modifications of this molecule. To examine the intracellular localization of p200RhoGAP, N1E-115 cells were immunostained with anti-p200RhoGAP antibody followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit antibody. In naïve cells, p200RhoGAP was seen predominantly co-stained with cortical actin (Fig. 6B). When the N1E-115 cells were allowed to differentiate by a 24-h serum withdrawal, p200RhoGAP was detected in the ends of neurite extensions, which were also heavy in actin content (Fig. 6B). Moreover, overexpression of the RhoGAP domain or the full-length p200RhoGAP, but not their respective catalytic arginine residue mutants, could induce differentiation of N1E-115 cells (Fig. 7). Therefore, it is possible that p200RhoGAP acts as a Rho GTPase regulator that is involved in the differentiation of neuronal cells during the formation of neurite extensions.

**Interaction of p200RhoGAP with SH3 Domains and Active Src Kinase**—Because the C-terminal region of p200RhoGAP contains multiple proline-rich motifs that confer to the class II SH3 domain binding consensus (Fig. 1C) (21), we examined the possibility that p200RhoGAP may interact with various SH3-containing proteins. To this end, HA-tagged p200RhoGAP was transiently expressed in COS-7 cells, and the cell lysates were incubated with fluorescein isothiocyanate-conjugated anti-rabbit antibody. In naïve cells, p200RhoGAP was seen predominantly co-stained with cortical actin (Fig. 6B). When the N1E-115 cells were allowed to differentiate by a 24-h serum withdrawal, p200RhoGAP was detected in the ends of neurite extensions, which were also heavy in actin content (Fig. 6B). Moreover, overexpression of the RhoGAP domain or the full-length p200RhoGAP, but not their respective catalytic arginine residue mutants, could induce differentiation of N1E-115 cells (Fig. 7). Therefore, it is possible that p200RhoGAP acts as a Rho GTPase regulator that is involved in the differentiation of neuronal cells during the formation of neurite extensions.

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raise the possibility that in response to extracellular stimuli such as nerve growth factor, activated Src or Src-like kinases could recruit p200RhoGAP through the SH3 domain-proline-rich motif interaction that leads to p200RhoGAP phosphorylation, contributing to its regulation.

**DISCUSSION**

The RhoGAP family is defined by the presence of a conserved RhoGAP domain in the primary sequences that consist of ~150 amino acids and share at least 20% sequence identity with other RhoGAPs (5, 6). Previous studies have established that RhoGAP domain is sufficient for the binding to GTP-bound Rho proteins and for accelerating their GTPase activity. To identify novel RhoGAP family members, we have performed a data base search in both cDNA and genomic DNA data banks using the conserved features of RhoGAP domain as a probe. Among several potentially interesting clones, we analyzed the p200RhoGAP sequences in detail and further identified its murine homolog. Further characterization of p200RhoGAP led us to conclude that 1) p200RhoGAP is specifically expressed in the brain; 2) p200RhoGAP is capable of stimulating the GTPase activities of RhoA and Rac1 in *in vivo* and *in vitro*, and a conserved catalytic arginine residue in its RhoGAP domain is necessary for the GAP activity; 3) endogenous p200RhoGAP in N1E-115 cells colocalizes with cortical actin in the undifferentiated state and at the ends of the neurite extension in the differentiated state; 4) overexpression of the RhoGAP domain or full-length of p200RhoGAP induces a differentiation phenotype in N1E-115 cells; and 5) p200RhoGAP binds to class II SH3 domains and is tyrosine-phosphorylated upon association with active Src. Taken together, our studies suggest that this novel RhoGAP is involved in the regulation of neurite outgrowth by exerting its GAP activity and that its cellular activity may be regulated through interaction with Src or Src-like tyrosine kinases.

One of the established physiological roles of Rho GTPases is the regulation of actin cytoskeleton during neuronal migration, axonal growth and guidance, and formation of synapses (11). Consequently, regulators and effectors of Rho GTPases are found to play key roles in neuronal morphogenesis. Recent studies of p190RhGAP-deficient mice showed that this RhoGAP plays an important role in axon outgrowth, guidance and fasciculation, and neuronal morphogenesis (14). In cells of the neural tube floor plate of p190RhoGAP mutant mice, excessive accumulation of polymerized actin were found, suggesting a role in the regulation of Rho-mediated actin assembly within the neuroepithelium (23). p190RhoGAP was co-enriched with F-actin at the distal end of axon, and overexpression of p190RhoGAP induced neurite formation in a neuronal cell line (14). Furthermore, deletion of neuronal adhesion molecules in mice causes similar defects as seen in p190RhoGAP null mice, and p190RhoGAP appears to be one of the major Src kinase substrates in neuron (14). These studies implicate p190RhoGAP in the neuronal development and neurotogenesis by mediating Src-dependent adhesion and Rho GTPase regulation. Such a role is further strengthened by an RNA interference study of p190RhoGAP in *Drosophila*, where blockage of p190RhoGAP leads to the retraction of axonal branches by affecting the RhoA-Drok-MRLC signaling chain (13). The exclusive presence of p200RhoGAP in the brain suggests a neuronal-specific role. Although p200RhoGAP is capable of inducing neurite formation in N1E-115 cells similar to p190, apparently it is non-redundant to p190 in *in vivo*. One way to address the issue of the *in vivo* function of p200RhoGAP would be to decipher its physiological role in neurons through gene targeting approach.

