REGULATION OF RHOA DURING NGF-INDUCED NEURONAL DIFFERENTIATION

Ph.D. Thesis

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Ph.D. program: Biochemistry and Molecular Biology

Ph.D. Subprogram: The Role of ras Protooncogenes in Signal Transduction

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Pécs, 2004

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I. INTRODUCTION

The first Rho protein was identified as a member of the Ras superfamily of monomeric, small GTP-binding proteins. The best studied members of the Rho family, RhoA, Rac1 and Cdc42, gained attention as key regulators of the cytoskeleton and of cellular processes that are dependent on the actin cytoskeleton, such as cytokinesis, phagocytosis, pinocytosis, cell migration, axon guidance. Later, Rho GTPases have been found to also regulate a variety of other biochemical pathways including transcription factors and kinase pathways, G_1 cell-cycle progression, cell transformation and neuronal differentiation (for review see Narumiya 1996).

To understand the mechanism by which Rho family GTPases regulate the cytoskeleton and cell adhesion, enormous effort has been made over the past several years to identify upstream regulators and downstream effectors of the Rho family GTPases. As a result, a number of regulatory proteins and targets have been isolated. Intensive analyses of their function have provided some insight into the modes of activation and action of the Rho family GTPases at the molecular level. Moreover, several studies have been conducted to understand more clearly the role of RhoA in signal transduction pathways that regulate the cytoskeleton and cell proliferation. In contrast, until recently the molecular mechanisms involved in the role of Rho proteins in NGF-induced neuronal differentiation have been largely unknown.

This thesis is the continuation of the Rho project conducted in Dr. Tigyi's laboratory at University of Tennessee Memphis in association with Dr. Szeberényi's project at he University of Pécs. During the first phase of the project, a model was established in which RhoA has a dual role during the initiation and elongation of neurite outgrowth in PC12 cells (Sebok 1999). Then we turned to the molecular mechanisms involved in upstream regulation of RhoA during NGF-induced neuronal differentiation, and we also wanted to study the effector pathway downstream of RhoA.

During the second phase of our project, we wanted to study the members of the signalling pathway mediating NGF effect on RhoA inactivation. We found that TrkA, independently from the Ras-mediated signaling, regulates PI3-K, and PI3-K through Rac1 mediates inactivation of RhoA during neurite outgrowth. Regarding the downstream effectors, we found that NGF has an opposite effect on the RhoA effectors, PKN and ROK. We were also able to explore the mechanism of this: we showed that in addition to GDP-GTP binding, phosphorylation of RhoA at serine 188 by PKA regulates the association of RhoA to its effector proteins Rho-kinase and PKN.

II. THEORETICAL BACKGROUND

1. RhoA

1.1. *The Rho family*

Rho GTPases are members of the Ras superfamily of monomeric, small GTP-binding proteins. The mammalian Rho family proteins share significant (more than 50%) sequence homology to each other (Nobes 1994). According to their sequence and functional similarities, the mammalian Rho GTPases can be grouped into six different classes consisting of the following members: Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, Rac3, RhoG), Cdc42 (Cdc42Hs, G25K, TC10), Rnd (Rnd1/Rho6, Rnd2/Rho7, Rnd3/RhoE), RhoD and TTF (Aspenstrom 1999). The most extensively characterized subfamilies are Rho, Rac and Cdc42. They regulate signal transduction pathways linking various membrane receptors to the assembly of actin-myosin filaments, lamellopodia and filopodia, respectively. It is not surprising, therefore, that Rho GTPases have been found to play a role in a variety of cellular processes that are dependent on the actin cytosceleton, such as cytokinesis, phagocytosis, pinocytosis, cell migration, axon guidance (for review see Narumiya1996). They also regulate a variety of other biochemical pathways including transcription factor (serum response factor and nuclear factor kappaB), and kinase pathways, G_1 cell-cycle progression and cell transformation.

1.2. *Regulation of RhoA*

Like all other regulatory GTP-binding proteins, Rho family members act as molecular switches cycling between GDP- and GTP-bound states which is regulated by at least three classes of cellular proteins.

– Regulatory proteins

There are guanine nucleotide exchange factors (GEFs), also known as GDP dissociation stimulators (GDSs), that facilitate the release of GDP from the Rho family GTPases. First in 1991, Dbl was shown to release GDP from Cdc42 *in vitro* (Hart 1991). Since then, more than 30

mammalian GEFs for various Rho family members have been identified. All are characterized by the presence of a Dbl-homology (DH) domain wich encodes the catalytic activity (Cherfils 1999, Hart 1994) and by the pleckstrin homology (PH) domain wich mediates membrane localization through lipid binding (Rahmen 1997), and might also directly affect the activity of the DH domain (Soisson 1998). In addition to the DH and PH domains, GEFs often contain other protein-protein interaction motifs that vary between different family members (Cerione 1996).

There are Rho GTPase-activating proteins (GAPs) that stimulate the intrinsic GTPase activity of the Rho GTPases, leading to their conversion to the inactive GDP-bound state (Lamarche 1996). About 20 GAPs have been identified to date. GAPs share a related GAP homology domain. In addition to this, GAPs, relatively specific for Rho *in vitro* (p190 Rho-GAP, p122 Rho-GAP and myr5), have functional domains (Narumiya 1996). For example p190 has a Ras GAP-binding domain, which can bind to the N-terminal SH2 domain of P120 Ras GAP (Settleman 1992). This may indicate that Ras signalling can influence Rho activity in the cell *via* interaction of the respective GAPs. Domains of p122 Rho GAP and myr5 also indicate connection of Rho to other pathways, such as PLCδ and actin, respectively (Homma 1995, Reinhard 1995).

Another important molecule regulating Rho activation is Rho guanine nucleotide dissociation inhibitor (GDI). GDI interacts specifically with the GDP-bound form of Rho famiiy members and inhibits the dissociation of GDP from them (Olofsson 1999). GDI also prevents the binding of GDP-Rho, but not GTP-Rho, to cell membranes and can extract GDP-Rho from the membrane (Bishop 2000).

– *The GTPase cycle*

In resting cells, the Rho family GTPases exist mostly in the GDP-bound, inactive form and in complexes with Rho GDI in the cytosol. When cells are activated by a certain extracellular signals, on one hand, Rho GDI dissociation factors (GDFs) such as the ERM (ezrin, radixin and moesin) family proteins bind to the GDP-Rho/Rho GDI complex and dissociate Rho GDI from GDP-Rho. Thereby they enhance the ability of GEFs to bind to GDP-Rho. On the other hand, there are experimental proves that receptor activation can also stimulate GEF's action through $G\alpha_{13}$ binding, in the case of p115 Rho GEF (Kozasa 1998, Hart 1998) and phosphorylation in the case of Vav (Crespo 1997, Han 1997). Then the activated GEFs convert the GDP-bound form of

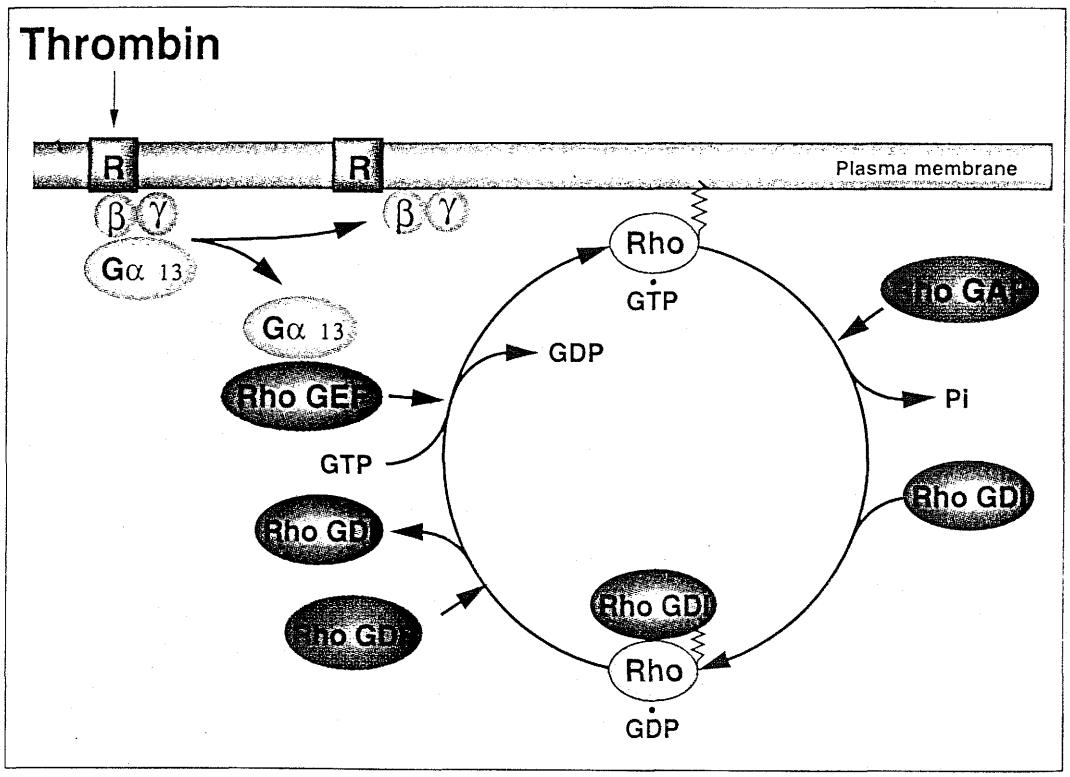

Figure 1 Mode of activation of the Rho family GTPases. GEF, Guanine nucleotide exchange factors; GAP, GTPase-activating proteins; GDI, GDP dissociation inhibitor; GDF, GDI dissociation factor.

Rho to the GTP-bound form. The GTP-bound form of Rho family GTPases is targeted to cell membranes by its COOH-terminal prenyl group and interacts with specific targets. GAPs act as negative regulators by enhancing the GTPase activity of the Rho family GTPases and reconverting them to the inactive GDP-bound form that then can form a complex with Rho GDI and translocate from the membrane to the cytosol (Figure 1, based on Kaibuchi 1999). This model shows that in addition to being in a GTP-loaded form, RhoA must be in the right signaling compartment in the plasma membrane, where it can interact with its regulators and targets.

1.3. Localization of RhoA

As mentioned above the activated RhoA is associated with the plasma membrane and activation is terminated when it is removed from this compartment and complexes with Rho-GDIs in the cytoplasm (Narumiya 1996). We have also shown earlier that during neurite initiation, NGF treatment elicited a translocation of RhoA from the plasma membrane into the cytosol (Sebok 1999). Furthermore, to learn more about RhoA localization, caveolae were studied. Caveolae, which are cholesterol rich specialized microcompartments of the plasma membrane are also rich in receptors and G proteins (Okamoto 1998). Caveolin, an important structural protein found in caveolae, can directly associate with G-proteins, Ras, protein kinase C (PKC) and tyrosine kinase receptors (Huang 1999; Okamoto 1998). In PC12 cells, both low and high affinity NGF receptors, p75 and TrkA respectively, have been shown to associate with caveolae-like membrane domains (CLM) and NGF binding to both receptors was enriched in CLM (Bilderback 1999; Grimes 1997; Huang 1999). Two related caveolin-binding motifs are found in most caveolin-associated protein (Okamoto 1998) and are also found in RhoA. In fibroblasts, C3 exoenzyme treatment has been shown to shift RhoA from caveolae to non-caveolar membranes (Michaely 1999), suggesting that inactivation of RhoA might result in a change in its subcellular localization. In PC12 cells, RhoA was shown to translocate from caveolar membrane fraction to noncaveolar membran fraction during NGF-induced neuronal differentiation (my unpublished data). Taken together, these suggest that both NGF receptor and Ras GTPase may play role in the pathway leading to the inactivation of RhoA in NGF-treated PC12 cells.

1.4. Effectors of RhoA

To date 30 or so potential effectors for Rho, Rac and Cdc42 have been identified using yeast two-hybrid system and ligand overlay assay (Bishop 2000). Here I will focus on effectors of RhoA. For Rho binding proteins, two distinct sequence motifs have been described: homology region 1 (HR1) and Rho binding domain (RBD). Although HR1 and RBD do not share primary sequence homology, they are both highly charged, they are predicted to contain significant α-helical content, and predominantly bind RhoA in the GTP-bound state (Zong 1999).

Using Rho/Rac chimeras, multiple domains of Rho are shown to be involved in effector binding. Surprisingly, the "insert domain" (residues 123-137), a region unique to Rho family GTPases, is not the specificity determinant during binding. It was shown, however, to be required for Rho-kinase activation during Rho-induced transformation of NIH3T3 cells (Zong 2001) and it may be involved in recognition of other, unknown, effectors. A determinant for effector binding was identified between residues 75-92, within loop 6 (Zong 1999), and between residues 27/34-38, switch I region, also known as effector loop. Switch I region was shown to play a key role in effector binding. First, it senses the GTP-bound state of Rho leading to the GTP dependent recognition of Rho by its effectors (Zong 1999). Second, residues within it have different affinity towards different Rho effectors, enabling selective interaction between RhoA and its effectors. For example, residues Lys^{27} and Gln^{29}, make strong contacts with the HR1 Rho binding domain of PKN (Bishop 2000). In contrast, special point mutations of residues 39 and 40 (F39L, E40L) disrupt the binding of Rho to Rho kinase but not PKN, suggesting their special role in Rho and Rho kinase interaction (Sahai 1998).

Based on their Rho binding motifs, several Rho effector molecules can be classified into three groups. 1.) The PRK family includes the HR1 domain containing proteins: PRK1/PKN, PRK2, rhofilin1, rhofilin2 and rhotekin. 2.) The Rho kinase family includes RDB domain containing proteins: Rho-kinase and ROCK-I. 3.) In the third group of Rho effectors, little is known about the sequence of Rho-binding domain, which is different from HR and RDB. Citron kinase (citron-K), citron and mDia are the members of this group.

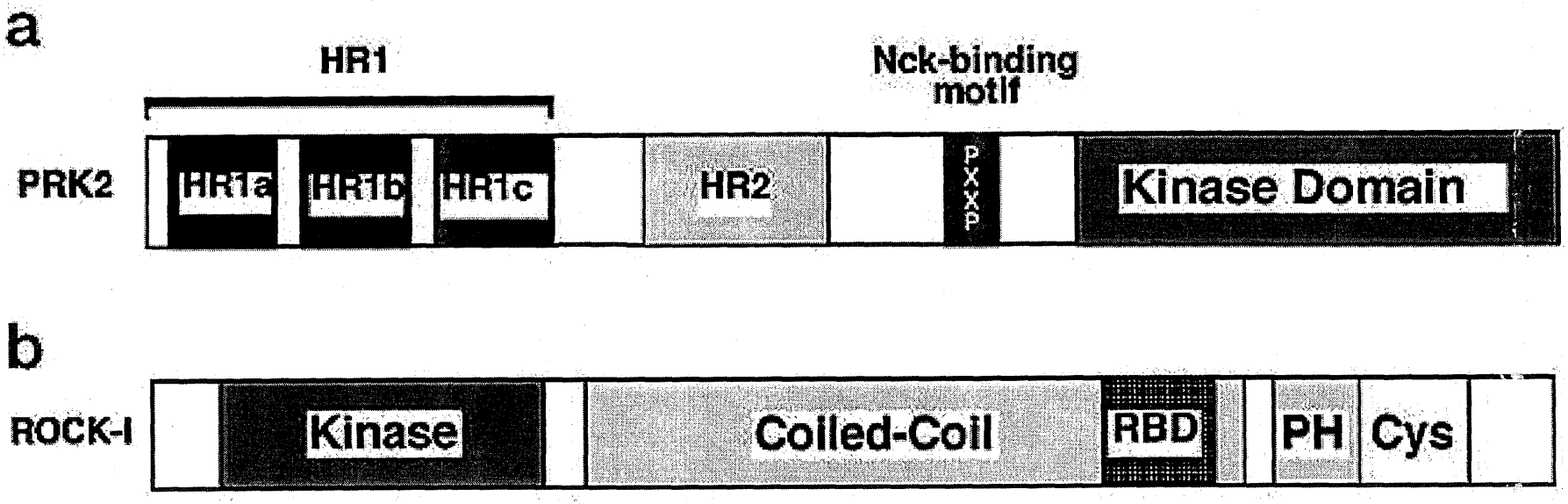

Fig. 2. Rho-GTP specifically interacts with the HR1a domain of PRK2 and the RBD of ROCK. a, diagram indicating the presence of HR1a, HR1b, and HR1c putative Rho-binding domains, at the N terminus of PRK2. HR2, catalytic domain and a Nck-binding domain are also shown. b, diagram showing the RBD of ROCK-I, located between the coiled-coil and pleckstrin homology (PH) domain. The catalytic domain and cysteine-rich region are also shown.
(Zong et al. (1999) J. Biol. Chem. 274, 4551- 4560)

– PRK family

The protein kinase C-related protein kinases (PRKs) are a subfamily of serine/threonine-specific kinases. The best studied member of this family is PRK1, also termed as protein kinase N (p120PKN). The N-terminal regulatory region of PRKs contains a polybasic region followed by a Rho effector homology (REM) region, which contains three repeats of HR1 (Amano 1996, Flynn 1998). The C-terminal region contains a catalytic domain highly homologous to protein kinase C (PKC) (Mukai 1994). One of the functions of PKN to mediate Rho signaling, which regulate cytoskeleton and cell morphology. PKN was shown to inhibit the assembly of intermediate filaments *in vitro* by association to and phosphorylation of head-rod domain of neurofilament protein (Mukai 1996). PKN also has role in controling endosomal and intracellular receptor traffic by interacting with RhoB (Mellor 1998, Zeng 2003). PKN phosphorylates and inhibits Cdc25C *in vitro*. Moreover, the active form of PKN delays the mitosis of *Xenopus* egg cycling extracts. These results indicate another important function of PKN: mediating RhoA signaling to regulate the cell cycle timing (Misaki 2001). PKN was also shown to mediate the signal from RhoA to SRF-dependent transcription (Sahai 1998, Gudi 2002).

