The Rho family of small GTPases has been shown to be involved in the regulation of neuronal morphology, and Rac and Rho exert antagonistic actions in neurite formation. In this study, we have examined the cross-talk between Rac and Rho in relation to the nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells. NGF induced a rapid activation of Rac1 and suppression of RhoA activity. Constitutively active RhoA, RhoAV14, or constitutively active Gαs-induced endogenous RhoA activation inhibited the NGF-induced Rac1 activation without any effect on the NGF-induced extracellular signal-regulated kinase activation. Moreover, Y-27632, an inhibitor of Rho-associated kinase, completely abolished the RhoA-induced down-regulation of the NGF-induced Rac1 activation. We also revealed that NGF induced a rapid recruitment of Rac1 to the cell surface protrusion sites and formed filamentous actin-rich protrusions. Activation of RhoA and Rho-associated kinase formed a thick ringlike structure of cortical actin filaments at the cell periphery and then inhibited the NGF-induced recruitment of Rac1 to protrusions. These results indicate that RhoA down-regulates the NGF-induced Rac1 activation through Rho-associated kinase, inhibiting the neurite formation.

In the developing nervous system, formation of appropriate connections between neurons is an essential process for the establishment and maturation of neural circuits. Neurite extension and retraction are very important processes in the formation of neuronal networks, and these dynamic morphological changes of neuronal cells are largely decided by the actin cytoskeletal organization (1). The Rho family of small GTPases, consisting of Cdc42, Rac, and Rho, has been implicated in the reorganization of the actin cytoskeleton and subsequent morphological changes in various cellular functions (2). Among them, Rac is involved in membrane ruffling and formation of lamellipodia, whereas Rho is responsible for regulating the assembly of focal adhesion and stress fiber formation in fibroblasts (3, 4). Similar to other GTPases of the Ras superfamily, they serve as a molecular switch by cycling between an inactive GDP-bound state and an active GTP-bound state. Activation of the Rho family proteins requires GDP-GTP exchange catalyzed by various guanine nucleotide exchange factors, and their activation is regulated by GTPase-activating proteins, which stimulate the intrinsic GTPase activities of the G proteins, leading to the cessation of their actions. In addition, guanine nucleotide dissociation inhibitors inhibit the exchange of GDP for GTP and might also serve to regulate their association with membranes (5). In neuronal cells, Rho family proteins are involved in axon and dendrite formation in various types of neurons (6–9), and effects in the regulation of these GTPase activities have been reported to affect the development of the nervous system (10–13). Rac has been shown to be involved in the formation of lamellipodia of a growth cone and to be required for the outgrowth of neurites (14). On the other hand, activation of Rho was reported to induce the collapse of the growth cone and the retraction of neurites and to inhibit the neurite outgrowth (14–16). As mentioned above, Rac and Rho are thought to counteract each other’s activity in neuronal cells (14, 17). Therefore, the balance between Rac and Rho activities is likely to be a crucial point for neuronal morphology. The cross-talk between Rac and Rho has been studied in a variety of cell lines, and the physiological significance of the cross-talk has been established in various cellular functions (18, 19). However, little is yet known about the regulatory mechanism of the cross-talk between Rac and Rho.

Rat pheochromocytoma PC12 cells have been used as a model system for neuronal differentiation and neurite outgrowth. After stimulation with nerve growth factor (NGF), the cells stop growing and begin to extend neurites. Rac plays an important role in the regulation of the cytoskeletal changes required for neurite outgrowth in response to NGF, whereas Rho has been shown to inhibit neurite outgrowth by NGF (16). In this study, we have examined the cross-talk between Rac and Rho in PC12 cells, and we showed that NGF induced the Rac1 activation but also the inhibition of RhoA activity and that the activation of RhoA down-regulated the NGF-induced Rac1 activation via Rho-associated kinase.

### EXPERIMENTAL PROCEDURES

**Materials**—Y-27632 was a generous gift from Yoshitomi Pharmaceutical Industries (Saitama, Japan). Agents obtained and commercial sources were as follows: NGF, Promega; mouse monoclonal anti-Rac1 antibody, Transduction Laboratories; mouse monoclonal anti-Rho antibody, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); mouse monoclonal anti-hemagglutinin (HA) antibody (clone 12CA5), Roche Molecular Biochemicals; rabbit antiphosphospecific extracellular signal-regulated kinase (ERK) antibody, New England Biolabs, Inc.; rabbit anti-ERK1 and mouse anti-ERK2 antibodies, Upstate Biotechnology, Inc.

