RA-RhoGAP, Rap-activated Rho GTPase-activating Protein Implicated in Neurite Outgrowth through Rho*1

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Rap1 and Rho small G proteins have been implicated in the neurite outgrowth, but the functional relationship between Rap1 and Rho in the neurite outgrowth remains to be established. Here we identified a potent Rap GTP-activating protein (GAP), RA-RhoGAP, as a direct downstream target of Rap1 in the neurite outgrowth. RA-RhoGAP has the RA and GAP domains and showed GAP activity specific for Rho, which was enhanced by the binding of the GTP-bound active form of Rap1 to the RA domain. Overexpression of RA-RhoGAP induced inactivation of Rho for promoting the neurite outgrowth in a Rap1-dependent manner. Knockdown of RA-RhoGAP reduced the Rap1-induced neurite outgrowth. These results indicate that RA-RhoGAP transduces a signal from Rap1 to Rho and regulates the neurite outgrowth.

Formation and extension of axons and dendrites, so-called neurite outgrowth, is a crucial event in neuronal differentiation and maturation during development of the nervous system (1, 2). As neurites extend further and acquire their final axonal and dendritic identities, neurons establish synaptic contacts and reach full maturation (3, 4). These morphological changes require reorganization of the neuronal actin and microtubule networks and its accompanying membrane expansion and contraction (5, 6). Regulation of actin dynamics via particular signaling pathways is known to be a crucial mechanism directing the morphological development of neurons, including axon growth, guidance, and branching.

One such group of actin-regulating pathway is directed by Rho small G protein (7). Overexpression of a constitutively active mutant of RhoA has been shown to induce neurite retraction and arrest growth in neuronal cell lines (8, 9) and primary cultured neurons (10, 11). Conversely, inhibition of the RhoA action by the C3 exoenzyme-catalyzed ADP-ribosylation (12) enhances neurite extension and growth cone movement (13, 14). Likewise, inactivation of ROCK, a well characterized downstream effector of Rho, produces a similar effect in cerebellar granule neurons and hippocampal neurons (10, 11). On the other hand, extension of neurites requires the SNARE-dependent fusion of plasmalemmal precursor vesicles with the plasma membrane of growth cones. ROCK activated by Rho phosphorylates syntaxin-1, which increases the affinity of syntaxin-1 for tomosyn and forms a stable complex with tomosyn, resulting in inhibition of the formation of the SNARE complex (15). Tomosyn localizes at the palm of growth cones and inhibits the fusion of the vesicles there, thus promoting transport of the vesicles to the plasma membrane of the leading edges of growth cones. In retraction of neurites, tomosyn localizes at all over the edges of the neurites and inhibits fusion of the vesicles with the plasma membrane. Thus, the capacity of Rho-directed pathways to control actin stability and vesicle transport is fundamental for neurite extension, guidance, and branching. An increase in Rho activity results in reduction of axon extension, guidance, and branching, whereas a decrease in Rho activity enhances axon extension, guidance, and branching. In contrast to these activities of Rho, activation of Rap1 through the angiotensin II receptor and cannabinoid receptors enhances neurite outgrowth in neuronal cell lines (16, 17). Activation of Epac, a Rap1 guanine nucleotide exchange factor (GEF), enhances neurite outgrowth in PC12 cells (18). Moreover, recent studies have shown that Rap1 is involved in the establishment of neuronal polarity and that Rap1 may play a general role as a positional signal and organize cell architecture (19). Rap1 determines which neurite will become an axon and directs the recruitment of Cdc42, which is essential for the formation of molecularly distinct dendrites and axon. For this Rap1- and Cdc42-induced selection of a single neurite to become an axon and to extend rapidly, inactivation of Rho is required for the extension of the axon. Supporting this idea that Rap1 negatively regulates Rho in the regulation of vascular endothelial barrier function, Rap1 has been shown to negatively regulate Rho activity and counteract the Rho-induced disruption of VE-cadherin-mediated cell-cell adhesion (20). However, the molecular mechanism underlying the functional relationship between Rap1 and Rho in the neurite outgrowth remains unknown.