An other kinase member of this family is p140PRK2, which has 58% sequence identity to PKN (Reid 1996). One important structural and probably functional difference should be mentioned between PKN and PRK2: PRK2, but not PKN, contains a proline-rich region just N-terminal of the kinase domain of PRK2, that functions as SH3-binding domain, linking PRK2 function to the adapter protein, NCK (Quilliam 1996). Suprisingly, the structural similarity between PKN/PRK1 and PRK2 does not correlate exactly with their GTPase binding properties. While PKN specifically binds to GTP-bound RhoA, PRK2 binds to and its activity can be significantly stimulated by either GDP- or GTP-bound RhoA *in vitro*. It is possible, however, that PRK2 only interacts with the GTP-bound form of RhoA *in vivo*. Furthermore, PRK2 can also interact with the GTP-bound form of Rac. Expression of a catalytically inactive mutant of PRK2 disrupts the actin stress fiber of fibroblasts suggesting that PRK2 participates in the Rho-mediated regulation of cytoskeleton (Vincent 1997). A constitutively active form of PRK2 induces SRF-dependent transcription in a cell type-specific fashion (Gudi 2002).

In addition to GTPases, other PRK interactions have been identified. Both of the PRKs can form a complex with 3-phosphoinositide-dependent protein kinase (PDK1) *in vivo*. This complex formation is Rho-dependent. Furthermore, PDK1 phosphorylates the activation loop threonine

of PRKs and this phosphorylation event is crucial for activation of them (Flynn 2000). Flynn in his paper proposed a model for the mechanism of the activation of PRKs. According to this model, initially, a GTP-bound Rho interaction exposes the C-terminal region of the catalytic domain of PRKs, permitting the binding of PDK1. This can, in turn, phosphorylate the PRKs in the presence of PtdIns(3,4,5)P_3, inducing intamolecular disruption, thus producing a molecule capable of autophosphorylation and further activation (Flynn 1998). This model raises a possible role of PI3-K in RhoA-mediated signaling events.

The scaffold-like members of this family, rhofilin1/2 and rhotekin contain only one N-terminal HR1 region , very similar to PKN HR1a (Watanabe 1996, Reid 1996, Peck 2002). Rhotekin and rhophilin lack any catalitic activity, but both contain putative protein-protein or protein-lipid association domains (Watanabe 1996). Based on this, they might be involved in translocation of RhoA to various compartments of the cytosceleton, serve as scaffolding proteins, or even negatively regulate rho function (Zhon 1998, Fu 2000).

– *Rho-kinase family*

Rho-kinase is a Ser/Thr protein kinase and was identified as a GTP Rho-binding protein (Matsui 1996). It was also identified as ROKα (Leung 95) and ROCK-II (Nakagawa 1996). $p160^{ROCK}$/ROKβ/ROCK-I is an isoform of Rho-kinase. They share a highly conserved (90% identical) kinase domain which is situated at the N-terminal end and has 72% sequence homology with the catalytic domain of myotic dystrophy kinase (Matsui 1996). Rho-kinase has a putative coiled-coil domain in its middle portion and a pleckstrin homology (PH)-like domain and a Cys-rich zinc finger at its C-terminal end. Rho binding domain is situated at the C-terminal portion of the coiled -coil region. Interaction with GTP-bound RhoA activates the phosphotransferase activity of Rho kinase. The loss of C-terminal portion of Rho-kinase makes it constitutively active, whereas the kinase-deficient forms or various C-terminal portions of Rho-kinase function as dominant negative forms in the cells. This suggests that the C-terminal region is a putative negative regulatory region of Rho-kinase (Amano 1999).

The distribution of the two isoforms of Rho-kinase is different. Rho-kinase is highly expressed in the brain and weekly expressed in the lung, whereas ROCK-I is expressed at high levels in the heart, lung, skeletal muscle, kidney and pancreas, at medium level in placenta and liver and hardly at all in the brain (Matsui 1996, Ishizaki 1996). It was also reported that

expression levels of Rho-kinase are gradually increased in the mouse brain during postnatal development, indicating its role during neuronal differentiation (Komagome 2000). In cells, most of the Rho-kinase is recovered in the cytosol. When the cells are activated with growth factors, Rho-kinase is partly translocated to the plasma membrane. It has been also reported that Rho-kinase accumulates at the cleavage furrow during cytokinesis and colocalizes with stress fibers (Amano 2000).

Rho-kinase is able to regulate several different cellular functions through the activation of a number of substrates. In figure 2 (from paper: Amano 2000), the substrates and the cellular events regulated by Rho-kinase are summarized. Shortly, Rho-kinase has been shown to mediate the formation of stress fibers, focal adhesions, and c-fos expression (for review, see Amano 2000). Most recently, Rho-kinase, in heart and cardiac myocyets, was also shown to regulate cell cycle protein expression such as cyclin D3, CDK6 and p27(KIP1), which is required for initiation of cell cycle and G_1/S phase transition (Zhao 2003).

Here I will concentrate on Rho-kinase effectors involved in neuronal differentiation only. Myosin binding subunit (MBS) of myosin phosphatase, which is also an effector of Rho, was the first identified substrate of Rho-kinase. Phosphorylation of MBS inactivates myosin phosphatase and contributes to the elevation of myosin light chain (MLC) phosphorylation and thereby increases myosin-based contractility (Kimura 1996). Accumulation of MBS phosphorylated by Rho-kinase is observed in the membrane ruffling area, at the leading edge and at the cleavage furrows during cytokinesis and on stress fibers (Kawano 1999). In addition to the regulation of MLC by indirect phosphorylation, Rho-kinase regulates MLC by direct phosphorylation at the same sites which are phosphorylated by MLC kinase. The enhancement of MLC phosphorylation is associated with the formation of stress fibers and focal addhesions, smooth muscle contraction and LPA-induced neurite retraction (Amano 1998). Another signal transduction pathway was described by Maekawa *et. al.* which also mediates LPA-induced neurite retraction. They showed that Rho-kinase also directly phosphorylates LIM-kinase and thereby activates it, which in turn phosphorylates cofilin during LPA-induced, RhoA-Rho-kinase mediated neurite retraction in N1E-115 neuroblastoma cells (Maekawa 1999). Rho-kinase was also found to phosphorylate collapsin response mediator protein-2 (CRMP-2) in chick dorsal root ganglion neurons during LPA-induced growth cone collapse (Arimura 2000). Moreover, activation of ROK is sufficient to induce neurite retraction in NGF-differentiated PC12 cells (Katoh 1998). Taken together, these

results show that Rho-kinase, which is highly expressed in neuronal cells, plays a major role in the LPA-induced, Rho-mediated neurite retraction. Furthermore, microinjection of kinase-dead, dominant negative ROKα-KD construct was shown to induce neurite outgrowth in the absence of NGF in PC12 cells. The neurite outgrowth induced by ROKα-KD expression or by NGF treatment involves common components such as Cdc42 and Rac1 (Chen 1999).

– *Other effectors*

Their Rho-binding domain is different from HR and RDB, although, little is known about the sequence of this domain. Here, I will discuss mDia shortly, because from this group only mDia was used in my experiments.

mDia is a mammalian homologue of the *Drosophila* diaphanus and belongs to the formin homology (FH) family. It contains a Rho-binding domain in its N-terminal region, an FH1 region containing polyproline stretches in the middle, and FH2 region in its C terminus. Through its FH1 region, mDia binds to the actin-binding protein profilin and this interaction induces actin polymerization in the cell (Watanabe 1997). Rho-binding domain deleted, active mutants of mDia induce well-organized, parallel actin stress fibres in trasfected cells and correct the actin alignment induced by ROCK, indicating that mDia and ROCK cooperatively act as downstream targets of Rho in the Rho-induced reorganization of the actin cytoskeleton (Nakano 1999) and in the alignment of actin bundles (Watanabe 1999). Further studies showed that mDia coordinates F-actin and plays crucial role in the localization of adherent junction components to the cells periphery (Sahai 2002) through its FH1 region, while it coordinates microtubules, cytokinesis and establishment of cell polarity through its FH2 region (Nakano 1999, Ishizaki 2001).

In addition to these molecules for which direct binding of GTP-Rho was shown, some molecules were shown to be activated by GTP-Rho in crude membrane fractions or cell lysates. They include PI-4-phosphate 5-kinase, PI 3-K and phospholipase D, although their direct physical interaction with Rho have not been established yet (Narumiya 1996).

2. NEURONAL DIFFERENTIATION

2.1. The main elements of the signal transduction pathway of neuronal differentiation

– Receptors

Two kinds of membrane receptors mediate NGF signaling in PC12 cells: TrkA and $p75^{NTR}$. TrkA binds to NGF with high affinity. After neutrophin binding, TrkA receptors undergo dimerization and autophosphorylation followed by activation of their intrinsic tyrosine kinase activity. Tyrosine phosphorylation of specific intracellular residues of TrkA creates docking sites for adaptor and signaling molecules (Friedman 1999). The low affinity NGF receptor, $p75^{NTR}$ is a single transmembrane glycoprotein, member of the tumor necrosis factor (TNF) receptor family. It has cystein-rich repeats in the extracellular domain and an intracellulardeath domain (Leipinsh 1997, Raoul 2000). The p75 receptor appears to regulate TrkA signaling through, in part, direct interaction as well as to mediate neutrophin-induced cell death (Carter 1997).

Thus far, several downstream cascading pathways have been identified that play major roles in NGF signaling. Foremost among these are the Ras/Raf/MEK/ERK and the Crk/C3G/Rap1/bRaf pathways leading to sustained ERK activation which is required for neurite outgrowth. A third pathway, including PI3-K, leading to survival is also known in PC12 cells. Although, PI3-K shows little direct interaction with activated TrkA, PI3-K pathway is at least partly, mediated by Gab1 and/or IRS1 (Friedman 1999). In details, see under *PI3-K*.

– Ras-Raf-MAP kinase pathway

Several small GTP-binding proteins have been shown to play important roles mediating NGF-induced differentiation in PC12 cells. Here, I would like to focus on the Ras mediated pathway . Later on, under part 2.3 (Cross-talk between RhoA, Rac, Ras), the possible interaction between small G-proteins also will be discussed. Ras has a clearly established role in NGF-induced neuronal differentiation. On one hand, expression of a constitutively active Ras protein leads to morphological differentiation of PC12 cells. On the other hand, expression of dominant negative Ras inhibits NGF-induced neuronal differentiation in PC12 cells (Szeberényi 1990). Boglári and Szeberényi showed that a Ras independent pathway also exists from TrkA, supporting to neurite formation (Boglári 2001).

Mammalian cells have three mitogen-activated protein kinase (MAPK) pathways, which regulate the activity of the extracellular signal-regulated kinase (ERK) MAPKs, the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) MAPKs, and the p38 MAPKs. Foremost among the NGFR-linked signaling pathways, the Ras/Raf/MEK/ERK pathway which plays a major role in mediating neurite outgrowth (for review see Vaudry 2002). This pathway appears to be regulated by a remarkable number of upstream Trk-activated elements, including Shc/Grb2/SOS, Crk/SOS and SH2-B/Grb2/SOS. Additionaly, a second pathway leads to sustained ERK activation and appears to involve Crk/C3G/Rap1/bRaf. TrkA-induced sustained Rap1-mediated B-Raf activation may be due to a differential recruitment of PI3-K and scaffolding proteins to activated TrkA (Kao 2001).

The ability of NGF to trigger neuronal differentiation instead of proliferation, as EGF does, is thought to depend, at least in part, on its ability to activate ERKs for a sustained period (for review see Szeberényi 1994). Sustained activation of ERKs may be required for the translocation of ERKs into the nucleus where they induce a distinct set of gene expression (Boglari 1998, 2001). Interestingly, although NGF induces sustained activation of ERKs, activation of Ras is terminated rapidly (Qui 1991), suggesting that sustained activation of ERKs by NGF involves pathways that are downstream or independent of Ras. One potential pathway involves the small G protein Rap1. Rap1 activation stimulates ERKs in PC12 cells via direct activation of the MAPKK kinase B-Raf, the only known effector of Rap1 (Vossler 1997). Furthermore, Rap1 was shown to be required for the sustained activation of ERKs by PKA in PC12 cells (Vossler 1997). Thus, PKA was shown to activate Ras-independent pathways that are not rapidly terminated.

In contrast to ERKs in the neuronal differentiation signaling pathway in PC12 cells, JNK and p38 have important role in mediating stress-activated signaling pathways. They are stimulated by DNA damage, heat and osmotic shock, cytokines and protein synthesis inhibitors (Minden 1997, Widmann 1999). Their role in the neuronal differentiation pathway is much less clear. Although NGF activates them in PC12 cells (Minden 1994, Morooka 1998), NGF withdrawal also leads to sustained activation of these kinases (Xia 1995). In fibroblasts, members of Rho family GTPases are also known to regulate both the JNK and p38 signaling pathways. Constitutively active Rac, Cdc42 and PAK, an effector for Rac, activate both JNK and p38 (Minden 1995, Bagrodia 1995), although none of them can activate ERKs. Furthermore, dominant negative Rac1 was able to inhibit the effect of constitutively active Ras on JNK, placing

Rac as being possibly downstream of Ras in the Ras-JNK pathway (Minden 1995). The activation of JNK by transfection of an activated Cdc42 mutant can also be blocked by dominanat negative Rac suggesting the hierarchical relation between Cdc42 and Rac1 with respect to JNK pathway (Minden 1995).

– PI3K

Under this section, I summarize the reasons why PI3K was chosen in my experiments as one of the candidates to mediate signalling from NGFR to RhoA. PI3K has been implicated in several responses to neurotrophins including neurite extension and promotion of survival (Crowder 1998, Dudek 1997). The protein kinase Akt has been identified as a key effector of PI3K in pathways leading to cell survival (Dudek 1997, Kauffmann-Zeh 1997). The PI3K/Akt pathway affects a conserved cell death program in which apoptosis results from activation of a cascade of cystein proteases termed caspases (Budihardjo 1999).

PI3K was also shown to be an important downstream element in neurite extension and differentiation signaling in PC12 cells through TrkA (Friedman 1999). Lately, a non-receptor protein-tyrosine kinase (Fes) was shown to mediate signal transduction between activated NGF receptor and PI3K by Ras-ERK-independent manner (Shibata 2003). PI3K also activates the c-Jun NH_2-terminal kinases (JNKs), which can promote differentiation or apoptosis, depending on the cell's history of exposure to NGF (Leppa 2001, Vaudry 2002).

Functional studies established PI3K as an effector of Ras, an upstream modulator of Rac1 activity (Rodriguez-Viciana 1997) and a candidate effector of RhoA (Zhang 1993). The PI3K-mediated signal from Ras to Rac1 is required for the oncogenic transformation of Rat-1 cells (Tang 1999). *In vitro* data suggest that Rac1 specifically interacts with phosphatidylinositol 3,4,5-trisphosphate, a product of PI3K (Missy 1998). RhoA also displays significant, although weaker, binding to this lipid (Missy 1998). Expression of an activated Ras mutant caused increased Rac1 GTP binding and PI3K activity in membranes of PC12 cells. Taken together, these data suggest the existence of Ras–›PI3K–›Rac1 signaling pathway. However, in Swiss 3T3 fibroblast cells, Wortmannin, specific inhibitor of PI3K, does not interfere with Ras-mediated actin remodelling (Nobes 1995). Thus, a secondary pathway may exists (RTK–›PI3K–›Rac1), which is Ras-independent, and is induced by the activation of receptor tyrosine kinases including TrkA. The Ras-independent pathway for Rac1 regulation is supported both by (i) the

phosphorylation-dependent association of PI3K with the Tyr751 residue of TrkA (Yamashita 1999) and (ii) by the finding that PI3K regulates at least two of the Rac1-GEFs, Vav and Sos-1 (Han 1998; Nimnual 1998).

– PKA

Cyclic AMP (cAMP), as an intracellular second messenger, an activator of PKA, plays important roles in many aspects of nerve system function. cAMP can promote neurite outgrowth (Rydel 1988), induce the switching of directional turning of nerve growth cones in response to a gradient of diffusible factors, such as brain-derived growth factor, netrin-1, and acetylcholine (Song 1997). Mark *et al.* showed that combination of EGF and low levels of cAMP stimulate differentiation of PC12 cells (Mark 1995). There are two possible explanation for this result. First, while EGF causes only short activation of ERKs, PKA was shown to be required for maximal sustained activation of ERK1 and maximal induction of gene expression by NGF (Yao 1998). Second, cAMP was shown to inhibit the EGF-stimulated increase in cell cycle progression factors as well as proliferation due to the antiproliferative activity of cAMP (Mark 1997).

There is an other set of data indicating the importance of PKA during the signal transduction of neuronal differentiation. Forskolin-induced differentiation of PC12 cells was mediated by the activation of PKA (Chijiwa 1990), followed by the activation of p38 MAPK (Hansen 2000). In response to thyroid-stimulating hormone, Rac was shown to be an intermediate in the PKA-dependent activation of p38 MAPK (Pomerance 2000), suggesting the role of Rac and PKA in the NGF-induced, Ras-independent signal transduction pathway in PC12 cells. Boglari in his paper also suggested the possible role of PKA in mediating signals between p90Rsk and CREB independently from the Ras-ERK pathway (Boglari 2002).

In the case of my research, it is very important finding that the catalytic subunit of PKA phosphorylates RhoA at Ser-188 *in vitro* (Lang 1996). Phosphorylation of RhoA significantly increases its interaction with GDI even in its GTP-bound state under physiological concentration of ions (Lang 1996, Forget 2002). Moreover, it was shown that phosphorylation of RhoA by PKA *in vitro* decreases the binding and the activation of its effector, Rho kinase (Dong 1998) leading to reduced tension of actin fibers and thus neurite outgrowth. Another downstream effector of RhoA, phospholipase D, was also shown to be inhibited by phosphorylation of RhoA

(Kwak 2000). Taken together, these results emphasize the physiological rate of phosphorylation of RhoA as a crucial element in the regulation of RhoA.