Received for publication, January 11, 2001, and in revised form, March 8, 2001
Published, JBC Papers in Press, March 15, 2001, DOI 10.1074/jbc.M100254200
Inc. (Lake Placid, NY); Alexa 488-conjugated phalloidin and rhodamine-conjugated phalloidin, Molecular Probes, Inc. (Eugene, OR); rhodamine-conjugated donkey anti-mouse immunoglobulin G (IgG) antibody, Chemicon International Inc.; horseradish peroxidase-conjugated goat anti-mouse IgG antibody and horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody, DAKO; and chemiluminescence ECL Western blotting system, Amersham Pharmacia Biotech. Mammalian expression vector pcDNA3 carrying cDNA for a variant of the Aequorea victoria green fluorescent protein (GFP) was obtained as described previously (20). cDNA for the catalytic domain of Rho-associated kinase, ROKa (CD-ROKα; Ref. 15), was inserted into mammalian expression vector pEF-BOS. The sources of other materials are as indicated.

Cell Culture and Transfection—PC12 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% horse serum, 5% fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin, and 0.2 mg/ml streptomycin under humidified conditions in 95% air and 5% CO2 at 37 °C. Inhibition of the NGF-induced Rac1 Activation by RhoA

After residual formaldehyde had been quenched with 50 mM NH4Cl in PBS, cells on coverslips were fixed with 3.7% formaldehyde/PBS for 20 min. Immunofluorescence Microscopy—PC12 cells were seeded onto poly-D-lysine-coated glass coverslips in 24-well plates at a density of 1 x 10⁴ cells/well and cultured for 15–18 h. Then cells were transfected with 0.8 μg of total DNA using LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer’s instructions. Cells were fixed 24 h after transfection.

Serum-starved PC12 cells were treated with 50 ng/ml NGF for the indicated times. After the beads had been washed with the respective ice-cold lysis buffers (Rac1 activity assay, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM MgCl₂, 0.1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, and 1 μg/ml leupeptin; RhoA activity assay, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 30 mM MgCl₂, 0.1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, and 1 μg/ml leupeptin; ERK activity assay, 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2.5 mM MgCl₂, 10 mM NaF, 1 mM Na₃VO₄, 10% glycerol, 1% Nonidet P-40, 250 μM phenylmethylsulfonyl fluoride, 1 μM aprotinin, and 1 μg/ml leupeptin). The cell lysates were immediately centrifuged for 5 min at 10,000 × g at 4 °C, and the supernatants were separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto a polyvinylidene difluoride membrane (Millipore Corp.). The membrane was blocked with 3% low fat milk in Tris-buffered saline and then incubated at 4 °C with primary antibodies. The immunoblots were detected using a chemiluminescence ECL Western blotting system with horseradish peroxidase-conjugated secondary antibodies.

Effects of NGF on Rac1 and RhoA Activities—Serum-starved PC12 cells were treated with 50 ng/ml NGF for the indicated times. A, the cell lysates were incubated with GST-CRIB, and the amounts of GTP-bound Rac1 were determined by immunoblotting using a monoclonal antibody against Rac1 (inset). Total amounts of Rac1 in cell lysates are also shown. B, the cell lysates were incubated with GST-RBD, and the amounts of GTP-bound RhoA were determined by immunoblotting using a monoclonal antibody against RhoA (inset). Total amounts of RhoA in cell lysates are also shown. Rac1 and RhoA activities are indicated by the amounts of GTP-bound Rac1 and GTP-bound RhoA normalized to the amounts of Rac1 and RhoA in whole cell lysates, respectively, and values are expressed as fold of the value of serum-starved cells at time 0 min. The results shown are the means ± S.E. of triplicate experiments.

RESULTS

Effects of NGF on Rac1 and RhoA Activities—Rac has been shown to be involved in the NGF-induced neurite outgrowth (17, 22), while active Rho has been reported to prevent the NGF-induced neuritogenesis (16). To assess more directly modulation of Rac1 and RhoA activities by NGF, we measured the amounts of cellular GTP-bound Rac1 and RhoA using the GST-fused CRIB domain of RhoA, which has been shown to be involved in the NGF-induced neurite outgrowth (15, 22, 23). Serum-starved PC12 cells were treated with 50 ng/ml NGF for the indicated times. A, the cell lysates were incubated with GST-CRIB, and the amounts of GTP-bound Rac1 and RhoA were determined by immunoblotting using a monoclonal antibody against Rac1 (inset). Total amounts of Rac1 in cell lysates are also shown. B, the cell lysates were incubated with GST-RBD, and the amounts of GTP-bound RhoA were determined by immunoblotting using a monoclonal antibody against RhoA (inset). Total amounts of RhoA in cell lysates are also shown. Rac1 and RhoA activities are indicated by the amounts of GTP-bound Rac1 and GTP-bound RhoA normalized to the amounts of Rac1 and RhoA in whole cell lysates, respectively, and values are expressed as fold of the value of serum-starved cells at time 0 min. The results shown are the means ± S.E. of triplicate experiments.