The activities of the Rho family are positively regulated by GEFs and negatively regulated by GTP-activating proteins (GAPs) (21). Translocation of the Rho family between the membrane and the cytosol is regulated by Rho GDP dissociation inhibitors (22). The activities of these regulators are controlled by upstream cell surface receptors for guidance cues or cell adhesion molecules. Several RhoGEFs and RhoGAPs have been shown to be important for the neurite outgrowth (7, 23). However, a clear understanding of what specific regulator(s) of the Rho family, which connects Rap1 and Rho, contributes to the neurite outgrowth is still missing.

We have identified here the KIAA1391 human gene product as a GTPase, guanosine 5’-O-(thiotriphosphate); RBD, Rho-binding domain; Bt2cAMP, dibutyryl cyclic AMP; GFP, green fluorescent protein; RNAi, RNA interference; Pi, phosphatidylinositol; PH, pleckstrin homology.
novel downstream target of Rap1, which has the RA and GAP domains in addition to the PH and annexin-like repeat domains. The KIAA1391 gene is also designated as ARHGAP20 on the advice of the Human Gene Nomenclature Committee. The KIAA1391/ARHGAP20 gene is predicted to be a tumor suppressor gene inactivated by deletion in breast cancer and also by chromosomal translocation in B-cell chronic lymphocytic leukemia (24–26). The mouse and rat counterparts were also deposited in GenBank™ and termed RA-RhoGAP (RhoGAP having the RA domain) (27). However, their biological or biochemical properties have not been studied. We show here that RA-RhoGAP is activated by Rap1 and induces inactivation of Rho, resulting in the neurite outgrowth.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screening**—V12Rap1B, in which a glycine residue (Gly) at 12 amino acid (aa) residue of bovine Rap1B was replaced by a valine (Val), was prepared using the QuikChange site-directed mutagenesis kit (Stratagene). V12Rap1B (aa 1–172) was subcloned into a pBTM116 bait vector; the expressed fusion protein contains a LexA DNA-binding domain. The yeast two-hybrid screening was performed using a human brain cDNA library (Clontech) and *Saccharomyces cerevisiae* strain L40. Approximately 5.7 × 10^6 clones were screened for HIS3 and β-galactosidase expression as described (28).

**Construction of Expression Vectors**—KIAA1391/RA-RhoGAP cDNA was obtained from Dr. Takahiro Nagase at the Kazusa DNA Research Institute (Chiba, Japan). Mammalian expression vectors were constructed in pCMV-FLAG (29) using standard molecular biology methods. Constructs of RA-RhoGAP contained the following aa: pCMV-FLAG-RA-RhoGAP, aa 1–1194; pCMV-FLAG-RA-RhoGAP-ΔRA, aa 200–349 deletion. The cDNA for a GAP activity-defective mutant, RA-RhoGAP-R399A, in which arginine (Arg) at 399 aa residue was replaced to alanine (Ala), was generated using the QuikChange site-directed mutagenesis kit and subcloned into pCMV-FLAG vector. The pEF-BOS-myc-Rap1GAP was constructed (30). The pEF-BOS-myc-Rap1GAP and pCMV-FLAG-RA-RhoGAP-ΔRA-RhoGAP (20) were co-transfected with pEFBOS-myc-RhoA wild-type and various constructs of RA-RhoGAP, and cultured for 24 h. The cells were then washed with 1 ml of ice-cold PBS and lysed inBuffer B (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 0.1% Nonidet P-40, 0.5 mM sodium deoxycholate, 1 mM sodium vanadate). The samples were centrifuged at 100,000 g for 30 min, and the supernatant was collected as the cell lysates. The cell lysate (600 μg of protein) was incubated with GST-retainin Rho-binding domain (RBD) (30 μg) immobilized on glutathione-Sepharose beads (50 μl) for 1 h. After the beads were washed three times with Buffer B, the GTP-bound form of RhoA was detected by immunoblotting with an anti-RhoA mAb (Santa Cruz Biotechnology, Inc.).