2.2. The role of RhoA in neuronal differentiation

– Neurite retraction

The role of RhoA during neuronal differentiation was first suggested by studies using the Rho-specific ADP-ribosyltransferase C3 toxin (*C. botulinum* C3 Rho-ADP-ribosylating exoenzyme). C3 toxin induces cell flattening followed by growth arrest and rapid neurite extension in undifferentiated N1E-115 and PC12 cells (Nishiki 1990). In differentiated N1E-115 and PC12 cells, C3 toxin prevents agonist-induced cytoskeletal contraction and ensuing neurite retraction (Jalink 1994, Tigyi 1996). Lysophosphatidic acid (LPA), – the best studied agonist which induces neurite retraction –, was identified as an albumin-bound phospholipid that is generated during blood clotting (Tigyi 1992). In PC12 cells, LPA inhibits NGF-induced neuronal differentiation and causes the rapid retraction of established neurites in a chemically defined medium through activation of its seven transmembrane domain receptor (Tigyi 1992). Using Rho activation assay, LPA was shown to rapidly activate RhoA in N1E-115 cells to induce growth cone collapse and neurite retraction through a $G_{12/13}$-initiated pathway that involves protein-tyrosine kinase activity (Kranenburg 1999). Using the same assay in PC12 cells, we also showed that LPA activates RhoA. Moreover, activation of RhoA during neurite retraction caused by LPA was independent of Ras (Tigyi 1996b) suggesting the possibility of Ras-independent signaling from NGF toward RhoA. *In vivo*, LPA-like lipids have been identified in the posthaemorrhagic cerebrospinal fluid (Tigyi 1995), raising the possibility that such mediators may have either an adverse effect on regeneration of neurites, or an enhanced effect on Rho-mediated neurite retraction after nervous system injury.

– Neurite regeneration

After injury in the central nerve system (CNS) regeneration is hindered by growth inhibition by CNS myelin, since it contains several different growth inhibitory proteins. McKerracher's group published very encouraging data on the role of RhoA during regeneration in CNS. They showed that PC12 cells treated with C3 toxin or transfected with a dominant negative RhoA

extended neurites on inhibitory substrates of myelin associated glycoprotein (MAG) or myelin. Furthermore, they also demonstrated that C3 toxin induced axon regeneration of injured optic nerve of mice *in vivo* (Lehmann 1999). Most recently they showed that treatment either with C3 toxin or with a Rho kinase inhibitor, Y27632 was sufficient to stimulate axon regeneration and recovery of hindlimb function after spinal cord injury (SCI) in adult mice (Dergham 2002). These findings provide evidences that the RhoA signaling pathway is a potential target for therapeutic interventions after CNS and spinal cord injury.

– *Neurite outgrowth*

In addition to the role of RhoA during neurite retraction and regeneration, RhoA has been shown to be involved in the regulation of neurite outgrowth (Tigyi 1992; Jalink 1994; Kozma 1997; Lehman 1999). In our earlier studies (Sebok 1999) we found that expression of the activated V14RhoA mutant prevented neurite outgrowth induced by NGF. In contrast, expression of a dominant negative RhoA (N19RhoA) led to an increase in neurite initiation and branching. thus, RhoA was shown to have a dual role during neuronal differentiation. Inactivation of RhoA appears to be necessary for the initiation of neuronal differentiation, although during later stages of neurite elongation, introduction of N19RhoA caused a formation of short neurites (Sebok 1999). Taken together, these resuls suggest an important role of RhoA in NGF-induced neuronal morphogenesis, but little is known about the signal transduction pathways that couple NGF signaling to RhoA.

2.3. Cross-talk among RhoA, Rac and Ras

A hierarchical cascade linking Ccd42, Rac1 and RhoA was first described based on studies conducted in fibroblasts (Ridley and Hall 1992). According to this hypothesis, activation of Cdc42 induces activation of Rac1, which in turn leads to activation of Rho (for review see Scita et al. 2000 and Kjoller and Hall 1999). In contrast to the Cdc42–›Rac1–›Rho hierarchical cascade described in fibroblasts, no evidence for such a relationship has been found in neuronal cells, however, separate Cdc42/Rac and Rac/Rho hierarchies exist. More likely, Cdc42 and Rho activities are competitive or even antagonist in neuronal cells. For example in N1E-115 cells, PAK5 mediates signals from Cdc42 and Rac1 leading to the inactivation of RhoA (Dan 2002). In

NIH3T3 cells, activation of Rac1 also leads to the inhibition of RhoA suggesting that Rac1 is upstream of RhoA (Sander 1999). In contrast, in PC12 cells, RhoA suppressed the NGF-induced Rac1 activation through Rho-associated kinase pathway, suggesting that Rho acts upstream to Rac1 (Yamaguchi 2001). Although, in the experiments of Yamaguchi's group, NGF treatment was performed following a 18h long serum starvation and growth factor deprivation of PC12 cells, suggesting that this effect is more pertinent to an anti-apoptotic rescuing effect of NGF rather than that of inducing neuronal differentiation.

Settleman *et al.* (1992) reported a cross-talk between Ras and Rho by showing that p190Rho-GAP was tyrosine phosphorylated and formed a complex with the SH2 domain of p120Ras-GAP, suggesting that the latter may act as a Ras effector regulating the activity of RhoA (Settleman et al., 1992). Booden et al. found that expression of constitutively activated Ras elicited neurite outgrowth which was prevented by the co-expression of inhibitory Rho but required increased amounts of inhibitory Rac1 (Booden et al., 2000). These results suggest that RhoA and Rac1 were downstream of Ras, however, these authors did not elicite differentiation using NGF but instead with an activated Ras mutant.

III. MATERIALS AND METHODS

Cell lines, cell culture. Wild type PC12 cells and the M-M17-26 clone, which stably expresses a dominant negative Ras mutant were kindly provided by Dr. G. Cooper (Boston University, Boston, USA). The TrkA-deficient nnr5 clone was kindly provided by Dr. L. A. Green (Columbia University, New York, USA). B5 cells that are derived from nnr5 overexpressing TrkA were a gift from Dr. Susan Meakin (Robarts Research Institute, Ontario, Canada). A123.7 cells, a stable transfectants with the HLREVB1+2neo plasmid, which encodes two point mutations in the B locus cAMP-binding site of the type 1 regulatory subunit of PKA. All of these clones were grown in RPMI medium supplemented with 10% horse serum, 5% fetal bovine serum (FBS), and 1% glutamine (normal medium). To initiate differentiation, 7 x 10^6 cells were plated in 100 mm dishes in normal medium and exposed to 100 ng/ml NGF (Alamone Labs, Jerusalem, Israel). Under these conditions more than 75% of the wild type PC12 and B5 cells extended neurites by day 2. HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Reagents. Forskolin, H89, PKI, db-cAMP, GDP, GTP, GTPγS, Protein Kinase Inhibitor (PKI), Wortmannin, LY294002, glutathione-agarose beads were from Sigma-Aldrich (St. Louis, MO, USA). Recombinant PKA catalytic subunit was obtained from Promega (Madison, WI, USA). NGF was purchased from Alamone Labs (Jerusalem, Israel).

Plasmids. pGEX-2T^{Ser1881AlaRhoA} plasmid was a kind gift of Dr. J. Bertoglio (INSERM, Unite 461, Paris, France). All other RhoA and effector constructs (His-RhoA, HA-RhoA, GST-ROK, GST-PKN, GST-mDIA, GST-rhotekin, ROK, RhoA/GFP) were generated by subcloning the respective cDNAs to either the *E. coli* expression vectors (pET15b for His-tagged expression and pGEX-KG for GST-tagged expression) or mammalian vectors (pCEFL for GST-tagged expression, pKH3 for HA-tagged expression, or pMX-IRES-GFP for bicistronic expression with GFP) have been described in our previous publications (36-38).

Transient transfection. Wild type PC12 cells were transiently transfected with either dominant negative Rac1 construct (pN19Rac1-IRES-GFP), empty vector (pMX-IRES-GFP), pIRES-GFP,

pIRES-S188ARhoA-GFP, pIRES-V14RhoA, pIRES-S188AV14RhoA-GFP, pHA-wtRhoA or pHA-Ser188AlaRhoA using the Cytofectene reagent (Bio-Rad, Hercules, CA, U.S.A.). 2 μg plasmid and 200μl of Cytofectene reagent was used for every ml of transfection solution. After exposing the cells to this mixture for 8 hours, cells were grown in normal medium. Expression of GFP was detected and pictures of cells expressing GFP were taken using fluorescent microscopy. Between day three and six after transfection 80% of cells expressed GFP; therefore, NGF treatment was carried out between postransfection days 3-6.

Isolation of total cell lysate. Total cell lysate was isolated as described previously by Kranenburg (1999). Briefly, after NGF treatment, cells were rinsed with 3 ml of ice-cold PBS and lysed with 450 μl of lysis buffer A containing 20 mM TRIS (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 10% (V/V) glycerol, 0.1% (V/V) Triton X-100, and Protease Inhibitor Cocktail (Sigma). This lysate was incubated for 10 min on ice and the crude cell debris was removed by centrifugation at 10,000 x *g* for 10 min. The supernatant was designated total cell lysate and used for the experiments.

Membrane fractionation. After NGF treatment, cells were rinsed with 3 ml of ice-cold PBS and lysed with 450 μl of lysis buffer B containing 50 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA and the Protease Inhibitor Cocktail (Sigma). The lysate was sonicated twice for 15 sec, the unbroken cells and nuclei were removed by centrifugation at 1500 x *g* for 10 min. The supernatant was further centrifuged at 15,000 x *g* for 10 min at 4°C to sediment the crude heavy membrane fraction (HMF). The supernatant of HMF was used as cytosol. The crude HMF was resuspended in lysis buffer B and recentrifuged at 15,000 x g for 10 min and designated as HMF.

Western blot analysis. Twenty μg protein applied per lane was separated by SDS-PAGE on 12% gels and transferred to PVDF membranes (Bio Rad) using a semidry transfer apparatus (Bio-Rad). To block non-specific binding, membranes were incubated overnight at room temperature in 10mM Tris-HCl (pH 8.0) 150mM NaCl, 0.05% Tween 20 (TBST) containing 5% Nonfat-Dry Milk (Bio-Rad). After blocking, the membranes were probed for 2 hours with the primary antibody diluted in TBST, and washed three times with TBST before incubation for 45 min with

either peroxidase conjugated secondary anti-mouse antibody (Sigma, 1:5000) or donkey anti-rabbit antibody (Promega Corporation, Madison, WI, USA, 1:7500). The SuperSignal Reagent (Pierce, Rockford, IL, U.S.A.) was used to visualize antibody binding.

The primary antibodies used in our experiment. Anti-RhoA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.; diluted 1:500); anti-Rac1 monoclonal antibody (Santa Cruz Biotechnology; diluted 1:500); anti-phospho-Akt Ser473 antibody (New England Biolabs, Beverly, MA; diluted 1:1000) and anti-Akt antibody (New England Biolabs; diluted 1:1000).

In vitro phosphorylation of RhoA isolated from PC12 cells. Total cell lysate was isolated from untreated and NGF-treated PC12 cells as described under *Isolation of total cell lysate.* Using these cell lysates, GDP/GTPγ-S loading assay, *in vitro* PKA phosphorylation assay with cold ATP and pull down assay with GST-ROK and GST-PKN were performed followed by Western blotting as described under *Isolation and in vitro phosphorylation of His-tagged RhoA.*

Isolation and in vitro phosphorylation of His-tagged RhoA. SF9 cells were infected by baculovirus expressing His-tagged wild-type and S188A mutant RhoA proteins, His-wtRhoA and His-RhoA$^{Ser188\square\square\square}$, respectively. Recombinant RhoA proteins were isolated using the 6 x His Expression and Purification Kit (Pharmingen; San Diego, CA). GDP and GTPγS loading was perform as described by Zhang et al. (36). The recombinant proteins were treated with 4 U of the catalytic subunit of PKA for 10 min at 30°C in the presence of 5 μCi [^{32}P]γATP (3000 Ci/mmol; Amersham; Piscataway, NJ, USA) in 20 mM MES (pH 6.5), 100 mM NaCl, 0.1 mM EDTA, 30 μM β-mercaptoethanol, and 50% (V/V) ethylene glycol. The final reaction mixture contained 30 μl beads, 8 μl assay buffer, 4 μl PKA (1 U/μl), and 1.5 μl [^{32}P]γATP. The reaction was stopped by washing the beads with 100 μl of 300 μM imidazole. The phosphorylated proteins were separated by SDS-PAGE and detected using a Cyclone phosphorimager (Molecular Devices; Sunnyvale, CA, USA). After the phosphorylation reaction, His-tagged Rho proteins were loaded with either GTPγS or GDP (36) and then eluted from the Ni-NTA agarose beads (Sigma-Aldrich). A pull down assay was performed on these recombinant Rho proteins with GST-ROK and GST-PKN beads. To detect the amount of ^{32}P-labeled, effector-bound Rho proteins after the pull-down

assay, the GST-beads were boiled and the proteins were separated by SDS-PAGE followed by detection with the phosphorimager.

RhoA and Rac1 activation assays. The glutathione agarose-immobilized (GST)-proteins, which contain either the Rho binding domain of Rho-kinase (GST-ROK), PKN (GST-PKN), or the p21-binding domain (PBD) of human PAK1 (GST-PAK), were expressed in *E. coli* by using the pGEX-KG vector. Bacteria were lysed by 2 x 30 sec sonication in 50 mM HEPES (pH 7.6); 5 mM $MgCl_2$; 100 mM NaCl and Protease Inhibitor Cocktail (lysis buffer C) containing 50 µg/ml lysozyme. The lysate was cleared by centrifugation at 20,000 x *g* for 30 min at 4 °C. The fusion protein was purified using glutathione beads (Sigma) from the supernatant. Beads were washed three times with lysis buffer C. GST-ROK, GST-PKN or GST-PAK1 immobilized on glutathione beads was added directly to either HMF, cytosol, or total cell lysate. After a 45 min shaking at 4 °C, beads were washed with lysis buffer C and the bound RhoA or Rac1 was detected by Western blotting using anti-RhoA and anti-Rac1 antibodies. Every pull down assay was repeated at least three times using HMF and two times using total cell lysate. For controls, RhoA and Rac1 were loaded with either GDP or GTP-γ-S in every pull down assay.

Statistical methods. The quantitative results represent the mean of at least three individual experiments. Student's *t*-test for paired variables was used to test for differences caused by NGF treatment or phosphorylation, and data were considered significantly different at $P < 0.05$.

IV. RESULTS AND DISCUSSION

1. The activation state of RhoA during the initiation and elongation phase of NGF-induced neuronal differentiation.

We showed that brief treatment with NGF leads to inactivation of RhoA, as shown by its rapid translocation from the plasma membrane to the cytosol and a decrease in its ability to associate with ROK, one of its effectors. In contrast, we found that prolonged, 2-day NGF treatment increased RhoA expression and its association with the plasma membrane during the elongation phase of neuronal differentiation. The amount of ROK-bound RhoA returned to the level seen in untreated cells (J. Biol. Chem. 277. p35840, Fig. 2). These results are in agreement with our previous study on the role of RhoA in morphological differentiation. Suprisingly, we found that in contrast to ROK, NGF treatment increased the amount of RhoA associated with PKN, mDia and Rhotekin, another effectors of RhoA during the initiation phase of neuronal differentiation (manuscript submitted to Current Biology, Fig. 1). The nonhydrolyzable GTPγS elicited increased RhoA binding to all of these effectors as pozitive control, while C3 toxin diminished the binding of all targets as negative control (manuscript submitted to Current Biology, Fig. 1).

The first important new finding in this set of experiments is that the activation state of RhoA changes during the time course of neurite outgrowth. This result supports our earlier hypothesis that RhoA has dual role during the initiation and elongation phase of NGF-induced neuronal differentiation. Moreover the activation state of RhoA at any given time might be different at different parts of the cell – eg. at the cell body and at the growth cones, where – as it was shown recently – RhoA plays a crucial role in neurite pathfinding, an important process in neuronal development (Yuan 03).

The second important new finding in this set of experiments is the disparate activation of ROK, PKN, mDia and Rhotekin, targets of RhoA, in NGF treated PC12 cells. This finding was the starting point of our experiments in which we showed a novel mechanism capable of selectively inhibiting the activation of GTP-loaded RhoA towards ROK. These results will be discussed in part 3 of this chapter.

2. The signal transduction pathway mediating the signal from NGF to RhoA during initiation of neurite outgrowth.

2.1. TrkA

To study the signal transduction pathway, wich mediates signal from NGF to RhoA, we started our experiments to determine the role of TrkA, the high affinity NGF receptor. Using the nnr5 cell line, a clone of PC12 cells lacking functional TrkA receptor, we showed that functional TrkA receptor is required for NGF-induced inactivaton of RhoA (J. Biol. Chem. 277. p35840, Fig. 3).

2.2. Ras

Since, Ras has a central role in mediating NGF-induced neuronal differentiation, and both, in growth factor stimulated and also in tyrosine-kinase transformed cells, Ras-GAP and p190 Rho-GAP were shown to form a complex (Chen 2002), it seemed plausible that NGF reaches Rho through Ras. To study this possibility we used PC12 cells expressing a dominant negative Ras mutant. Expression of a dominant negative Ras mutant did not affect the inhibitory effect of NGF on RhoA. This finding suggested that a Ras-independent signal transduction pathway links NGFR to RhoA (J. Biol. Chem. 277. p35840, Fig. 4c).