The growing neurites possess growth cones at the tip where both positive and negative molecular guidance cues are detected and by which the path to the target cells is found during neural development. Recently, Wong *et al.* (24) have obtained compelling evidence showing the involvement of a RhoGAP in the intracellular signaling pathway connecting the extracellular guidance cue to the actin cytoskeleton in neuronal cells. The Slit proteins, by binding to the Robo receptor, control the migration of neurons by repelling axons and migrating neurons. One of the Robo cytoplasmic domain interacting proteins is Slit-Robo GAP (srGAP). Binding of Slit to Robo leads to the activation of srGAP, which in turn inactivates Cdc42. The differential cellular localization of srGAP induced by recruitment to the Robo receptor could generate a gradient of Cdc42 activity and uneven actin polymerization, providing a mechanism of Slit-initiated repulsive effects in neuronal migration (24). It will be of particular interest to see whether p200RhoGAP is involved in a similar manner in modulating Rho and/or Rac activity in response to neurotransmitters and/or growth or differentiation factors such as nerve growth factor and to further examine whether it may have a role in neuronal guidance.

RhoGAP may also play essential roles in the neuronal synaptic transmission. Nadrin, a neuron-specific RhoGAP, is involved in the regulation of Ca2+-dependent exocytosis (25). Nadrin is colocalized in the neurite termini with cortical actin filaments. It has been proposed that Nadrin could regulate Rho GTPases to disassemble cortical actin filaments for the regulated exocytosis through its RhoGAP activity. Given the similar intracellular distribution pattern of p200RhoGAP to Nadrin, it will be of interest to determine whether, like Nadrin, p200RhoGAP could be involved in regulating neurotransmitter production.

Although we have observed potent GAP activity of the RhoGAP domain of p200RhoGAP toward RhoA, Rac1, and Cdc42 in purified protein assay systems (Fig. 3), we could detect the GAP activity on RhoA and Rac1, but not Cdc42, in COS-7 and Swiss 3T3 cells (see Figs. 4 and 5). Further, the actin stress fiber disassembly and neuronal differentiation phenotype induced by p200RhoGAP overexpression are consistent with a Rho-specific GAP activity by p200RhoGAP. Although the differences between the RhoGAP domain and full-length molecule may be explained by the potential regulatory role of the C terminus of p200RhoGAP (discussed below), the cause for the difference between the specificity of RhoGAP domain action in *in vitro* and in *in vivo* discrepances in GAP specificity were seen previously in the cases of the RhoGAP domain of Cdc42GAP and p190RhoGAP, which displayed a predominantly Rho-specific GAP activity in cells but acted well as a GAP on Cdc42 in *in vitro* (12).

It appears that many RhoGAPs, including p200RhoGAP, contain SH3 domain or SH3 domain binding motifs that may contribute to the regulation of the RhoGAP activity. Previously Cdc42GAP and p85α were shown to interact with multiple signaling SH3 domains through the proline-rich motifs (26, 27). CdGAP, possibly through its multiple proline-rich sequences, binds to the endocytic protein Intersectin (28). Graf and PS-GAP may interact with FAK or proline-rich tyrosine kinase 2 with their respective SH3 domains (29–31). Given the ability of p200RhoGAP to interact with multiple SH3 domains, one working hypothesis we are pursuing is that the proline-rich motifs in the C-terminal region of p200RhoGAP may be involved in connecting to the Src, Crk, PLCγ, or phosphatidylinositol 3-kinase network.

Accumulating evidence indicates that RhoGAP activities can be modulated by protein kinases (6). One prominent example is the p190RhoGAP regulation by Src family tyrosine kinases (32,
33). Activation of Src in cells leads to phosphorylation of tyrosines 1087 or 1105 of p190RhoGAP that are located close to the RhoGAP domain (34, 35). Upon phosphorylation, p190RhoGAP recruits p120RasGAP through an SH2-phosphotyrosine interaction (36). This effectively activates the Rho-specific GAP activity of p190, causing disruption of actin stress fibers, reduction of focal contacts, and impairing the ability of the cell to adhere to fibronectin, the cellular effects consistent with decreased active Rho GTPase species in cells. In this context, our observation that p200RhoGAP binds to active Src in cells and becomes tyrosine-phosphorylated upon association with active Src may provide an analogy to the case of p190 regulation by Src, although the initial recognition of p200RhoGAP by Src is likely through a proline-rich motif-SH3 domain interaction instead of the phosphotyrosine-SH2 domain interaction. It remains to be seen whether Src SH3 domain binding and/or phosphorylation by the activated Src may lead to an enhanced GAP biochemical activity of p200RhoGAP. However, it is possible that this interaction and modification simply alter the intracellular localization pattern of p200RhoGAP to fine-tune the local Rho GTPase activity in neuronal cells.

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