**Pull-down Assay for Binding of Rap1 to RA-RhoGAP**—To determine the GTP-dependent and substrate-specific binding of Rap1B to RA-RhoGAP, HEK293 cells were transfected with pCMV-FLAG-RA-RhoGAP or pCMV-FLAG-RA-RhoGAP-ΔRA. After 48-h incubation, the cells were harvested, homogenized in Buffer A (20 mM Tris/HCl at pH 7.6, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mM MgCl₂, and 0.1% Nonidet P-40) containing protease inhibitors (Roche Applied Science), and centrifuged at 100,000 × g for 1 h. The supernatant (the soluble fraction) was incubated with the glutathione-Sepharose beads (Amersham Biosciences) carrying immobilized GST-Rap1B or GST-Ha-Ras preloaded with GTPγS or GDP as described (34). After the beads were extensively washed with Buffer A, the bound proteins were eluted by boiling in the SDS sample buffer (60 mM Tris/HCl at pH 6.7, 3% SDS, 2% 2-mercaptoethanol, and 5% glycerol) for 10 min. The samples were then subjected to SDS-PAGE, followed by immunoblotting with the anti-FLAG mAb.

**Rho GAP Assay**—GAP assay was performed as described (35). Lipid-modified Rap1B, RhoA, Rac1, Cdc42, His-Ra-RhoGAP, His-RA-RhoGAP-ΔRA, His-RA-RhoGAP-R399A, and His-Ra-RhoGAP-ΔRA-R399A proteins were generated in Sf9 cells using the baculovirus expression system as described (36, 37). Lipid-modified RhoA, Rac1, or Cdc42 (3 pmol) was incubated at 30 °C for 10 min in a reaction mixture (10 μl) containing 20 mM Tris/HCl at pH 8.0, 10 mM EDTA, 5 mM MgCl₂, 0.4 mM dithiothreitol, 0.3% CHAPS, and 1.5 μM [γ-32P]GTP (1 × 10⁴ cpm/pmol). The reaction was stopped by adding 2.5 μl of 80 mM MgCl₂. To this mixture (12.5 μl), the sample containing His-Ra-RhoGAP (0.1 pmol) and the GDPβS- or GTPγS-bound form of Rap1B (0.5 pmol) was added in a total volume of 50 μl and further incubated at 30 °C for the indicated periods of time. The mixture was applied to a nitrocellulose filter, and the radioactivity retained on the filter was measured by Cerenkov counting.

**Knock-down of RA-RhoGAP by the RNA Interference (RNAi) Method**—The mammalian expression vector, pBS-H1 (38), was used for expression of small interfering RNA in NG108 cells. The following inserts were used: RA-RhoGAP gene-specific insert was a 21-nucleotide sequence corresponding to nucleotides 434–454 (5′-AGAGATACATCGCTCTAGAAA-3′) from the reverse complement of the same 21-nucleotide sequence. A control (Scramble) insert was a 21-nucleotide sequence (5′-TCTGAATTCCTGCAACAACTG-3′) with no significant homology to any mammalian gene sequence, which was separated by a 10-nucleotide non-complementary spacer (5′-TT-
GATATCCG-3' from the reverse complement of the same 21-nucleotide sequence.