2.3. Rac1

Numerous studies were conducted in fibroblasts to determine the relationship between RhoA, Rac and PI3-K. In contrast, no evidence for such a relationship has been found in neuronal cells and the existing data are very controversial. Although a Cdc42 –> Rac1 –> RhoA hierarchical cascade was described in fibroblasts, the relationship between Rac1 and RhoA is more complex and likely depends on the cell type. These data suggested that Rac1 also might have a role in mediating signal from NGF to RhoA. Expression of a dominant negative Rac1 mutant in PC12 cells interferes with the effect of NGF on RhoA, as neurite outgrowth (J. Biol. Chem. 277. p35840, Fig. 4a) and inactivation of RhoA was inhibited (J. Biol. Chem. 277. p35840, Fig. 4d), suggesting that Rac1 mediates an inhibitory signal from TrkA to RhoA.

2.4. PI3-K

In Swiss 3T3 fibroblasts, a Ras independent pathway (receptor tyrosine kinase –> PI3-K –> Rac1) is likely to exist. This finding raised the question if PI3-K has any role in the signaling pathway between NGF and RhoA. Using PI3-K inhibitors, Wortmannin and LY294002, the role of PI3-K was determined in this signaling event. Inhibition of PI3-K blocked the effect of NGF on RhoA (J. Biol. Chem. 277. p35840, Fig. 5), indicating the requirement of PI3-K activity for the NGF-induced regulation of RhoA.

2.5. The relatioship between Rac1 and PI3-K

Finally, we determined the hierarchical relationship between Rac1 and PI3-K in this sinaling event. On one hand, inhibition of PI3-K reduced the NGF-induced activation of Rac1 (J. Biol. Chem. 277. p35840, Fig. 6a). On the other hand, expression of the dominant negative Rac1 did not affect the NGF-induced activation of PI3-K (J. Biol. Chem. 277. p35840, Fig. 6b). Taken together, we found that PI3-K acts upstream of Rac1 in the NGF-induced signal transduction pathway inhibiting RhoA in PC12 cells.

3. The role of phosphorylation of RhoA in the regulation of its affinity to effectors.

3.1 PKA

The disperate activation of RhoA targets, namely ROK, PKN, mDia and Rhotekin, by NGF points to a novel mechanism capable of regulating the activity of RhoA, in addition of the switch between GDP and GTP binding (manuscript submitted to Current Biology, Fig. 1). We choose GST-ROK and GST-PKN for further characterisation of this novel regulatory mechanism. PKA can specifically phosphorylate RhoA on serine at the position 188 *in vitro* (Lang 1996). Phosphorylation of RhoA was shown to increase its interaction with RhoGDI (Forget 2002). Furthermore, Rho-kinase was found to bind less to RhoA upon phosphorylation of RhoA by PKA (Dong 1998). Taken together, these data suggested that phosphorylation could be an additional mechanism of regulation of RhoA. In addition, there is experimental evidence for the role of PKA during NGF-induced neuronal differentiation. Direct activation of PKA by cyclic AMP

promotes neurite outgrowth in a variety of neuronal cell lines and also induces switching in turning direction of nerve growth cones (Song 1997). Furthermore, PKA is required for sustained activation of ERK1, a key step of NGF-induced differentiation (Yao 1998).

3.1.1. Similarly to NGF, treatment of cells with dbcAMP, a cAMP analog which activates PKA, and forskolin, a direct activator of adenylyl cyclase and PKA, decreased the amount of ROK-associated and increased the amount of PKN-associated RhoA (manuscript submitted to Current Biology, Fig. 2). These experiments showed that activation of cAMP-PKA signaling mimics NGF-induced target selection of RhoA in PC12 cells.

3.1.2. To inhibit PKA, we used H89 or PKI, spesific inhibitors of PKA or PKA-defficient A123,7 cells. Inhibition of PKA blocked the effect of NGF on the activation state of RhoA (manuscript submitted to Current Biology, Fig. 3). These results suggested that activaton of PKA is necessary for NGF-induced decrease of the amount of membrane- and ROK-associated RhoA. It is also necessary to increase the affinity of RhoA to PKN.

3.2. Ser188 aminoacid of RhoA

It has been proposed that PKA phosphorylates RhoA on the Ser188 aminoacid in the C-terminal tail (Lang 1996, Dong 1998). Therefore, we choose to study the Ser188 residues of RhoA in our system.

3.2.1. *In vitro* phosphorylation of His-tagged wild-type and S188A mutant RhoA with radioactively labelled ATP [^{32}P-γATP] indicated that PKA phosphorylates RhoA *in vitro* on a single site at Ser188 (manuscript submitted to Current Biology, Fig. 4A lane 3 & 4). Moreover, GTP loading of RhoA did not affect its phosphorylation by PKA (manuscript submitted to Current Biology, Fig. 4A lane 1 & 2).

3.2.2. To test the role of Ser188 aminoacid of RhoA during NGF-induced neurite outhgrowth in PC12 cell, S188A-RhoA mutant was transfected into the cells. Three days after NGF treatment, nearly 80% of wtPC12 cells bore neurites, however less than 5% of the PC12 cells expressing S188A RhoA extended neurites (manuscript submitted to Current Biology, Fig. 6). This suggests that phosphorylation of RhoA on the Ser188 residue is a required and necessary step for neurite outgrowth.

3.3. phosphorylation as a novel secondary switch in regulating RhoA function

Finally, we studied the effect of phosphorylation and GTP-GDP binding on the affinity of RhoA towards ROK and PKN in more details *in vitro* and *in vivo*.

3.3.1. As expected, GTP binding enhanced significantly the amount of both wtRhoA and S188ARhoA associated with ROK and PKN (manuscript submitted to Current Biology, Fig. 4D). These results showed that GTP-binding is necessary for the activation of RhoA. Furthermore, S188A mutation in RhoA has no effect on the activation of RhoA by the well known GTP-GDP cycling.

3.3.2. PKA treatment of RhoA caused a significant reduction in binding to GST-ROK (manuscript submitted to Current Biology, Fig. 4D, lower left panel); however, it had no effect on His-RhoA^{S188A} (manuscript submitted to Current Biology, Fig. 4D, lower right panel). Treatment with PKA did not inhibit but slightly augmented the GTP-dependent complex formation between RhoA and PKN (Fig. 4D, upper left panel). As expected, the interaction of His-RhoA^{S188A} with PKN was not affected by PKA treatment (Fig. 4D upper right panel). Thus, we concluded that phosphorylation of RhoA on ^{188}Ser by PKA permits its interaction with PKN but attenuates interaction with ROK, thereby leading to a selective activation of one effector relative to another by the activated GTPase.

3.3.3. To extend our *in vitro* observations, HEK293 cells (manuscript submitted to Current Biology, Fig. 5A) and PC12 cells (manuscript submitted to Current Biology, Fig. 5B) were used. NGF treatment of PC12 cells elicited a time-dependent activation of GST-PKN interaction with HA-RhoA and also with HA-RhoA^{S188A} (Fig. 5B, lower panel). NGF treatment caused a time-dependent decrease in HA-RhoA binding to GST-ROK. As predicted, the HA-RhoA^{S188A} mutant did not show decreased binding to GST-ROK following NGF treatment (Fig. 5B, upper panel). These results, similarly to the results of the experiments with HEK293 cells, support our *in vitro* finding that phosphorylation of RhoA at Ser188 is an additional regulatory mechanism of RhoA which has an opposite effect on its affinity to ROK and PKN.

4. Summary

Taken together, we first described some member of the signalling pathway from NGF to RhoA during the initiation phase of neuronal differentiation of PC12 cells. This includes the NGF receptor TrkA, the PI3K and the small GTPase Rac1. PI3-K was shown to act upstream of Rac1, however we have not shown direct activation or physical association between these proteins. We also showed that NGF inactivates RhoA through Ras-independent pathway. Moreover, most likely there are many other possible participants of this pathway, for example p75 and Cdc42 as their role was shown in other cells. Further experiments are planned to study these connections.

During the course of our experiments we were surprised to see that the affinity of ROK and PKN towards RhoA changes in an opposite way upon NGF treatment. The experiments designed to clarify this controversy lead us to identify a novel way of regulation of RhoA, it's phosphorylation of ^{188}Ser, which serves as a "secondary switch" in addition to GTP-binding, capable of channeling signals among effector pathways. RhoA target selection mediated by PKA phosphorylation plays an essential role in the negative modulation of ROK required for NGF-induced neurite outgrowth. A number of Rho GTPases contain consensus phosphorylation sites that can be potentially recognized by serine and tyrosine kinases, and our proposed "secondary switch" model may thus be applied to other small G-proteins for such a regulation. I consider these result as the most important finding of my Ph.D. thesis.

V. SIGNIFICANCE OF THIS PROJECT

Being a medical doctor by training, and at this time, doing my residency, who was and still is engaged in basic research I feel compelled to relate my work to real life problems, to seek the physiological relevance of my research. However fundamental the research is; and regardless of methodology involved mainly *in vitro* experiments or the model system being immortalized rat cell line, I firmly believe that this project is related to real medical problems and might help to answer biologically important questions.

To begin with the most important general aspect this project deals with signal transduction mechanisms related to neuronal differentiation of neuronal cells. Understanding what is happening in the cell when a signal (a hormon or growth factor) reaches it will help us to implement new diagnostic procedures or therapeutic methods, to have clearer picture of pathophysiological background of diseases. Just to name an example, knowing some member of NGF-induced neuronal signaltransduction pathway towards RhoA give us the possibility to design and test more drugs, which can intervine in this process.

By better understanding the regulation of RhoA during these events we might be able to influence these processes. An especially important goal would be to influence RhoA during neuronal regeneration – eg. after a traumatic spinal cord injury or stroke. Drugs targeted to inhibit RhoA potentially could improve the condition of these patients. This is supported by that finding that an inhibitor of RhoA, C3 toxin, was shown to enhance the regeneration of the optic nerve, but failed to cause full regeneration of it (Lehmann 99). In the light of our results, influencing only the GTP-GDP binding state of RhoA by C3 toxin is most likely not enough to fully mimic the effect of RhoA during neuronal regeneration.

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VII. APPENDIX – PUBLICATIONS

1. Nusser, N., Gosmanova, E., Zheng, Y. and Tigyi, G. (2002) Nerve Growth Factor Signals through TrkA, Phosphatidylinositol-3-kinase, and Rac1 to Inactivate RhoA during the Initiation of Neuronal Differentiation of PC12 Cells. *J. Biol. Chem.* **277,** 35840-35846.

2. Nusser, N., Gosmanova, E., Guo, F., Luo, Y., Zheng, Y. and Tigyi, G. Phosphorylation-regulated Target Selection of RhoA Is Required for NGF-Induced Neurite Outgrowth. (submitted to Current Biology)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 39, Issue of September 27, pp. 35840–35846, 2002
Printed in U.S.A.

Nerve Growth Factor Signals through TrkA, Phosphatidylinositol 3-Kinase, and Rac1 to Inactivate RhoA during the Initiation of Neuronal Differentiation of PC12 Cells*

Received for publication, April 15, 2002, and in revised form, July 19, 2002
Published, JBC Papers in Press, July 19, 2002, DOI 10.1074/jbc.M203617200

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In PC12 rat pheochromocytoma cells, nerve growth factor (NGF)-induced neuronal differentiation is blocked by constitutively active dominant mutants of RhoA but augmented by negative ones, suggesting a not yet elucidated inhibitory signaling link between NGF receptors and RhoA. Here we show that NGF treatment rapidly translocates RhoA from the plasma membrane to the cytosol and simultaneously decreases RhoA affinity to its target Rho-associated kinase (ROK), a key mediator of neurite outgrowth. This effect was transient, because after 2 days of NGF treatment, RhoA relocated from the cytosol to the plasma membrane, and its GTP loading returned to a level found in undifferentiated cells. Inhibition of RhoA is mediated by activation of the TrkA receptor, because NGF failed to induce RhoA translocation and inhibition of ROK binding in nnr5 cells that lack TrkA, whereas the inhibition was reconstituted in receptor add-back B5 cells. In MM17-26 cells, which due to expression of dominant negative Ras do not differentiate, NGF-stimulated transient RhoA inhibition was unaffected. The inhibitory pathway from TrkA to RhoA involves phosphatidylinositol-3-kinase (PI3K), because the inhibitors LY294002 or wortmannin prevented NGF-induced RhoA translocation and increased RhoA association with ROK. Furthermore, inhibition of PI3K significantly reduced NGF-mediated Rac1 activation, whereas dominant negative Rac1 abolished the inhibitory signaling to RhoA. Taken together, these data indicate that NGF-mediated activation of TrkA receptor stimulates PI3K, which in turn increases Rac1 activity to induce transient RhoA inactivation during the initial phase of neurite outgrowth.

The small GTPases RhoA and Rac1 are members of the Rho subfamily within the Ras superfamily of GTPases. RhoA plays an important role in the organization of the actin cytoskeleton, gene transcription, cell cycle progression, cell transformation, and membrane trafficking (for reviews, see Refs. 1–3). RhoA cycles between the GDP-bound inactive and active forms. Three classes of molecules are known to interact and regulate GDP/GTP cycling of RhoA: guanine nucleotide exchange factors (GEFs)[1] catalyze the exchange of GDP for GTP, GTPase-activating proteins (GAPs) stimulate the intrinsic GTPase activity of RhoA, and guanine nucleotide dissociation inhibitors (GDIs) inhibit the exchange of GDP for GTP and also stabilize cytosolic RhoA form binding GTP (2). For RhoA to transmit signals, two criteria must be satisfied: first, it must in a GTP-loaded form; second, RhoA, which is geranylgeranylated, must be in the right signaling compartment attached to the plasma membrane, where it can interact with its regulators and targets. Thus, RhoA in its GTP-bound active form associates with the inner surface of the plasma membrane (4), whereas GDI-mediated inactivation of RhoA translocates it to the cytoplasm and prevents it from binding to GTP.

The role of RhoA during neuronal differentiation was first suggested by studies using the Rho-specific ADP-ribosyltransferase C3 toxin (*Clostridium botulinum* C3 Rho-ADP-ribosylating exoenzyme), which induces neurite outgrowth in naive (not treated with NGF) PC12 cells (5). Furthermore, RhoA has been shown to be involved in the regulation of neurite outgrowth (6–10). In our earlier studies (11), we found that expression of activated V14RhoA mutant prevented NGF-induced neurite outgrowth. In contrast, dominant negative RhoA (N19RhoA) expression led to an increase in neurite initiation and branching. Furthermore, RhoA was shown to have a dual role during neuronal differentiation. Inactivation of RhoA appears necessary for the initiation of neuronal differentiation, although during later stages of neurite elongation, introduction of N19RhoA causes the formation of short neurites (11). Although these findings suggest an important role of RhoA in NGF-induced neuronal morphogenesis, little is known about the signal transduction pathways that couple NGF signaling to RhoA.

In their active forms, RhoA proteins interact with and modulate the activity of effector proteins that include the serine/threonine protein kinase N (p120, PKN), p160 RhoA-associated kinase ROKα (ROK or ROCK-II), p150 RhoA-binding kinase ROKβ (ROCK-I), citron-K, and nonkinases rhophilin, rhotekin, citron-N, and p140mDia (12, 13). Among these, ROK has been shown to mediate the formation of stress fibers, focal adhesions, regulation of myosin phosphorylation, and c-*fos* expression (for review, see Ref. 14). Moreover, activation of ROK is

* This work was supported by Research Grant IBN-9728147 from the National Science Foundation and Research Grant HL-61469 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a postdoctoral training award from the Center of Excellence in Neuroscience of the University of Tennessee Health Sciences Center.

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[1] The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; NGF, nerve growth factor; C3 exoenzyme, *Clostridium botulinum* Rho-ADP-ribosyltransferase; TBST, Tris-buffered saline with Tween 20; GST, glutathione *S*-transferase; HMF, heavy membrane fraction; TrkA, NGF receptor; GTPγS, guanosine 5′-*O*-thiotriphosphate.

sufficient to induce neurite retraction in the NGF-differentiated PC12 cells (15).

Rac1 is an essential mediator of axonal growth, guidance, and branching (8, 9, 16–18). In fibroblasts, a coupled activation of Rho by Rac and Cdc42 has been described (19), linking these GTPases into a system that regulates almost all aspects of cytoskeletal organization. However, this activation cascade appears to be a cell type-specific phenomenon; and in neuronal cells, Rac1 and RhoA, although both activable by Cdc42, have opposing effects on neurite outgrowth (8).

In the present study, we investigated the effect of NGF treatment on the GTP loading and membrane association of RhoA during the initiation and elongation phase of neuronal differentiation. We show that NGF through its TrkA receptor transiently inhibits RhoA signaling, as evidenced by its cytoplasmic translocation and diminished ability to interact with ROKα. However, during the elongation phase of neuronal differentiation, 2 days after NGF treatment, RhoA was relocated to the plasma membrane and its activation state returned to the control level. We provide evidence that TrkA, independently from Ras-mediated signaling, regulates PI3K, which through the small GTPase Rac1 mediates RhoA inactivation, which is a permissive signal for the initiation of neurite outgrowth. The present results establish an inhibitory coupling between the TrkA and RhoA through a novel negative regulatory mechanism connecting Rac1 to RhoA in PC12 cells.

MATERIALS AND METHODS

Cell Culture—Wild-type PC12 cells and the M-M17-26 clone, which stably expresses dominant negative Ras mutant, were kindly provided by Dr. G. Cooper (Boston University, Boston, MA). The TrkA-deficient nnr5 clone was provided by Dr. L. A. Green (Columbia University, New York). B5 cells derived from nnr5 overexpressing TrkA were a gift from Dr. Susan Meakin (Robarts Research Institute, Ontario, Canada). All of these clones were grown in RPMI medium supplemented with 10% horse serum, 5% fetal bovine serum, and 1% glutamine (normal medium). To initiate differentiation, 7×10^6 cells were plated in 100-mm dishes in normal medium and exposed to 100 ng/ml NGF (Alamone Laboratories, Jerusalem, Israel). Under these conditions, more than 75% of the wild-type PC12 and B5 cells extended neurites by day 2.