Inhibition of NGF-induced Rac1 Activation by RhoA—Active Rho has been known to prevent NGF-induced neuritogenesis (16). We next examined the effect of RhoA activation on NGF-induced Rac1 activation, using the PC12 cell line expressing constitutively active HA-tagged RhoA, HA-RhoAV14, under the control of IPTG, which we had previously established (referred
The expression of RhoAV14 by IPTG completely inhibited the NGF-induced Rac1 activation without any effect on the basal Rac1 activity in RhoAV14-inducible PC12 cells (Fig. 2). We recently demonstrated that the Ga12 family of heterotrimeric G proteins induced activation of endogenous RhoA, causing neurite retraction in PC12 cells (21). We then examined the effect of endogenous RhoA activation on the NGF-induced Rac1 activation using the PC12 cell line expressing constitutively active Ga12, Ga12QL, under the control of IPTG (21). The expression of Ga12QL by IPTG completely inhibited the NGF-induced Rac1 activation (Fig. 3), indicating that the RhoA activation pathway is a negative regulator of the NGF-induced Rac1 activation.

Recent studies have shown that cell adhesion to the extracellular matrix induces activation of Rac (23, 24). On the other hand, activation of Rho has been shown to induce cell rounding in PC12 cells, rendering the cells less adherent (21, 25). Therefore, down-regulation of the NGF-induced Rac1 activation by RhoAV14 might be a secondary effect of RhoAV14-induced reduction of cell adhesion activity. To address this issue, we examined the effect of RhoAV14 on the NGF-induced Rac1 activation in suspended RhoAV14-inducible PC12 cells. NGF induced a rapid increase in the amount of GTP-Rac1 in suspended PC12 cells as well, but the level reached a maximum at 1 min (data not shown). As shown in Fig. 4, the expression of RhoAV14 by IPTG inhibited the NGF-induced Rac1 activation in the suspended PC12 cells. Thus, the RhoAV14-induced down-regulation occurred irrespective of the cell adhesion activity.

NGF has been known to differentiate PC12 cells through the ERK signal transduction pathway (26, 27). RhoAV14-induced down-regulation of the Rac1 activation might result from perturbation of the NGF-induced ERK activation. To exclude this possibility, we examined the effect of RhoAV14 on the NGF-induced ERK activation by visualizing phosphorylation of endogenous ERK with an antiphospho-ERK antibody. As shown in Fig. 5, the expression of RhoAV14 by IPTG did not affect the NGF-induced ERK activation in RhoAV14-inducible PC12 cells, indicating that the RhoAV14-induced down-regulation occurred irrespective of the ERK signal transduction pathway.

Involvement of Rho-associated Kinase in RhoAV14-induced Down-regulation of the Rac1 Activation—Rho-associated kinase, one of the downstream targets of RhoA, has been shown to be involved in a variety of actions of Rho (28). To determine whether Rho-associated kinase was involved in the RhoAV14-
induced down-regulation of the Rac1 activation, the cells were treated with a Rho-associated kinase-selective inhibitor, Y-27632 (29). As shown in Fig. 6, Y-27632 had little effect on the basal and NGF-induced Rac1 activation. However, Y-27632 completely abolished the RhoAV14-induced down-regulation of the Rac1 activation in both adherent and suspended RhoAV14-inducible PC12 cells. In addition, Y-27632 also abolished the Go13QL-induced down-regulation of the Rac1 activation in Go13QL-inducible PC12 cells (data not shown). On the other hand, Y-27632 did not affect the time course profile of the NGF-induced Rac1 activation (Fig. 6E). These results indicate that the RhoAV14-induced down-regulation of the Rac1 activation is mediated by Rho-associated kinase.