RESULTS

Requirement of Inactivation of Rho in the Rap1-induced Neurite Outgrowth—To analyze the functional relationship between Rap1 and Rho in the neurite outgrowth, we first performed gain- and loss-of-function experiments using a neuroblastoma cell line, NG108 cells, which are known to be differentiated into neuron-like cells by the action of dibutyryl cyclic AMP (Bt2cAMP) (39). NG108 cells were co-transfected with GFP (Control) or co-transfected with GFP as a morphological marker along with Myc-V14RhoA (RhoA-CA), or both Myc-V12Rap1B and Myc-V14RhoA (RhoA-CA), cultured in DMEM for 48 h, and allowed to extend neurites. In the lower panels NG108 cells were transfected with GFP (Control) or co-transfected with GFP as a morphological marker along with Myc-Rap1GAP (Rap1GAP) alone or both Myc-Rap1GAP and HA-C3 (C3), cultured in DMEM with 0.6 mM 8CPT-cAMP for 48 h, and allowed to extend neurites. The transfected cells were identified by the expression of GFP (green), and their microtubule structures were examined by immunostaining of β-tubulin (red). Bars, 20 μm. B, quantitative analysis of the neurite outgrowth of the co-transfected NG108 cells. Bb, quantitative analysis of the number of neurite per transfected cell. An average neurite number (± S.D.) of between 40 and 60 analyzed cells. Bb, quantitative analysis of the length of neurite per transfected cell. An average neurite length (± S.D.) of between 40 and 60 analyzed cells. Bc, quantitative analysis of the number of branch tips per neurite of the transfected cells. An average branch tips number (± S.D.) of between 40 and 60 analyzed cells. Asterisks indicate statistical significance (Student’s t test; *, p < 0.01).
8CPT-cAMP-induced neurite outgrowth was reduced by overexpression of Rap1GAP in NG108 cells, and this inhibitory effect of Rap1GAP was rescued by co-expression with C3. To neglect the possibility that the rescue effect of C3 (inactivation of Rho) on the neurite outgrowth is attributable to activation of Rap1, Rac, or Cdc42, we examined the effect of inactivation of Rap1, Rac, or Cdc42 on the C3-induced neurite outgrowth. Co-expression of Rap1GAP, an enzyme hydrolyzing the GTP-bound form of Rap1 and inactivating it, did not affect the C3-induced neurite outgrowth (supplemental Fig. 1). Co-expression of a dominant negative mutant of Rac did not affect the C3-induced neurite outgrowth. Co-expression of N-WASP-CRIB, a specific inhibitor of the GTP-bound form of Rho.
Cdc42, did not affect the C3-induced neurite outgrowth. Thus, the rescue effect of C3 on the neurite outgrowth is mediated by inactivating Rho, not by activating Rap1, Rac, or Cdc42, in NG108 cells. These results indicate that the Rap1-induced neurite outgrowth requires inactivation of Rho, and Rap1 may be an upstream regulator of Rho in the neurite outgrowth.

Identification of RA-RhoGAP as a Connector between Rap1 and Rho—To identify a molecule which connects these two small G proteins, we performed the yeast two-hybrid screening using a human brain cDNA library with Rap1B-CA as a bait. We isolated several clones, one of which encoded a partial fragment (aa 96–382) of a human KIAA1391/ARHGAP20 gene, which has the RA and GAP domains in

FIGURE 4. Enhancement of GAP activity of RA-RhoGAP by Rap1. A, schematic structure of RA-RhoGAP and its truncated and point mutants. B, substrate specificity of RA-RhoGAP. [γ-32P]GTP bound to Cdc42, Rac1, or RhoA was incubated with various concentrations of recombinant His-RA-RhoGAP protein. After incubation, the hydrolysis of [γ-32P]GTP bound to Cdc42, Rac1, or RhoA was assayed by measuring the radioactivity of [γ-32P]GTP bound to each small G protein using the nitrocellulose-filtration method. C, enhancement of GAP activity of RA-RhoGAP by Rap1. [γ-32P]GTP bound to RhoA was incubated with 2 nM recombinant His-RA-RhoGAP protein with or without 10 nM GDP-bound or GTP-bound form of Rap1B, recombinant His-RA-RhoGAP-ΔRA protein with or without 10 nM GDP-bound or GTP-bound form of Rap1B, recombinant His-RA-RhoGAP-R399A protein, recombinant His-RA-RhoGAP-ΔRA-R399A protein, or buffer (Control) for the indicated periods of time. After incubation, the hydrolysis of [γ-32P]GTP bound to RhoA was assayed by measuring the radioactivity of [γ-32P]GTP bound to RhoA, using the nitrocellulose-filtration method. The results shown are representative of three independent experiments.
addition to the PH and annexin-like repeat domains (24) (Fig. 2A). The mouse and rat counterparts were also deposited in GenBank™ and termed RA-RhoGAP (RhoGAP having the RA domain) (27). However, their biological or biochemical properties have not been studied. We used the same terminology but RA-RhoGAP means Rap-activated RhoGAP.

We then confirmed the binding of Rap1 to RA-RhoGAP in a pull-down assay using recombinant proteins. FLAG-tagged full-length RA-RhoGAP (FLAG-RA-RhoGAP) bound to the GST fusion protein of Rap1B (GST-Rap1B) but not to the GST fusion protein of Ha-Ras (GST-Ha-Ras) (Fig. 2B). FLAG-RA-RhoGAP bound the GTPγS-bound form of GST-Rap1B preferentially to the GDP-bound form of GST-Rap1B. To confirm the binding of Rap1B to RA-RhoGAP through the RA domain, we generated the RA domain-truncated mutant of RA-RhoGAP, RA-RhoGAP-ΔRA, as shown in Fig. 2A. RA-RhoGAP-ΔRA did not bind either the GTPγS-bound form of GST-Rap1B or the GDP-bound form of GST-Rap1B. These results indicate that RA-RhoGAP binds Rap1 specifically through the RA domain and that the binding is GTP-dependent.