Transient Transfection—Wild-type PC12 cells were transiently transfected with either dominant negative Rac1 construct (pN17Rac1-IRES-GFP) or empty vector (pMX-IRES-GFP) using the Cytofectene reagent (Bio-Rad). Plasmid (2 μg) and 20 μl of Cytofectene reagent were used for every ml of transfection solution. After exposing the cells to this mixture for 8 h, cells were grown in normal medium. Expression of GFP was detected, and pictures of cells expressing GFP were taken using fluorescence microscopy. Between days 3 and 6 after transfection, 80% of cells expressed GFP; therefore, NGF treatment was carried out between postransfection days 3–6.

Isolation of Whole Cell Lysate—Briefly, after NGF treatment, cells were rinsed with 3 ml of ice-cold phosphate-buffered saline and lysed with 450 μl of lysis buffer A containing 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, and protease inhibitor mixture from Sigma. This lysate was incubated for 10 min on ice, and the crude cell debris was removed by centrifugation at $10,000 \times g$ for 10 min. The supernatant was designated "whole cell lysate" and used for the experiments.

Membrane Fractionation—After NGF treatment, cells were rinsed with 3 ml of ice-cold phosphate-buffered saline and lysed with 450 μl of lysis buffer B containing of 50 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, and protease inhibitor mixture. The lysate was sonicated twice for 15 s, and the unbroken cells and nuclei were removed by centrifugation at $1,500 \times g$ for 10 min. The supernatant was further centrifuged at $15,000 \times g$ for 10 min at 4 °C to sediment the crude heavy membrane fraction (HMF). The supernatant of the HMF was used as cytosol. The crude HMF was resuspended in lysis buffer B and recentrifuged at $15,000 \times g$ for 10 min and designated "HMF."

Western Blot Analysis—Protein (20 μg applied per lane) was separated by SDS-PAGE on 12% gels and transferred to polyvinylidene difluoride membranes (Bio-Rad) using a semidry transfer apparatus (Bio-Rad). To block nonspecific binding, membranes were incubated overnight at room temperature in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 (TBST) containing 5% nonfat dry milk (Bio-Rad). After blocking, the membranes were probed for 2 h with the primary antibody diluted in TBST and washed three times with TBST before incubation for 45 min with either peroxidase-conjugated secondary anti-mouse antibody (Sigma, 1:5,000) or donkey anti-rabbit antibody (Promega, Madison, WI; 1:7,500). The SuperSignal Reagent (Pierce) was used to visualize antibody binding. The following primary antibodies were used: anti-RhoA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:500), anti-Rac1 monoclonal antibody (Santa Cruz Biotechnology; diluted 1:500), anti-phospho-Akt Ser473 antibody (New England Biolabs, Beverly, MA; diluted 1:1,000), and anti-Akt antibody (New England Biolabs; diluted 1:1,000).

RhoA and Rac1 Activation Assays—The glutathione agarose-immobilized (GST) Rho-kinase (GST-ROK), which contains the Rho binding domain of ROK and GST-PAK1, which contains the p21-binding domain of human PAK1 (residues 51–135), was expressed in *Escherichia coli* by using the pGEX-KG vector. Bacteria were lysed by 2×30 s sonication in 50 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 100 mM NaCl, and protease inhibitor mixture (lysis buffer C) containing 50 μg/ml lysosyme. The lysate was cleared by centrifugation at $20,000 \times g$ for 30 min at 4 °C. The fusion protein was purified using glutathione beads (Sigma) from the supernatant. Beads were washed three times with lysis buffer C. GST-ROK or GST-PAK1 immobilized on glutathione beads was added directly to either the HMF, cytosol, or whole cell lysate. After 45 min of shaking at 4 °C, beads were washed with lysis buffer C; the bound RhoA or Rac1 was detected by Western blotting using anti-RhoA or anti-Rac1 antibodies. Every pull-down assay was repeated at least three times using the HMF and two times using whole cell lysate. For controls, RhoA and Rac1 were loaded with either GDP or GTPγS in every pull-down assay.

Statistical Methods—Student's t test for paired variables was used to test for differences elicited by NGF treatment, and data were considered significantly different at $p < 0.05$.

RESULTS

NGF Treatment Induces a Rapid and Transient Translocation and Inactivation of RhoA during Neurite Initiation Phase—RhoA was detected in the HMF of control naive PC12 cells (Fig. 1*A*). A brief 20-min treatment with 100 ng/ml NGF caused a translocation of RhoA from the membrane to the cytosol, and the membrane-associated fraction remained low up to 2 h (Fig. 1, *A* and *B*). As a positive control, we used C3 toxin, which inactivates RhoA by ADP-ribosylating it on Asn^{41} and blocks its ability to activate downstream targets. C3 treatment almost completely removed RhoA from the HMF (Fig. 1*A*) and diminished its binding to ROK both in the membrane fraction (Fig. 1*A*) and in whole cell lysates (data not shown). The RhoA blots were stripped and reprobed with an antibody to Rac1 (Fig. 1*B*). In contrast to RhoA, the amount of Rac1 increased slightly in the same HMF samples. RhoA showed a slight increase in the cytoplasm, whereas when the same blot was probed for Rac1 after stripping, a modest decrease was observed. To quantify the amount of RhoA detected by Western blotting, the area and intensity of bands were integrated and normalized to the untreated control sample (Fig. 1*C*). Membrane-bound RhoA is accessible to GEFs and presumed to be predominantly in a GTP-bound activated state, whereas cytosolic RhoA is in an inactivated GDP-bound state associated with GDI. Although a dissociation of RhoA from the plasma membrane is suggestive of its inactivation (Fig. 1, *A–C*), to obtain direct evidence for a reduced activation state elicited by NGF treatment, we measured the GTP-dependent binding affinity of RhoA to its effector, ROK, which has previously been shown to be inactivated during neurite outgrowth (15). In the HMF, the amount of GTP-bound RhoA decreased within the first 2 h of NGF treatment (Fig. 1*D*), as evidenced by its diminished ability to bind to ROK. The decrease was rapid because ROK-bound activated RhoA in the HMF decreased by as much as 40 ± 18% within 20-min treatment. After 2-h NGF treatment, the decrease in activated RhoA diminished further to as low as 30 ± 10% of the untreated control cells (Fig. 1*D*). Similar results were obtained when whole cell lysate was used

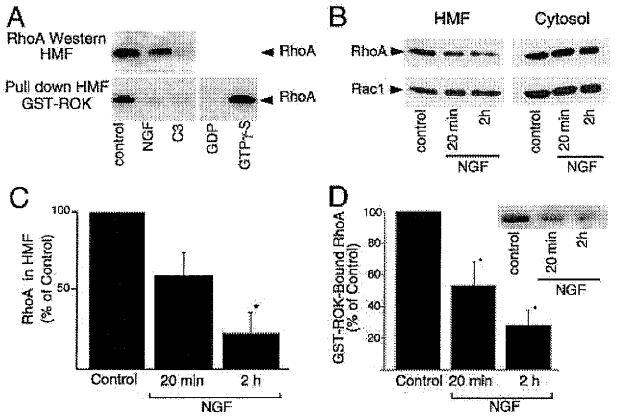

FIG. 1. **NGF induces translocation and inactivation of RhoA during neurite initiation.** *A*, the HMF was isolated from untreated controls or cells treated with either NGF (100 ng/ml) or C3 (10 μg/ml) for 20 min. Protein (20 μg per sample) was analyzed by Western blotting with an anti-RhoA antibody. For the pull-down assay with GST-ROK, 100 μg of HMF protein was used. For controls, a 10 μM concentration of either GDP or GTPγS was added to the HMF for 20 min prior to incubation with GST-ROK. NGF and C3 treatment of the membranes decreased the amount of membrane-associated RhoA and its ability to bind to ROK. *B–D*, PC12 cells were treated with NGF (100 ng/ml) for the times indicated, and the HMF was isolated and analyzed by Western blotting or GST-ROK pull-down assay. For Rac1 determination, blots were stripped of the anti-RhoA antibody and reprobed with an anti-Rac1 antibody. The intensity of the bands was measured and normalized to an untreated control sample. Data shown are the mean values (±S.E.) from at least three separate experiments (*, $p < 0.05$). Note in *B* and *C* that the amount of RhoA decreased in the HMF, whereas Rac1 showed a modest increase (*B*). Concomitant but opposite changes were seen in the RhoA and Rac1 immunoreactivity in the cytosol. The decrease in the membrane-associated RhoA was accompanied by diminished binding to ROK-GST (*D*).

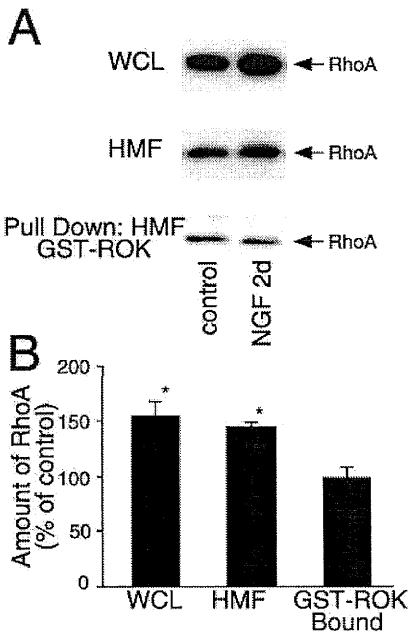

FIG. 2. **NGF-elicited changes in membrane-associated RhoA and RhoA activity during neurite elongation.** *A*, whole cell lysate (*WCL, upper panel*) and the HMF (*middle panel*) were isolated from untreated (control) or differentiating cells treated with NGF (100 ng/ml) for 2 days. Protein (20 μg/lane) was analyzed by Western blotting with an anti-RhoA antibody. HMF protein (100 μg) was used in the pull-down assay with GST-ROK (*lower panel*). The amount of RhoA immunoreactivity increased in both whole cell lysate and the HMF, whereas no changes were detected in its level of association with ROK. *B*, quantification of RhoA in whole cell lysate, in the HMF, or available to bind ROK after a 2-day NGF treatment. Band intensity was normalized to that of the untreated control sample; data are the means (±S.E.) of three experiments (*, $p < 0.05$).

in the pull-down assay (data not shown).

The cytosol was abundant in RhoA immunoreactivity (Fig. 1*B*). Despite this, no RhoA was pulled down from the cytoplasm by ROK (data not shown). However, when GTPγS was added exogenously, RhoA was pulled down by ROK from the cytosol of untreated control and NGF-treated cells, suggesting that it remained readily activable (data not shown). Addition of GDP did not have any effect on the binding of cytosolic RhoA to ROK (data not shown). Taken together, these data confirm that RhoA resident to the cytoplasm is in an inactivated but activable state and that NGF treatment translocates RhoA to this pool. Addition of GDP to the HMF diminished RhoA activation, whereas GTPγS increased it, validating the sensitivity of the assay (Fig. 1*A*).

NGF Increases RhoA Activation during Neurite Elongation—Wild-type PC12 cells were exposed to either NGF or vehicle for 2 days. The PC12 cells used in the present study responded rapidly to NGF; after 1 day, the cells began extending neurites that reached the diameter of a cell body on the second day of NGF treatment. Thus, after 2 days of NGF exposure, these cells were in the elongation phase of differentiation when whole cell lysate and the HMF were isolated and the amount of RhoA determined. A 2-day NGF treatment caused a 55 ± 15% and 46 ± 4% increase in the amount of total RhoA in whole cell lysate and membrane-associated RhoA in the HMF, respectively (Fig. 2, *A* and *B*). The GST-ROK pull-down assay with the HMF showed no significant change as compared with untreated control cells. Taken together, the present results (Figs. 1 and 2) showed that NGF increases RhoA expression but not activation during neurite elongation.

TrkA Receptor Is Required for RhoA Activation and Translocation—NGF activates TrkA and p75 receptors in PC12 cells. The nnr5 cell line, a clone of the PC12 cell line, lacks functional TrkA, whereas the B5 cell line is an nnr5 clone that stably overexpresses TrkA. These two clones were used to assess the role of the TrkA receptor in the regulation of RhoA. In contrast to wild-type PC12 cells, NGF exposure did not significantly change the amount of membrane-associated RhoA in nnr5 cells (Fig. 3). NGF treatment caused no detectable change in the amount of ROK-bound RhoA in nnr5 cells (Fig. 3). In the B5 cells, NGF treatment decreased the amount of membrane-associated RhoA (Fig. 3), as was seen in wild-type PC12 cells (Fig. 1). The amount of ROK-bound RhoA decreased after brief NGF treatment; thus, overexpression of TrkA in nnr5 cells restored the effect of NGF on RhoA signaling. These findings indicate the requirement for functional TrkA receptor in NGF-induced inactivation of RhoA.

Expression of the Dominant Negative N17Ras Mutant Does Not Inhibit the Effect of NGF on RhoA Translocation and Inactivation—Because Ras activation is a necessary and sufficient signaling event for neuronal differentiation, we tested NGF-induced inactivation of RhoA in M-M17-26 cells, which stably express high amounts of the dominant negative mutant N17-Ras. In M-M17-26 cells, a brief 20-min NGF treatment led to a 48 ± 7.8% decrease in membrane-bound RhoA. Furthermore, 2 h of NGF treatment reduced the amount of RhoA in the HMF to 27 ± 14.2% of that in untreated control cells (Fig. 3). This effect of NGF did not differ significantly from that found in wild-type PC12 cells (Fig. 1). The amount of ROK-associated RhoA also decreased during the first 2 h of NGF treatment (Fig. 3). These data indicate that Ras activity is not necessary for the inactivation of RhoA during the initial phase of NGF-

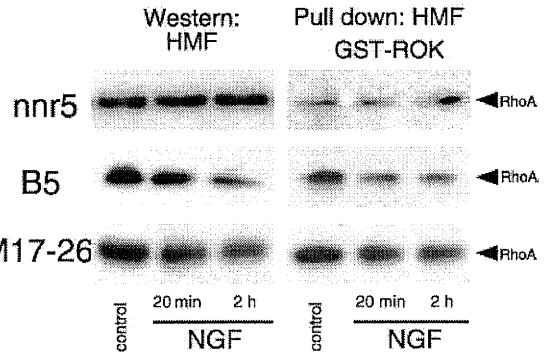

FIG. 3. **Functional TrkA but not Ras is required for NGF-induced RhoA translocation and decreased ROK binding.** *Top*, nnr5 cells lacking a functional TrkA receptor show no translocation of RhoA and have decreased association with ROK. *Middle*, B5 cells are nnr5 cells that overexpress the TrkA receptor. They show restored responses, as seen in wild-type cells. *Bottom*, M-M17-26 cells expressing dominant negative Ras show translocation of RhoA and decreased ROK binding after NGF treatment, as seen in wild-type cells. Protein (20 μg/lane) was analyzed by Western blotting with an anti-RhoA antibody (*left panels*). HMF protein (100 μg) was used in the pull-down assay with GST-ROK, followed by Western blotting with anti-RhoA antibody (*right panels*). Results shown are representative of three experiments.

induced differentiation.

Expression of Dominant Negative Rac1 (N17Rac1) Inhibits the Effect of NGF on RhoA Activity—The Rac1 GTPase has been found capable of regulating RhoA activity in fibroblasts (19) and neuroblastoma cells (8). To test the role of Rac1, the dominant negative N17Rac mutant was transiently expressed in PC12 cells using a tandem vector that also expresses GFP. An empty vector plasmid expressing GFP only was used as a negative control. Expression of N17Rac, but not of GFP, inhibited neuronal differentiation in NGF-treated cells, as these cells developed no neurites (Fig. 4*A*). To further control the effect of transient expression of N17Rac protein, NGF-induced activation of Rac1 was measured using a PAK pull-down assay (Fig. 4*B*). NGF treatment caused rapid activation of Rac1 in vector-transfected cells but did not cause a detectable activation of Rac1 in N17Rac1-expressing cells (Fig. 4*B*). These experiments confirmed that the expression of N17Rac1 inhibited the function of Rac1 in these cells. In cells expressing N17Rac1, NGF did not induce RhoA translocation from the membrane to the cytosol (Fig. 4*C*). Moreover, the amount of ROK-associated RhoA showed no detectable decrease after NGF treatment. As in wild-type PC12 cells, NGF decreased the amount of both membrane- and ROK-associated RhoA in vector-transfected cells (Fig. 4, *B* and *C*), suggesting that Rac1 mediates an inhibitory signal from TrkA to RhoA.

Yamaguchi *et al.* (37) reported recently that expression of constitutively active V14RhoA blocked NGF-induced activation of Rac1. However, these authors used an 18-h-long serum starvation, which in our hands induces apoptosis. We have tried to reproduce these findings but with a shorter 4-h serum starvation in PC12 cells transiently transfected with V14RhoA. As shown in Fig. 4*D*, under these conditions, activation of Rac1 following a 10-min exposure to NGF measured by PAK pull-down was not attenuated in PC12 cells transfected with V14RhoA as compared with vector-transfected cells.

PI3K Inhibitors, Wortmannin and LY294002 Block NGF-induced Inactivation of RhoA—Signals from the TrkA-receptor are transmitted in part by PI3K, which in other cell types has been linked to the regulation of Rac1 (20). To test the role of PI3K in the NGF-induced inactivation of RhoA, a brief 2-h LY294002 treatment was applied before exposure to NGF. Pretreatment of the cells with 30 μM LY294002 alone did not change the amount of membrane- or ROK-associated RhoA (data not shown). In cells pretreated with LY294002, there was no detectable change in the amount of RhoA in the HMF after NGF treatment (Fig. 5). Furthermore, using the ROK pull-down assay, we found that the amount of ROK-associated RhoA also remained unchanged after NGF treatment (Fig. 5). Similar results were obtained when the same experiments were carried out in cells pretreated with another PI3K inhibitor, wortmannin (Fig. 5). These results indicate that PI3K activity is required for the NGF-induced regulation of RhoA.