Inhibition by RhoAV14 and CD-ROKα of NGF-induced Recruitment of Rac1 to Protrusions—We examined the subcellular distribution of Rac1 and F-actin after the stimulation with NGF. Immunofluorescence staining of Rac1 using an anti-Rac1 antibody revealed that in unstimulated RhoAV14-inducible PC12 cells, Rac1 was present mainly in the cytoplasm (Fig. 7).
RhoAV14-inducible PC12 cells were treated with or without 5 mM IPTG for 12 h in the absence or presence of 10 μg/ml NGF for 3 (A) or suspended (B), quantification of the effect of Y-27632 on Rac1 activity in attached (C) or suspended (D) RhoAV14-inducible PC12 cells. The Rac1 activity is indicated by the amount of GST-CRIB-bound Rac1 normalized to the amount of Rac1 in whole cell lysates, and values of Rac1 activity are expressed as fold increase over the value of cells that were not treated with either IPTG or NGF or Y-27632. Data are the means ± S.E. of triplicate experiments. E, after serum-starved PC12 cells had been exposed to vehicle or 10 μg/ml Y-27632, they were treated with 50 ng/ml NGF for the indicated times. The cell lysates were incubated with GST-CRIB, and the amounts of GTP-bound Rac1 were determined by immunoblotting using a monoclonal antibody against Rac1 (upper panels). Total amounts of Rac1 in cell lysates (middle panels) and expression of HA-tagged RhoAV14 (bottom panels) are also shown.

promotes contractility by activation of Rho-associated kinase, leading to neurite retraction, cell rounding, and the inhibition of neurite outgrowth, while activation of Rac enhances cell spreading and neurite extension and interferes with Rho-mediated cell rounding through phosphorylation of the myosin-II heavy chain, suggesting antagonistic roles for Rho and Rac in the control of neuronal morphology (35). In contrast to the signal-convergent system, we here demonstrated that constitutively active RhoA or GTPase-activated Rac1 activation down-regulated the NGF-induced Rac1 activation via a Rho-associated kinase-dependent pathway. This down-regulation of Rac1 activity by RhoA can be categorized into the different regulatory mechanisms, upstream signal regulation, referred to as signal divergence (34). Concerning this system, down-regulation of Rho activity by Rac has been reported in NIH3T3 cells (36). However, this is the first report of down-regulation of Rac1 activity by RhoA. Rho activation induces cell rounding, decreasing cell adhesion activity in PC12 cells (21, 25). However, the RhoA-induced down-regulation of Rac1 activity is not a secondary effect of this RhoA-mediated reduction of cell adhesion activity, because the down-regulation was observed with suspended PC12 cells as well. Therefore, the site of action of RhoA is on a step(s), involved in the NGF-induced Rac1 activation signaling pathway. Rac is a critical component in the signaling pathway of the NGF-induced neurite outgrowth in PC12 cells (37–39), and activation of Ras was shown to induce neurite outgrowth (20, 40, 41). Recently, Rac1 was revealed to act downstream of Ras in neurite outgrowth independent of Ras-mediated ERK activation in N1E-115 neuroblastoma cells (42). Furthermore, NGF was shown to activate ERK in PC12 cells in the presence of RhoAV14 expression (16). We also observed here that active RhoA did not perturb the NGF-induced ERK activation. Therefore, active RhoA may modulate the signaling pathway from Ras to Rac1 but not from Ras to ERK. The activity of Rac1 is known to be regulated by a variety of proteins, such as guanine nucleotide exchange factors, GTPase-activating proteins, and guanine nucleotide dissociation inhibitors (28). A Rac guanine nucleotide exchange factor, Sos, was shown to be implicated in coupling Ras to Rac (43, 44). RhoA may modulate the activities of these molecules, such as Sos. Molecular mechanisms of the RhoA-induced down-regulation of Rac1 activity are currently under investigation in our laboratory.

We further investigated the effect of active RhoA on actin reorganization. We revealed here that NGF recruited Rac1 to the protrusion sites and accumulated F-actin, initiating the neurite formation. Active RhoA inhibited the NGF-induced Rac1 recruitment to the protrusion sites and resultant process formation through Rho-associated kinase. Our result showed that the cytoskeletal action of RhoA and its downstream effector, Rho-associated kinase, was the stabilization of cortical actin filaments in the cell periphery of PC12 cells. Since Rho-associated kinase is known to increase phosphorylation of myosin light chain, consequently elevating contractile activity of myosin, this myosin activation may enhance the stabilization of cortical actin filaments. In neuronal cells, disruption of the cortical actin filament network was shown to induce neurite formation, suggesting that contractile activity by cortical actin
filaments inhibits the neuritogenesis and neurite extension (45, 46). Therefore, Rho-associated kinase-induced formation of the ringlike structure of cortical actin filaments in PC12 cells may interfere with the NGF-induced Rac1 recruitment and F-actin reorganization, which are initial events for the neurite formation. In this study, we showed that Y-27632, a Rho-associated kinase inhibitor, completely abolished the RhoA-induced down-regulation of the NGF-induced Rac1 activation, suggesting that the RhoA-induced down-regulation is mediated by Rho-associated kinase. The inhibition of Rac1 recruitment by Rho-associated kinase may contribute to the RhoA-induced down-regulation of the Rac1 activation.