**Tissue Distribution and Localization of RA-RhoGAP in the Growth Cone of the Neurite**—We made an anti-RA-RhoGAP pAb and examined the tissue distribution of RA-RhoGAP in rat by use of this pAb. Immunoblot analysis revealed that RA-RhoGAP was dominantly expressed in the brain, the liver, and the testis (Fig. 3A). We next examined the localization of RA-RhoGAP in Bt2cAMP-induced differentiated NG108 cells. NG108 cells were fixed after being cultured in DMEM for 24 h and stained with the combinations of the anti-RA-RhoGAP pAb and an anti-β-tubulin mAb. The immunofluorescence signal for RA-RhoGAP was found in the cell body and the growth cones of neurites (Fig. 3B).

**Enhancement of GAP Activity of RA-RhoGAP by Rap1**—To examine whether RA-RhoGAP indeed shows RhoGAP activity, we first generated recombinant proteins of RA-RhoGAP and its truncated and point mutants as shown in Fig. 4A. His-tagged full-length RA-RhoGAP (His-
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RA-RhoGAP showed GAP activity toward RhoA, while it showed much less effect on Rac or Cdc42 (Fig. 4B). The GTPys-bound form of Rap1B enhanced the RA-RhoGAP activity, whereas the GDPβS-bound form of Rap1B was less effective (Fig. 4C). The apparent $K_a$ value of the GTPys-bound form of Rap1B for His-RA-RhoGAP (the concentration necessary for the half-maximal activation) was about 10 nM (data not shown). These results indicate that RA-RhoGAP is a direct downstream target of Rap1. In addition, His-RA-RhoGAP-ΔRA showed more potent RhoGAP activity than wild-type His-RA-RhoGAP. The activity of His-RA-RhoGAP-ΔRA was similar to that of wild-type His-RA-RhoGAP which bound the GTPys-bound form of Rap1B. In addition, the GAP activity of RA-RhoGAP-ΔRA was not affected by the presence of the GTPys-bound form of Rap1B. To confirm the GAP activity of RA-RhoGAP through the RhoGAP domain, we generated a GAP activity-defective mutant, RA-RhoGAP-R399A, as shown in Fig. 4A. Both His-RA-RhoGAP-R399A and His-RA-RhoGAP-ΔRA-R399A abolished their GAP activity toward RhoA. These results suggest that the RA domain inhibits the GAP activity of the GAP domain and that when the RA domain binds Rap1, this inhibitory effect is released to induce the full activation of RA-RhoGAP.

Involvement of RA-RhoGAP in the Neurite Outgrowth in a Manner Dependent on its GAP Activity—We then examined whether RA-RhoGAP is indeed involved in the neurite outgrowth regulated by Rap1 and Rho in NG108 cells. Expression of RA-RhoGAP enhanced neurite outgrowth (Fig. 5A). Statistically, the number, length, and branching of neurites of the cells transfected with RA-RhoGAP were more than those of the control cells transfected with GFP alone (Fig. 5B). The RA-RhoGAP-induced neurite outgrowth was further enhanced by co-expression with Rap1B-CA. These effects of Rap1B-CA and RA-RhoGAP are selectively on undifferentiated NG108 cells but not on differentiated NG108 cells already bearing neurites (supplemental Fig. 2). Rap1 and RA-RhoGAP seem to be mainly involved in the early phase of neurite formation (neurite sprouting) in the undifferentiated cells. Expression of RA-RhoGAP-ΔRA enhanced neurite outgrowth to extents more than those induced by wild-type RA-RhoGAP alone and similar to those induced by wild-type RA-RhoGAP and Rap1B-CA. Expression of RA-RhoGAP-ΔRA-R399A did not enhance the neurite outgrowth. The effect of RA-RhoGAP on the neurite outgrowth is dependent on its GAP activity.