In PC12 Cells PI3K Acts Upstream from Rac1 Mediating the Inactivation of RhoA—We have also investigated the hierarchical relationship between Rac1 and PI3K in signaling events leading to the inactivation of RhoA. First, we inhibited PI3K with LY294002 and then measured the NGF-induced activation of Rac1 by using the PAK pull-down assay. In the control cells, without LY924002 pretreatment, NGF increased the amount of activated Rac1 4-fold (Fig. 6*A*). In contrast, in PC12 cells pretreated with 30 μM LY924002 for 2 h prior to 5 min NGF treatment, PAK activation was reduced to 2-fold but not abolished (Fig. 6*A*). Next, to test whether Rac1 mediates PI3K activation, a dominant negative N17Rac1 mutant was transiently transfected in PC12 cells. To monitor the inhibitory effect of N17Rac1, the GST-PAK pull-down assay was used (see Fig. 4*B*). To determine whether N17Rac1 inhibited the activation of PI3K, we measured the phosphorylation of Akt by Western blot using anti-phospho-Akt antibody. There was no detectable phosphorylated Akt in serum-starved PC12 cells (Fig. 6*B*). NGF caused a rapid increase in the phosphorylation of Akt in vector- and N17Rac1-transfected PC12 cells (Fig. 6*B*). Using anti-Akt antibody, we found that the amount of Akt was same in every sample (Fig. 6*B*). Thus, our data indicate that NGF-induced activation of PI3K in PC12 cells was independent of Rac1.

DISCUSSION

In the present report, we sought to establish the mechanism underlying our earlier observations using constitutively active and dominant negative RhoA mutants (11), which provided circumstantial evidence for a dual role of RhoA in NGF-induced differentiation of PC12 cells. Activated RhoA prevented the withdrawal of the cell from the cell cycle and abolished neurite outgrowth, whereas dominant negative RhoA enhanced differentiation. This observation implied that NGF signaling should negatively couple to RhoA to promote the early events of differentiation, which include the cessation of cell proliferation and the initiation of neurite outgrowth. In contrast to naive PC12 cells, in differentiated cells, we found that the expression of activated RhoA accelerated the rate of neurite elongation, whereas dominant negative RhoA reduced it. In the present study, we first investigated the effect of NGF signaling on RhoA by measuring its translocation and GTP-loading as measured by the affinity to its target, ROK.

Here we provide evidence that a brief treatment with NGF leads to RhoA inactivation, as shown by its rapid translocation from the membrane to the cytosol and a decrease in its ability to associate with its downstream target, ROK (Fig. 1). These results provide a mechanism for our earlier phenomenological observation that NGF somehow inactivated RhoA-ROK signaling during the initiation phase of neuronal differentiation. We extended our study to determine not only the localization but also the activation state of RhoA during neurite elongation. Again, in agreement with the previous study using RhoA mutants, we found that a prolonged 2-day NGF treatment increased RhoA expression and its association with the plasma membrane during the neurite elongation phase. Although prolonged NGF treatment caused no elevation in the GTP-bound

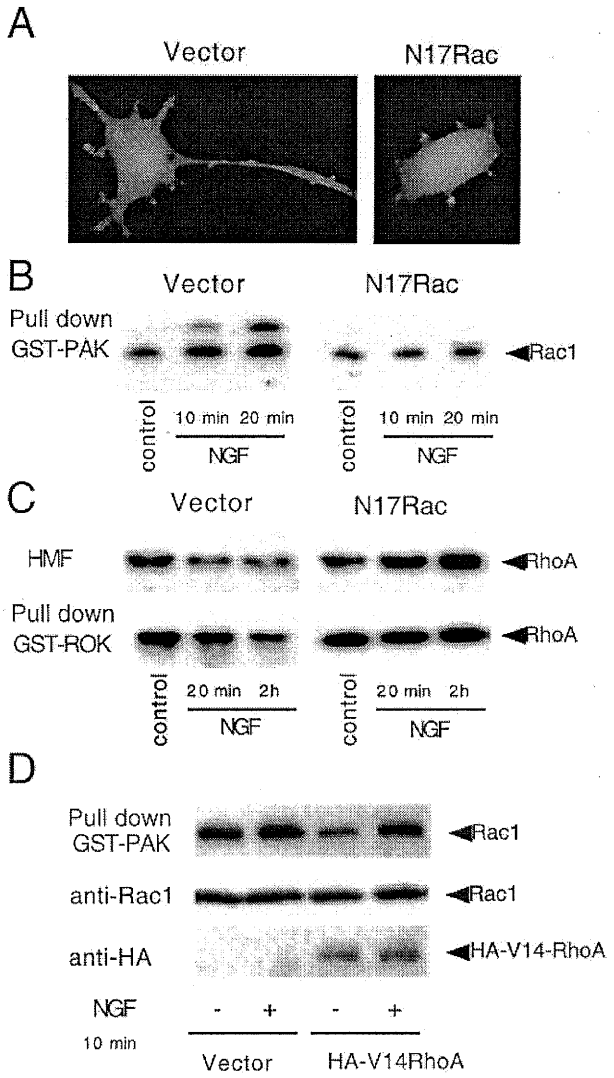

FIG. 4. **Expression of dominant negative N17Rac1 inhibits the effect of NGF on RhoA inactivation.** PC12 cells were transiently transfected with either the pMX-IRES-GFP vector or pN17Rac1-IRES-GFP plasmid encoding the N17Rac1 gene. *A* shows two representative pictures of PC12 cells expressing either GFP vector or GFP with N17Rac1. Three days after transfection, cells were treated with 100 ng/ml NGF for another 4 days to induce neuronal differentiation. N17Rac1-expressing cells extended no neurites. Calibration *bar*, 20 μm. *B*, PC12 cells were transiently transfected with either pMX-IRES-GFP vector or pN17Rac1-IRES-GFP plasmid expressing the dominant negative N17Rac1. Three days after transfection, cells were treated with 100 ng/ml NGF for the times indicated. Whole cell lysate was isolated and used in the pull-down assay with GST-PAK, followed by Western blotting with anti-Rac1 antibody. NGF induced Rac activation in vector-transfected, but not in N17Rac-transfected, cells. The intensity of the bands was measured and normalized to a control sample. Data shown are the mean values (±S.E.) from at least three separate experiments (*, $p < 0.05$). *C*, expression of N17Rac inhibited the effect of NGF on inactivation of RhoA. On the third day after transfection, cells were treated with 100 ng/ml NGF for the times indicated and the HMF was isolated. Protein (20 μg/lane) were analyzed by Western blotting with an anti-RhoA antibody (*upper panels*). HMF proteins (100 μg of HMF) were also used in the pull-down assay with GST-ROK, followed by Western blotting with anti-RhoA antibody (*lower panels*). *D*, expression of V14RhoA does not inhibit NGF-induced Rac1 activation as measured by GST-PAK pull down. PC12 cells were transfected with V14RhoA or empty vector for 36 h. Cells were withdrawn from serum by replacing the medium with serum-free RPMI 1640 for 4 h. Cells were exposed to 100 ng/ml NGF for 10 min. Cell lysates were prepared and the expression of hemagglutinin-tagged V14RhoA was determined using anti-hemagglutinin antibody. Rac1 activation was determined using GST-PAK, and the amount of activated Rac1 was monitored by an anti-Rac-1 antibody by Western blotting. NGF-elicited Rac1 activation was indistinguishable in vector-transfected and V14Rho-expressing cells.

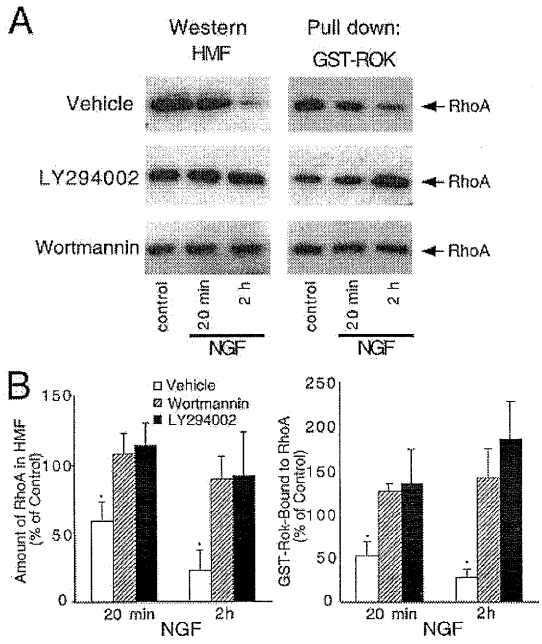

FIG. 5. **Wortmannin and LY294002, inhibitors of PI3K, block NGF-induced inactivation of RhoA.** *A*, PC12 cells were treated with either 30 μM LY294002 compound or 500 nM wortmannin for 2 h prior to treatment with 100 ng/ml NGF for the times indicated. HMF protein (20 μg/lane) was analyzed by Western blotting with an anti-RhoA antibody (*left panels*). HMF protein (100 μg) was used in the pull-down assay with GST-ROK, followed by Western blotting with anti-RhoA antibody (*right panels*). Wortmannin and LY294002 abolished NGF-induced translocation and decreased GST-ROK binding of RhoA. *B*, quantification of RhoA content in the HMF and association with ROK relative to vehicle-treated control. Data represent the means (±S.E.) of at three experiments (*, $p < 0.05$). Both inhibitors abrogated the changes seen in vehicle-treated controls and even caused a modest rise in the level of activated RhoA associated with ROK.

state of RhoA because the amount of ROK-bound RhoA returned to the a level seen in naive cells, this change represents a 70% increase as compared with the initiation phase (compare Fig. 1 and Fig. 2). Taken together, these results support our dual-role model proposed earlier: NGF treatment rapidly inactivates RhoA during the initiation phase, but it returns to the plasma membrane compartment with a basal level of GTP loading during the elongation phase of neuronal differentiation.

Which NGF receptor mediates the inhibitory effect to RhoA? Using nnr5 cells deficient in functional TrkA receptor and a receptor add-back clone the B5 cells, we found evidence that TrkA is necessary for the inhibitory coupling to RhoA.

We next turned our focus to TrkA-linked signaling pathways, specifically the Ras pathway because it has a central role in mediating NGF-induced neuronal differentiation. Settleman *et al.* (21) reported a cross-talk between Ras and Rho by showing that p190Rho-GAP was tyrosine-phosphorylated and formed a complex with the SH2 domain of p120Ras-GAP, suggesting that the latter may act as a Ras effector negatively regulating the activity of RhoA. Booden *et al.* (22) found that expression of constitutively activated Ras elicited neurite outgrowth that was prevented by the co-expression of inhibitory Rho only in

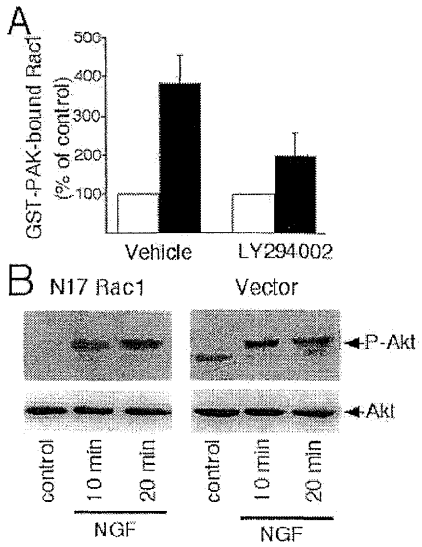

FIG. 6. **PI3K regulates Rac1 in the NGF-activated inhibitory signal transduction pathway to RhoA.** *A*, PC12 cells were pretreated with 30 μM LY294002 compound for 2 h prior to exposure to 100 ng/ml NGF for the times indicated. Whole cell lysate (100 μg) was also used in the pull-down assay with GST-PAK, followed by Western blotting with anti-Rac1 antibody (*upper panels*). The intensity of the bands was measured and normalized to the control. Data shown are the mean values (±S.E.) from at least three separate experiments (*, $p < 0.05$). Although LY294002 substantially reduced Rac1 activation, it did not abolish it. *B*, PC12 cells were transiently transfected with either pMX-IRES-GFP vector or pN17Rac1-IRES-GFP plasmid expressing the dominant negative N17Rac1. Three days after transfection, cells were treated with NGF for the times indicated, and whole cell lysate was isolated. Protein (20 μg/lane) was analyzed by Western blotting with either an anti-phospho-Akt (Ser^{473}) antibody (*upper panels*) or an anti-Akt antibody (*lower panels*). N17Rac did not interfere with Akt activation, as indicated by an increase in Akt phosphorylation.

the presence of increased amounts of inhibitory Rac1. These results suggest that RhoA and Rac1 were coupled to Ras; however, these authors did not elicit differentiation using NGF, but rather with an activated Ras mutant. We reported that activation of RhoA during neurite retraction caused by lysophosphatidic acid was independent of Ras (23), suggesting the possibility of Ras-independent signaling from NGF toward RhoA. Recently, Boglari and Szeberenyi (24) showed that a TrkA-independent Ras pathway exists that is sufficient to mediate neurite outgrowth. Using a PC12 subclone, the M-M17-26 line that expresses a dominant negative N17Ras (25), we found no significant difference between the effect of NGF on the inactivation of RhoA in wild-type and N17Ras-expressing cells. The M-M17-26 cell line has been well characterized, and there is no evidence for NGF-induced ERK1/2 activation in these cells (data not shown), whereas NGF activates PI3K and consequently Akt (26). Taken together, our results provide further evidence for a dichotic signaling originating from TrkA: a Ras-dependent branch that activates the MAPK cascade and a Ras-independent one that mediates the inactivation of RhoA and is linked to the PI3K-Akt pathway.

The lipid kinase PI3K can mediate signals from Ras to Rac1 required for the oncogenic transformation of Rat-1 cells (27). *In vitro* data suggest that Rac1 specifically interacts with phosphatidylinositol 3,4,5-trisphosphate, a product of PI3K (28). RhoA also displays significant, although much weaker, binding to this lipid (28). Expression of an activated Ras mutant caused increased Rac1 GTP binding and PI3K activity in membranes of PC12 cells (29).

A hierarchical cascade linking CDC42, Rac1, and RhoA was

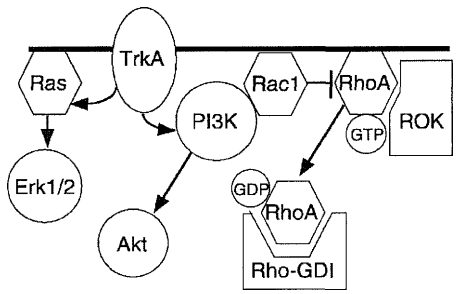

FIG. 7. **A scheme of the signaling pathways linking TrkA to the inhibition of RhoA.** TrkA, independently of Ras, activates PI3K. PI3K activates Rac1, and active Rac1 induces the translocation of RhoA to the cytoplasm, where it may complex with Rho-GDIs and also inhibits its ability to bind to ROK.

first described in fibroblasts (19). According to this hypothesis based on studies conducted in fibroblasts, activation of Cdc42 induces activation of Rac1, which in turn leads to activation of Rho (for review, see Refs. 2 and 30). To extend this model, functional studies established PI3K as an effector of Ras and an upstream modulator of Rac1 activity (20). However, in Swiss 3T3 fibroblast cells, wortmannin does not interfere with Ras-mediated actin remodeling (31). Hence, a secondary pathway is likely to exist (receptor tyrosine kinase → PI3K → Rac1) that is Ras-independent and turned on by the activation of receptor tyrosine kinases, including TrkA. The Ras-independent pathway for Rac1 regulation is supported by the phosphorylation-dependent direct association of PI3K with the Tyr^{751} residue of TrkA (32). PI3K has been shown to regulate at least two of the Rac1-GEFs, Vav and Sos-1 (33, 34). We found no change in the amount of RhoA in the HMF after NGF treatment of cells pretreated with either LY294002 or wortmannin (Fig. 5). These results suggest that PI3K activation is required for the NGF-induced regulation of RhoA. In cells expressing dominant negative Rac1, NGF did not induced translocation of RhoA from the membrane to the cytosol (Fig. 4). Furthermore, the amount of ROK-associated RhoA also showed no detectable decrease in response to NGF treatment in N17Rac1-expressing cells (Fig. 4, *B* and *C*). Finally, LY294002 or wortmannin substantially reduced NGF-induced activation of Rac1 in PC12 cells (Fig. 6*A*). In contrast, NGF caused a rapid increase in phosphorylation of Akt in both vector- and N17Rac1-transfected PC12 cells (Fig. 6*B*). Hence, our data suggest that PI3K acts upstream from Rac1 in the NGF-activated signal transduction pathway inhibiting RhoA (Fig. 7).

However, the relationship between Rac1 and Rho is more complex and likely depends on the cell type. In contrast to the Cdc42 → Rac1 → Rho hierarchical cascade described in fibroblasts, no evidence for such a relationship has been found in neuronal cells. For example, in N1E-115 cells, PAK5 mediates signals from Cdc42 and Rac1 leading to the inactivation of RhoA (35). Sander *et al.* (36) showed in NIH3T3 cells that activation of Rac1 leads to the inhibition of RhoA, suggesting that Rac1 is upstream of RhoA. Yamaguchi *et al.* (37) reported recently that RhoA suppressed NGF-induced Rac1 activation through the Rho-associated kinase pathway in PC12 cells, suggesting that Rho acts upstream to Rac1. In their experiments, however, NGF treatment was performed following an 18-h serum starvation and growth factor deprivation of PC12 cells, suggesting that this effect is more pertinent to an anti-apoptotic rescue effect of NGF rather than that of inducing neuronal differentiation. Under the conditions used in our assays, we found no inhibiton of NGF-induced Rac1 activation in response to NGF, suggesting that the prolonged serum starvation used by those authors may have affected the outcome of

the assay. In our hands an 18-h-long serum withdrawal has adverse effect of the adherence of the cells, and cells are beginning to undergo apoptosis (data not shown).

Taken together, our results in naive PC12 cells support a Ras-independent signal transduction pathway linking TrkA to Rac1 through PI3K, which mediates the inhibition of RhoA during the early stages of neuronal differentiation (Fig. 7). The signaling complexes involved in coupling these molecules is subject of ongoing studies.