Studies using a dominant negative Rac1 show that Rac1 is involved in the NGF-induced neurite outgrowth in PC12 cells (17, 22). Furthermore, activation of Rac was shown to produce a loss of contractility associated with cell spreading and the formation of neurite-like extensions in N1E-115 cells (42). On the other hand, active Rho suppresses the NGF-induced neurite outgrowth in PC12 cells (16). Therefore, Rac and Rho exert antagonistic actions in neurite formation. We here demonstrated that NGF activated Rac1 but inversely suppressed the RhoA activity in PC12 cells. Effects of RhoA and Y-27632 appear to be restricted to the NGF-induced Rac1 activation. Although the regulation of this basal Rac1 activity is not understood, the activation of Rac1 by NGF and the basal activity may be regulated by different signaling mechanisms. Suppression of RhoA activity by NGF may enhance the duration of the NGF-induced Rac1 activation. Recently, NGF was shown to abolish Rho activation by binding to the neurotrophin receptor p75 (47). Considering the expression of p75 in PC12 cells (48), the NGF-induced down-regulation of RhoA may be mediated by p75. Furthermore, inactivation of Rho signaling pathway has been reported to promote axon regeneration in the central nervous system (8), and inhibition of the Rho/Rho-associated kinase pathway was suggested to act as a gate critical for the initiation of axonal outgrowth (49). Thus, negative regulation of Rho activity is critical for neurite outgrowth. In sharp contrast to NGF, ephrin-A5 was shown to induce collapse of growth cone by opposite regulation of Rho and Rac, Rac activation and Rac down-regulation (50). Neuronal morphology appears to be critically balanced between Rho and Rac activities.

In conclusion, we demonstrate here that NGF oppositely regulates Rac1 and RhoA activities, activation of Rac1 and suppression of RhoA, and that activation of RhoA blocks the NGF-induced neurite formation by the inhibition of Rac1 activity via Rho-associated kinase in PC12 cells. This work takes

![Figure 7](image1.png)

**FIG. 7.** Effect of RhoA<sup>V14</sup> on the NGF-induced subcellular distribution of endogenous Rac1 and F-actin. Serum-starved RhoA<sup>V14</sup>-inducible PC12 cells were treated with or without 5 mM IPTG for 12 h. The cells stimulated or not stimulated with 50 ng/ml NGF for 3 min were fixed and double-stained with an anti-Rac1 monoclonal antibody (left panels) and Alexa 488-conjugated phalloidin (right panels). The results shown are representative of three independent experiments. *Bar*, 10 μm.

![Figure 8](image2.png)

**FIG. 8.** Effect of CD-ROKα on the NGF-induced subcellular distribution of endogenous Rac1 and F-actin. PC12 cells were transiently transfected with an empty vector (a–d) or an expression vector encoding CD-ROKα (e–h) along with an expression vector encoding GFP. At 24 h after transfection, cells were stimulated with 50 ng/ml NGF for 3 min and were fixed. Transfected cells were identified by the fluorescence of GFP (a, c, e, and g). Cells were stained with an anti-Rac1 monoclonal antibody (b and f) or rhodamine-conjugated phalloidin (d and h). The results shown are representative of three independent experiments. *Bar*, 10 μm.
RhoAV14-induced inhibition of endogenous Rac1 and F-actin distribution. Serum-starved RhoA V14-inducible PC12 cells were treated with or without 5 mM IPTG for 12 h in the presence of 10 µM Y-27632 and F-actin elution.

The results shown are representative of three independent experiments. Bar, 10 µm.

A close-up of an important role of Rho family GTPases in neurite formation and will help to elucidate the molecular mechanism of neurite outgrowth.

Acknowledgments—We thank Yoshitomi Pharmaceutical Industries and Dr. S. Nagata of Osaka University for supplying Y-27632 and pEF-BOS, respectively.

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RhoA Inhibits the Nerve Growth Factor-induced Rac1 Activation through Rho-associated Kinase-dependent Pathway

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J. Biol. Chem. 2001, 276:18977-18983.
doi: 10.1074/jbc.M100254200 originally published online March 15, 2001

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