Under these conditions, we performed Rho-GTP pull-down assays to further confirm the level of the GTP-bound form of RhoA altered in the...
presence of Rap1B-CA, RA-RhoGAP, or both together in vivo. We carefully titrated RA-RhoGAP expression levels of each sample and performed the pull-down assay. Rap1B-CA alone was sufficient to cause a decrease in the amount of the GTP-bound form of RhoA in NG108 cells (Fig. 6). RA-RhoGAP alone caused a significant decrease in the amount of the GTP-bound form of RhoA. RA-RhoGAP together with Rap1B-CA caused a decrease in the amount of the GTP-bound form of RhoA more potently than either RA-RhoGAP or Rap1B-CA alone. RA-RhoGAP-ΔRA-R399A indeed abolished its GAP activity toward RhoA. These results are consistent with those of the cell level experiments as shown in Fig. 5. These results indicate that RA-RhoGAP is involved in the neurite outgrowth dependent on its GAP activity which is regulated by Rap1.

Involvement of RA-RhoGAP in the 8CPT-cAMP-induced Neurite Outgrowth—To confirm that RA-RhoGAP is involved in the 8CPT-cAMP-induced neurite outgrowth, we examined the effect of RA-RhoGAP knock-down by the RNAi method. We knocked down endogenous RA-RhoGAP by the RNAi method (Fig. 8Aa). RA-RhoGAP knocked down cells reduced the Rap1B-CA-induced neurite outgrowth (Fig. 8Ab). Statistically, the number, length, and branching of neurites of the cells transfected with RA-RhoGAP siRNA and Rap1B-CA were less than those of the control cells transfected with scramble RNA and Rap1B-CA (Fig. 8B). Taken together, these results indicate that RA-RhoGAP transduces a signal from Rap1 to Rho and is involved in the neurite outgrowth regulated by Rap1 and Rho.

DISCUSSION

Many downstream targets of Rap1 have been identified: they include Raf1, B-Raf, RafGDS, afadin/AF-6, PI3-kinase, and RAPL (40–43). Raf and B-Raf are protein kinases connecting Ras small G protein to ERK; RafGDS is a regulator of another small G protein Raf; afadin/AF-6 is an actin filament- and nectin-binding protein at adherens junctions; PI3-kinase is a phosphatidylinositol kinase, and RAPL is a small protein that transduces a signal from Rap1 to integrin. In this study, we have identified RA-RhoGAP as another direct downstream target of Rap1. RA-RhoGAP has the RA and GAP domains in addition to the PH and annexin-like repeat domains. It indeed shows a GAP activity specific for Rap1B-CA, RA-RhoGAP, or both together in vivo. We carefully titrated RA-RhoGAP expression levels of each sample and performed the pull-down assay. Rap1B-CA alone was sufficient to cause a decrease in the amount of the GTP-bound form of RhoA in NG108 cells (Fig. 6). RA-RhoGAP alone caused a significant decrease in the amount of the GTP-bound form of RhoA. RA-RhoGAP together with Rap1B-CA caused a decrease in the amount of the GTP-bound form of RhoA more potently than either RA-RhoGAP or Rap1B-CA alone. RA-RhoGAP-ΔRA-R399A indeed abolished its GAP activity toward RhoA. These results are consistent with those of the cell level experiments as shown in Fig. 5. These results indicate that RA-RhoGAP is involved in the neurite outgrowth dependent on its GAP activity which is regulated by Rap1.

Involvement of RA-RhoGAP in the Neurite Outgrowth Downstream of Rap1—In the last set of experiments, to further confirm that RA-RhoGAP is involved in the neurite outgrowth downstream of Rap1, we performed a loss-of-function analysis by use of the RNAi method. We knocked down endogenous RA-RhoGAP by the RNAi method (Fig. 8Aa). RA-RhoGAP knocked down cells reduced the Rap1B-CA-induced neurite outgrowth (Fig. 8Ab). Statistically, the number, length, and branching of neurites of the cells transfected with RA-RhoGAP siRNA and Rap1B-CA were less than those of the control cells transfected with scramble RNA and Rap1B-CA (Fig. 8B). Taken together, these results indicate that RA-RhoGAP transduces a signal from Rap1 to Rho and is involved in the neurite outgrowth regulated by Rap1 and Rho.