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Submitted to Current Biology:

Phosphorylation-regulated Target Selection of RhoA Is Required for NGF-Induced Neurite Outgrowth

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Abstract

Extension of neurites is inhibited by activated RhoA GTPase, which is considered a molecular switch, turned on upon binding of GTP and off when GTP is hydrolyzed to GDP. When monitoring NGF-induced RhoA activation of multiple effectors in PC12 cells, that according to the molecular switch hypothesis, would have to be uniformly activated by GTP-loaded RhoA, we noted differential interactions with its effectors. NGF elicited a protein kinase A-mediated phosphorylation of RhoA on serine^{188}, which rendered it unable to bind to Rho-associated kinase (ROK), whereas it retained the ability to interact with other RhoA targets including rhotekin, mDia and PKN. We show that phosphorylation of serine^{188} represents an additional switch, capable of channeling signals among effector pathways. NGF-induced phosphorylation of RhoA prevents ROK activation, which would otherwise inhibit neurite outgrowth, hence it is an obligatory step for the initiation of neurite outgrowth in PC12 cells. Therefore, phosphorylation of ^{188}serine serves as a novel secondary switch in regulating RhoA function, capable of overriding GTP-binding-elicited target activation.

Introduction

Extension of neuronal processes is a fundamental process in the establishment of neural networks. Neurite initiation and outgrowth in the developing nervous system is regulated by chemoattractants and chemorepellants, whose signals are mediated by the Rho family GTPases. RhoA is a member of the Ras superfamily of low-molecular-weight GTPases essential for the regulation of the actin cytoskeleton, gene transcription, cell cycle progression, cell transformation, and membrane trafficking (1-6). RhoA, like other GTPases, is considered a molecular switch turned on upon binding of GTP and turned off when GTP is hydrolyzed to GDP (4).

Activators of RhoA, including myelin-associated glycoprotein (7), lysophosphatidic acid (8-10), and serum (8, 11), are chemorepellants that inhibit nerve growth factor (NGF)-induced neuronal differentiation and cause neurite retraction in PC12 pheochromocytoma cells. Conversely, selective inhibition of RhoA by *Clostridial* toxins (12, 13) or the introduction of dominant negative mutants (13, 14) promotes neuronal differentiation and neurite outgrowth and renders neuronal cells resistant to neurite retraction induced by chemorepellant activators of RhoA. Previous work has established that the constitutively active ^{14}Val-RhoA mutant

abolishes NGF-induced neuronal differentiation, preventing the cell's withdrawal from the cell cycle and the initiation of neurite outgrowth (13). Nonetheless, treatment of PC12 cells expressing ^{14}Val-RhoA with dibutyryl-cAMP (db-cAMP), a membrane-permeable cAMP analogue, restores NGF-induced differentiation and neurite outgrowth. Furthermore, cAMP and forskolin, activators of protein kinase A (PKA), are capable of preventing neurite retraction induced by chemorepellants (12, 13, 15-18).

It is now well established that RhoA activation can mediate opposite effects on neurites: in the developing neurite, it has an inhibitory and repellant function; but during the stage of elongation of established neurites, it enhances the rate of neurite outgrowth (13, 19-21). The opposite effects of RhoA on neurite initiation and elongation suggest that the role of RhoA signaling changes during different stages of neuronal differentiation (13). This hypothesis implies that the inhibitory effect of RhoA activation on neurite initiation must be temporarily overcome during the period of neurite initiation by an NGF-induced signal. Alternatively, it is also conceivable that specific RhoA targets are differentially modulated by, for example, PKA signaling, thereby allowing the selective temporary activation of some effectors while inhibiting others.

In pursuit of the first hypothesis and because the activation of the PKA signaling pathway appears to overcome the effects of ^{14}Val-RhoA as well as those of RhoA activation elicited by chemorepellants, we hypothesized that a temporary NGF-induced activation of PKA signaling might negatively modulate the interaction of RhoA with a subset of its downstream targets.

RhoA can interact with over ten different effectors that include the serine-threonine kinases p160 Rho-associated kinase (ROK or ROCKII, (22)) and p120 protein kinase N (PKN), and the non-kinases rhotekin and p140mDIA (23). Some of these effectors inhibit, whereas others promote, neurite outgrowth and neuronal differentiation. For example, ROK has been shown to mediate the formation of stress fibers, focal adhesions, regulation of myosin phosphorylation, and c-fos expression (24). In neuronal cells, activation of ROK-mediated activation of myosin II kinase, LIM kinase, and phosphatidylinositol 4-phosphate 5-kinase inhibits neurite outgrowth and causes neurite retraction (25-29). On the other hand, PKN promotes neuronal differentiation by regulating SRE-mediated gene expression and also interacts with α-actinin (30). To examine possible selective interaction of effectors with RhoA, we decided to monitor RhoA-mediated activation of several of the RhoA effectors,

which, according to the on/off switch hypothesis for GTPase signaling, would have to be uniformly activated by the GTP-loaded RhoA.

Results and Discussion

We determined the effects of a 20-min NGF treatment of PC12 cells on Rho activity using a pull-down assay in which GTP-loaded RhoA binds to the Rho-interacting domain of ROK, PKN, mDIA, or rhotekin fused to glutathione *S*-transferase (GST). The NGF treatment resulted in a rapid and marked decrease in GST-ROK binding to RhoA (Fig. 1A). The extent of RhoA inactivation revealed by the decrease in GST-ROK binding was comparable to that caused by C3 toxin (2 μg/ml, overnight), which ADP-ribosylates RhoA in the switch I effector domain. There was RhoA present in the cell lysate that could be readily activated by the non-hydrolyzable GTPγS, indicating that the assay was sensitive to GTP loading of RhoA. Thus, the decreased GST-ROK binding to RhoA might indicate that NGF treatment diminished the amount of the GTP-loaded RhoA, leading to reduced activation of this target. Unexpectedly, NGF treatment increased the binding of RhoA to GST-PKN, GST-mDIA, and GST-rhotekin (Fig. 1A). GTPγS elicited increased RhoA binding to all of these effectors. The disparate activation of these RhoA targets by NGF points to a novel mechanism capable of selectively inhibiting the activation of GTP-loaded RhoA with GST-ROK. Because of their robust interaction with GTPγS-bound RhoA, we chose GST-ROK and GST-PKN for further characterization of the NGF effect.

Similar to the NGF effect, treatment of cells with db-cAMP (500 μM) caused a disparate regulation of the two RhoA targets by inhibiting ROK while simultaneously enhancing PKN binding (Fig. 2A). We also determined the effects of forskolin (1 μM), a direct activator of adenylyl cyclase and PKA, on Rho activity by using the pull-down assay (Fig. 2B). NGF and forskolin each resulted in a rapid decrease in GST-ROK binding that was evident by 20 min and sustained up to 120 min after treatment. In the same cell lysates, GST-PKN binding to RhoA showed a time-dependent increase. Addition of GTPγS to the lysates increased RhoA binding to both targets. These results set up an apparent paradox contradicting the current dogma that GTP-loading of the GTPase is sufficient to cause its interaction with its effectors and predict that a yet unidentified signal triggered by NGF and cAMP might exert a selective negative modulation of RhoA interaction with ROK.

The cAMP-dependent PKA is involved in NGF-induced gene regulation leading to neuronal differentiation in PC12 cells (31). Because forskolin and cAMP regulate PKA and RhoA activity, we examined NGF-induced RhoA translocation and ROK- and PKN-binding in A123.7 cells, a clonal variant of PC12 cells with expression of a dominant negative regulatory subunit unable to bind cAMP and activate PKA I and PKA II (32). In A123.7 cells, NGF treatment did not cause a significant change in the interaction of RhoA with ROK or PKN (Fig. 3A). These results suggest that PKA could act as a modulator of RhoA target interaction.

To obtain further evidence for the modulatory effect of PKA on Rho/target interaction we treated wild-type PC12 cells with H89 (10 μM, 30 min pretreatment), which is a specific inhibitor of PKA, or with the myristoylated pseudosubstrate nonapeptide Myr—GRTGRRNAI (PKI, 5 μM, 30 min pretreatment) prior to NGF treatment and measured RhoA binding to GST-ROK and GST-PKN (Fig. 3B). Each agent abolished the NGF-induced decrease in RhoA binding to GST-ROK and the increase in PKN binding, complementing our observations obtained in PKA-deficient A123.7 cells. The results provide pharmacological evidence for the role of PKA in the differential regulation of RhoA effector interaction. NGF treatment of PC12 cells leads to a rapid decrease in the amount of RhoA in the plasma membrane (supplemental material Fig. 1A). Forskolin mimics this effect that is inhibited by H89, PKI and also absent in A123.7 cells (supplemental Fig. 1B). Hence, PKA signaling affects the binding of RhoA to its targets and also its translocation, the latter of which has been previously described (33).

It has been proposed that PKA phosphorylates RhoA, and the site of phosphorylation has been tentatively assigned to ^{188}Ser in the C-terminal tail (33, 34). To verify this and evaluate the effect of GTP-loading of RhoA on its phosphorylation by PKA, we purified His-epitope-tagged RhoA GTPase (His-RhoA) using Ni-Sepharose affinity chromatography from baculovirus transduced Sf-9 insect cells and incubated it with the purified recombinant catalytic subunit of PKA in vitro in the presence of γ-[^{32}P]ATP and GDP or GTPγS. We also performed the same treatments using a ^{188}Ser-Ala mutant of RhoA that has a mutated putative PKA consensus phosphorylation site. As shown in figure 4, PKA treatment caused an intense labeling of His-RhoA with [^{32}P] (Fig. 4A, lanes 1 & 2), whereas His-RhoA^{S188A} was not labeled (Fig. 4A, lanes 3 & 4). This indicates that PKA phosphorylates RhoA in vitro on a

single site at ^{188}Ser. Moreover, GTP-loading of RhoA did not affect its phosphorylation by PKA.

Next, we tested the interaction of phosphorylated RhoA with GST-ROK or GST-PKN using the pull-down assay. Addition of GTPγS to phosphorylated His-RhoA caused a 3.7-fold increase in GST-PKN binding as compared to GDP-loaded RhoA (Fig. 4B, lanes 5 & 6). In contrast, phosphorylated His-RhoA/GST-ROK binding showed only a 30% increase in RhoA binding (Fig. 4B, lanes 7 & 8) upon the addition of GTPγS. This net increase was smaller than the 45% nonspecific increase in binding observed when GST without a RhoA binding domain was incubated in the presence of GTPγS (Fig. 4B, lanes 9 & 10). Therefore, GTPγS caused no net increase in the binding of phosphorylated RhoA to GST-ROK. Taken together, these results indicate that phosphorylation of ^{188}Ser on RhoA differentially affects the interaction of PKN and ROK with RhoA in a cell-free system. Moreover, non-phosphorylated wild type RhoA, when loaded with GTP, showed increased binding to both ROK and PKN, thus underlining that GTP-loading is sufficient and necessary to trigger binding of both effectors and that phosphorylation only modulates ROK binding but not PKN binding to the GTP-bound RhoA.

These results lead us to further hypothesize that phosphorylation of RhoA on ^{188}Ser by PKA permits its interaction with PKN but attenuates interaction with ROK, thereby leading to a selective activation of one effector relative to another by the activated GTPase. This hypothesis was tested first in vitro by using purified recombinant His-tagged RhoA or the ^{188}Ser-Ala mutant treated with the catalytic subunit of PKA; and each was subjected to pull-down assay using GST-PKN or GST-ROK. GTPγS loading caused increased binding to GST-PKN for both His-RhoA and His-RhoA^{S188A} (Fig. 4D, upper panels). Treatment with PKA did not inhibit but slightly augmented the GTP-dependent complex formation between RhoA and PKN (Fig. 4D, upper left panel). As expected, the interaction of His-RhoA^{S188A} with PKN was not affected by PKA treatment (Fig. 4D upper right panel). GTPγS loading of His-RhoA or His-RhoA^{S188A} increased the complex formation with GST-ROK as compared to the GDP-loaded control (Fig. 4D, lower panels). PKA treatment of RhoA caused a significant [$p < 0.05$] reduction in binding to GST-ROK (Fig. 4D, lower left panel); however, it had no effect on His-RhoA^{S188A} (Fig. 4D, lower right panel). Thus, GTP-dependent binding of the phosphorylation deficient His-RhoA^{S188A} to ROK was similar to that seen for PKN, indicating

the pivotal role of this PKA phosphorylation site of RhoA in the inhibition of binding to ROK.

Next, by introducing epitope-tagged RhoA and its targets, we sought to determine whether activation of cAMP signaling would differentially regulate the interaction of RhoA with its targets ROK and PKN in intact cells. To extend our in vitro observations for the PKA-mediated effector selection of RhoA, HEK293 cells were cotransfected with hemaglutinin (HA)-epitope tagged RhoA or RhoA^{S188A} and GST-ROK or GST-PKN. Two days after transfection, forskolin (1 μM) was applied for 15 or 30 min to the cells to evoke cAMP production and PKA activation. The cells were lysed and GST-fusion proteins were purified and analyzed for the presence of HA-tagged RhoA using Western blotting (Fig. 5A). Forskolin treatment caused a time-dependent decrease in HA-RhoA interaction with GST-ROK indicating that interaction of ROK with RhoA is negatively regulated by cAMP signaling in vivo (Fig. 4A, upper panel). In contrast, forskolin treatment of the cells transfected with HA-RhoA^{S188A} did not decrease, but rather increased, the binding of GST-ROK (Fig. 5A, upper panel). These in vivo results fully agree with our observations made in the cell-free system and support the presumed role of PKA phosphorylation of RhoA on ^{188}Ser in mediating the inhibition of ROK binding. Similarly to endogenous RhoA in PC12 cells (Fig. 1), HA-RhoA transfected into HEK293 cells also showed an enhanced interaction with GST-PKN following forskolin treatment (Fig. 5A, lower panel). In contrast, forskolin treatment did not enhance the complex formation between GST-PKN and HA-RhoA^{S188A} (Fig. 4A, lower panel). These experiments provide proof that the interaction of RhoA with ROK and PKN in intact cells is selectively modulated by forskolin activation of cAMP signaling.

Our hypothesis predicts that NGF treatment will lead to activation of PKA, which in turn phosphorylates RhoA, thereby selectively inhibiting the interaction between RhoA and ROK, whereas it enhances the complex formation with PKN. This hypothesis was tested following NGF treatment of PC12 cells transfected with HA-tagged RhoA or RhoA^{S188A}. To ascertain the role of PKA activation in response to NGF, PKI (5 μM) was added to the cells 30 min prior to NGF treatment. The cells were lysed, and the activation state of the two RhoA targets was determined by pull-down assay. Just as for endogenous RhoA, NGF treatment elicited a time-dependent activation of GST-PKN interaction with HA-RhoA and also with HA-

$RhoA^{S188A}$ (Fig. 5B, lower panel). Pretreatment with the PKI slightly attenuated the NGF-induced increase in HA-RhoA and binding to GST-PKN (Fig. 5B, lower panel). As seen for endogenous RhoA (Fig. 2), NGF treatment caused a time-dependent decrease in HA-RhoA binding to GST-ROK (Fig. 5B, upper panel). This inhibition was abrogated and reversed by the PKI treatment (Fig. 5B, upper panel). As predicted, the HA-$RhoA^{S188A}$ mutant did not show decreased binding to GST-ROK following NGF treatment, but rather an increase, which was not abolished by the PKI (Fig. 5B, upper panel). These experiments are consistent with the hypothesis that NGF-induced activation of PKA inhibits the interaction of RhoA with ROK but enhances its interaction with PKN. The mechanism for PKA's enhancement of RhoA-PKN interaction remains unclear but could involve phosphorylation-induced conformational changes at the switch regions of RhoA.

In the context of NGF-induced differentiation, previous studies have shown that activation of RhoA signaling and the ROK pathway inhibits the initiation of neurite outgrowth and leads to retraction of established neurites. In view of the selective inhibition of RhoA-ROK interaction elicited by NGF-induced activation of PKA, we hypothesized that the inhibition of ROK activation might be a required and necessary step for the initiation of neurite outgrowth. This hypothesis implies that the $RhoA^{S188A}$ mutant, which cannot be phosphorylated by PKA, might exert a dominant inhibitory effect on NGF-induced neurite outgrowth in PC12 cells. To test it, we cotransfected PC12 cells with constitutively active $RhoA^{S188A}$ using the pIRES plasmid, which provides the simultaneous expression of the green fluorescent protein (GFP) aiding the identification and morphological analysis of transfected cells (Fig. 6A). Three days after NGF treatment, nearly 80% of PC12 cells transfected with the pIRES-GFP plasmid bore neurites that were at least two cell-diameters long. However, less than 5% of the PC12 cells expressing $RhoA^{S188A}$ extended neurites following NGF treatment. A similarly low percentage of $RhoA^{14Val}$ expressing cells extended neurites, and the percentage of neurite-bearing cells was identical to that of cells transfected with the $RhoA^{V14S188Ala}$ double mutant (Fig. 6B). Thus, the $RhoA^{S188A}$ mutant, similarly to $RhoA^{14Val}$, renders PC12 cells unable to differentiate, as indicated by the abolished neurite outgrowth following NGF treatment. This suggests that increased activation of other RhoA targets without inactivation of ROK signaling is not sufficient to cause neurite outgrowth; hence, the ROK pathway exerts an overriding negative control on neurite initiation.