FIGURE 8. Involvement of RA-RhoGAP in the neurite outgrowth downstream of Rap1. A, effect of RA-RhoGAP knock-down by the RNAi method. NG108 cells were transfected with scramble RNA (Scramble) or RA-RhoGAP siRNA. The whole cell lysates (50% transfection efficiency) were subjected to SDS-PAGE, followed by immunoblotting with the anti-RA-RhoGAP pAb and the anti-actin mAb. The immunoblot band intensity was quantified in a lower panel. B, NG108 cells were co-transfected with GFP as a morphological marker along with both scramble RNA (Scramble) and FLAG-Rap1B-CA or both RA-RhoGAP siRNA and FLAG-Rap1B-CA, cultured in DMEM for 48 h, and allowed to extend neurites. The cells expressing the RA-RhoGAP siRNA or the scramble RNA were identified by the expression of GFP (green). Their microtubule structures were examined by immunostaining of β-tubulin (red). Bars, 20 μm. a, quantitative analysis of the neurite outgrowth of the co-transfected NG108 cells. a, quantitative analysis of the number of neurite per transfected cell as in Fig. 1Ba. b, quantitative analysis of the length of neurite per transfected cell as in Fig. 1Bb. c, quantitative analysis of the number of branch tips per neurite of the transfected cells as in Fig. 1Bc. Asterisks indicate statistical significance (Student’s t test; *, p < 0.01).
Rho and this GAP (the Rap1 activity) is enhanced by the GTP-bound form of Rap1. The RA domain (the Rap1-binding region)-deficient truncated form of RA-RhoGAP, RA-RhoGAP-ΔRA, shows more potent RhoGAP activity than the wild type, suggesting that the RA domain negatively regulates the RhoGAP activity. In the steady state, RA-RhoGAP is likely to form a closed conformation by an intramolecular interaction between the RA domain and the RhoGAP domain and thereby negatively regulates the RhoGAP activity. In the active state, the binding of Rap1 to the RA domain may reduce the intramolecular interaction and stabilize an open conformation of RA-RhoGAP.

We have then shown here that RA-RhoGAP is dominantly expressed in the brain and localizes at the growth cones of extending neurites of NG108 cells and that RA-RhoGAP indeed transduces a signal from Rap1 to Rho and inactivates Rho. It has been shown that repression of the Rho-mediated signaling pathway induces rapid actin depolymerization of the growth cone and thereby extends the incipient neurite further (13, 14). Consistently, we have shown here that the Rap1-induced, RA-RhoGAP-mediated inactivation of Rho enhances neurite outgrowth of NG108 cells. It has been proposed that the localization and activation of Rap1B determine which neurite will be a single elongated neurite formation, a neurite formation signal(s) may recruit and activate the Rap1-RA-RhoGAP-Rho system, eventually allowing neurite formation (13), 14). Thus, the Rap1-RA-RhoGAP-Rho system could serve as a positional signal in neurite outgrowth.

Rap1 is activated by RapGEFs, and many RapGEFs have been identified: they include C3G regulated by Crk adaptor protein, Epacs regulated by cAMP, CalDAG-GEFs regulated by diacylglycerol and Ca²⁺, and PDZ-GEFs regulated by unknown factors (40). It remains unknown which RapGEF is involved in the activation of Rap1 to induce the neurite outgrowth, but the recruitment of a Rap1GEF to the growth cone may be required primarily. It remains unknown which RapGEF is involved in the activation of Rap1 to induce the neurite outgrowth of NG108 cells. It has been proposed that the localization and activation of Rap1B determine which neurite will be a single elongated neurite formation, a neurite formation signal(s) may recruit and activate the Rap1-RA-RhoGAP-Rho system, eventually allowing neurite formation (13). For the case of RA-GEF/PDZ-GEF, the localization of RA-RhoGAP at the growth cones of neurites and the cell body in NG108 cells (supplemental Fig. 3). The localization of expressed RA-RhoGAP containing proteins there. After the PI 3-kinase activation at the growth cone, resulting in recruiting the group of PH domain-containing proteins there. After the PI 3-kinase activation at the growth cone may be required primarily. It remains unknown which RapGEF is involved in the activation of Rap1 to induce the neurite outgrowth.

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