The molecular switch concept for GTPase function distinguishes only the GTP-loaded/active and GDP-loaded/inactive state of RhoA in controlling effector interaction and does not take into consideration other modulatory mechanisms. Our present results identify a novel mechanism in the regulation of the interaction of RhoA with its effectors. This regulation involves the phosphorylation of a C-terminal serine residue by PKA. Phosphorylation of ^{188}Ser selectively inhibits binding of ROK to RhoA but does not impair but in vivo tends to augment its interaction with other effectors, including PKN, mDIA, and rhotekin, providing a mechanism for differential target selection by the activated GTPase. This is different from the ADP ribosylation-mediated modification of the switch I domain brought about by C3 toxin, which abrogates the activation of all RhoA targets (Fig. 1). Two distinct regions in Rho, amino acids 23-40 and 75-92, have been identified previously in target interactions (35). Class I targets of RhoA that include PKN, rhophilin, and rhotekin interact with the 75-92 domain. Class II targets that include ROK I and II interact with both domains, whereas the class III target citron interacts with the 23-40 domain (35). Our results modify this concept with a third site, the C terminal region, and provide evidence for the specific inhibitory regulation of class II Rho effectors determined by the phosphorylation state of ^{188}Ser, which serves as a "secondary switch" in addition to GTP-binding. It has previously been suggested that PKA and the cGMP-dependent protein kinase modulate RhoA signals (30, 33, 34), and the latter has been shown to inhibit serum stimulation of the transcription factor SRF (30). We found that RhoA target selection mediated by PKA phosphorylation plays an essential role in the negative modulation of ROK required for NGF-induced neurite outgrowth. A number of Rho GTPases contain consensus phosphorylation sites that can be potentially recognized by serine and tyrosine kinases, and our proposed "secondary switch" model may thus be applied to other small G-proteins for such a regulation.

Acknowledgements

Supported by USPHS grants NHLBI-61469, CA-92160, and NSF IBN-9728147. We thank Drs. Jacques Bertoglio (INSERM, Faculte de Pharmacie) and John Wagner (Harvard University) for sharing reagents.

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Figure Legends

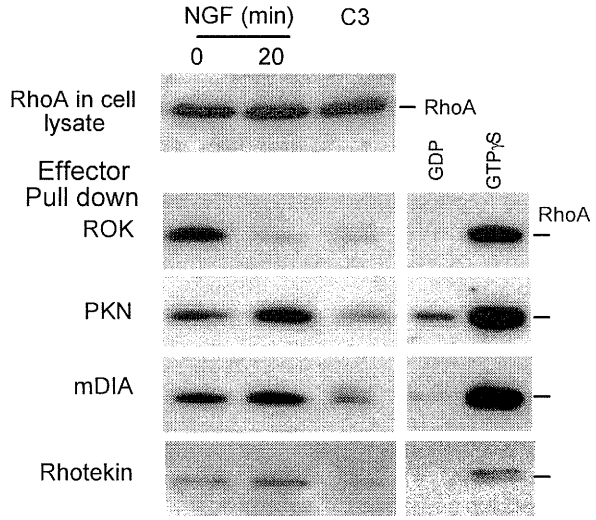


Fig. 1

Figure 1. Differential regulation of RhoA and its targets by NGF in PC12 cells. The amount of Rho in whole cell lysates (50 μg/lane) was determined by Western blotting using a monoclonal anti-RhoA antibody (top). Cells were exposed to NGF for 20 min or C3 toxin (2 μg/ml, overnight), and active RhoA was determined using pull-down assays with the GST-fusion proteins of the Rho-binding domain of ROK, PKN, mDIA, and rhotekin purified from *E. coli* lysates. To monitor the effect of GTP-loading on RhoA, pull-down assays were performed using cell lysates (100 μg protein) from vehicle-treated cells (0.1 % BSA) after treatment with either 10 μM GDP or GTPγS for 20 min prior to assay. NGF treatment caused a decrease in ROK binding, whereas it increased the binding of PKN, mDIA, and rhotekin. C3 toxin diminished the binding of all targets.

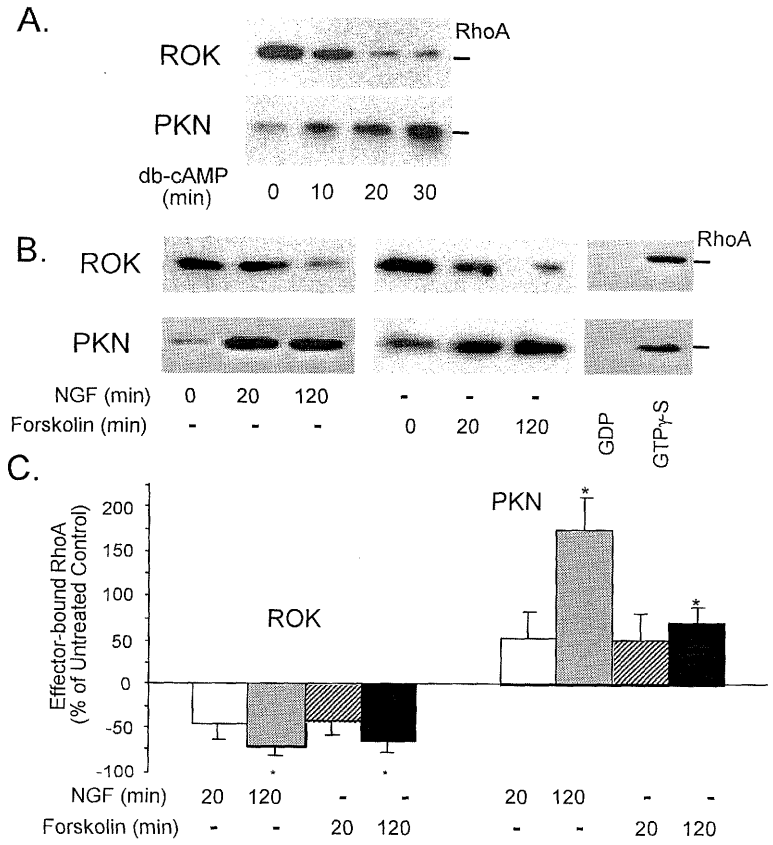


Fig. 2

Figure 2. Activation of cAMP-PKA signaling mimics NGF-induced target selection of RhoA. (**A**) Treatment of PC12 cells with 500 μM db-cAMP caused a time-dependent decrease in RhoA binding to ROK but increased binding to PKN in the same lysate. (**B**) NGF (100 ng/ml) and forskolin (1 μM) differentially regulate ROK and PKN binding to RhoA. Heavy membrane fractions (HMF; 100 μg) was used for pull-down assays with GST-ROK and GST-PKN followed by Western blotting. For control, HMF were treated with either 10 μM GDP or GTPγS for 20 min prior to assay (upper panel). **(C)** The intensity of the RhoA band was measured by densitometry in the different pull-down assays and normalized to an untreated control sample. Data shown are the mean values ± sem (n = 3,* $P < 0.05$ relative to untreated control).

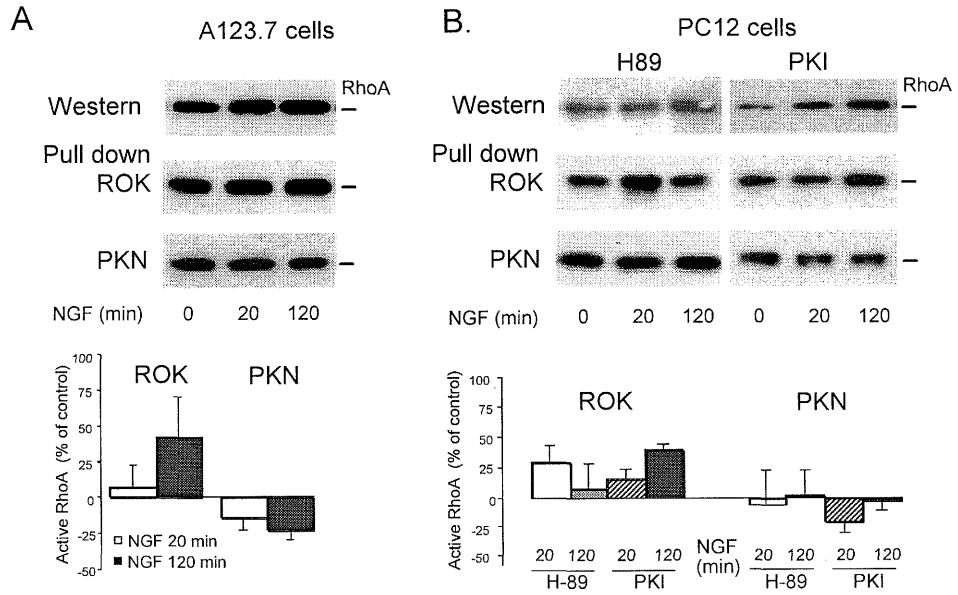


Fig. 3

Figure 3. Genetic or pharmacological inhibition of PKA blocks NGF-induced target selection of RhoA. **(A)** The A123.7 clonal derivative of PC12 cells, which expresses the type 1 regulatory subunit of PKA encoding two point mutations in the B locus cAMP-binding site, was also treated with 100 ng/ml NGF for the times indicated. The heavy membrane fraction (HMF) was isolated, and pull-down assays were performed with GST-ROK and GST-PKN. In A123.7 cells, NGF treatment did not elicit a decrease in ROK binding to RhoA; it evoked a non-significant increase. PKN binding was slightly reduced following NGF treatment, whereas the opposite trend is seen in wild-type cells (*cf.* Figs. 1 & 2). **(B)** PC12 cells were pretreated with the PKA inhibitor H89 (10 μM) or the myristoylated PKA inhibitory pseudosubstrate peptide (PKI, 5 μM) for 30 min prior to treatment with 100 ng/ml NGF for the times indicated, and pull-down assays were performed to monitor the interaction of RhoA with GST-ROK or GST-PKN. As in A.123.7 cells lacking PKA, pharmacological inhibition of PKA reversed the trend of the interaction of RhoA with ROK and PKN. Control represents the amount of RhoA pulled down at time 0, and bars represent the mean and sd for 3 experiments.

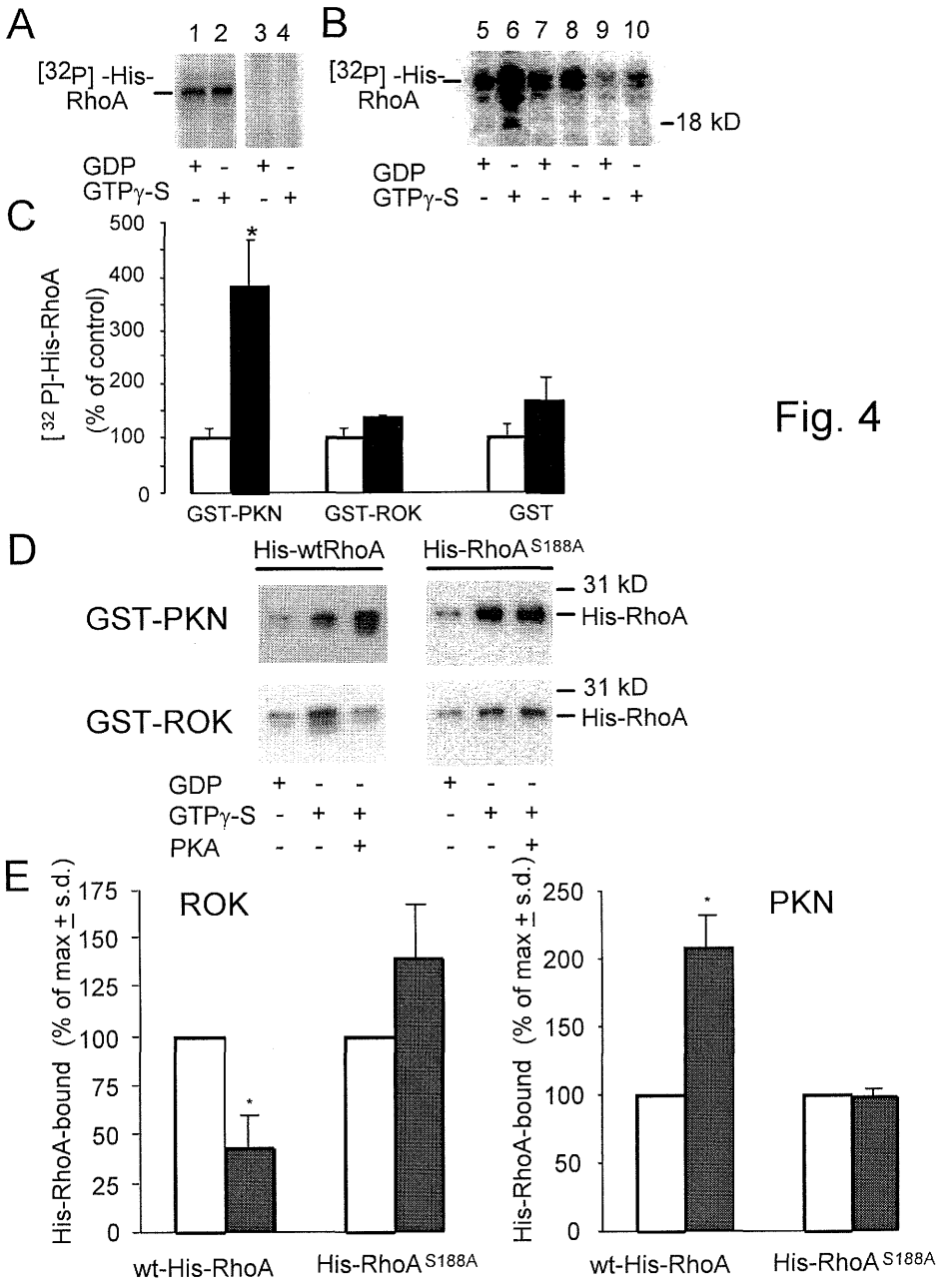


Fig. 4

Figure 4. Phosphorylation of RhoA by PKA differentially regulates interaction with ROK and PKN in a cell-free system. (**A**) SF9 cells were transduced with baculovirus expressing His-tagged wild-type RhoA (lanes 1, 2, 5-10) or the S188A mutant (lanes 3 & 4). Recombinant RhoA proteins were isolated and loaded with 10 μM GDP or GTPγS as described under Experimental Procedures. The recombinant proteins were phosphorylated by the catalytic subunit of PKA in the presence of [^{32}P]γATP, separated by SDS-PAGE, and

detected using phosphorimaging (lanes 1-4). Note that the RhoA^{S188A} was not phosphorylated. (**B**) The GDP- or GTPγS-loaded [^{32}P]His-RhoA was tested in pull-down assays for binding to GST-PKN (lanes 5 & 6), GST-ROK (lanes 7 & 8), or GST without a Rho-binding domain (lanes 9 & 10). The amount of [^{32}P]His-RhoA pulled down was determined by phosphorimaging. **(C)** Quantification of the target-bound [^{32}P]His-RhoA. Open bars are GDP-, filled bars are GTPγS-loaded [^{32}P]His-RhoA. The bars represent the mean ± sem (n = 3; $*P < 0.05$ relative to GDP-loaded control). (**D**) Cold ATP was used to phosphorylate His-RhoA or His-RhoA^{S188A} with the catalytic subunit of PKA as described for the experiment shown in panel A. PDA was performed with GST-ROK and GST-PKN after GDP or GTPγS loading. The effector-associated His-tagged RhoA proteins were detected using anti-His antibody. **(E)** Quantification of GST-ROK and GST-PKN bound to phosphorylated or non-phosphorylated His-RhoA (open bars, non-phosphorylated; filled bars, phosphorylated recombinant His-RhoA or His-RhoA^{S188A}). The results show normalized binding to GTPγS-loaded maximal binding obtained for each His-RhoA construct mean % of max ± sd, n = 4). Note that phosphorylation of wild type His-RhoA decreased ROK and enhanced PKN binding. In contrast, the His-RhoA^{S188A} mutants showed no significant change in binding to either ROK or PKN.

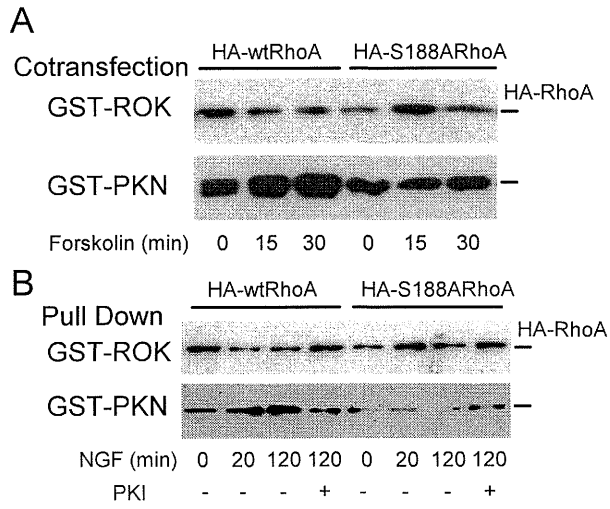


Fig. 5

Figure 5. Activation of PKA by NGF mediates target selection for RhoA in vivo. (**A**) HEK293 cells were cotransfected with hemaglutinin (HA) epitope-tagged wild-type (HA-wtRhoA) or S188A mutant RhoA (HA-RhoA^{S188A}) and with GST-ROK or GST-PKN. Two days after transfection, cells were treated with 25 μM forskolin for the times indicated. The expressed GST-fusion proteins were isolated using GST-beads, and the amount of associated RhoA was detected using Western blotting with monoclonal anti-RhoA antibody. The interaction of epitope-tagged RhoA with ROK and PKN was subject of differential target selection following NGF treatment. In contrast, RhoA ^{S188A} was not subject to differential target recognition after NGF treatment. (**B**) PC12 cells were transfected with either a HA-RhoA or HA-RhoA^{S188A} construct. Two days after transfection, cells were exposed to 100 ng/ml NGF. In some cases, 30 min pretreatment with 5 μM PKI was applied prior to NGF treatment. Pull-down assays were performed with the cell lysate using GST-ROK and GST-PKN fusion proteins. The amount of RhoA bound to GST-ROK and GST-PKN beads was detected using Western blot with monoclonal anti-RhoA antibody. The results shown in both panels are representative of three independent experiments. The NGF-induced target selection was abolished by PKI treatment for RhoA, but its trend was not altered for RhoA^{S188A}.

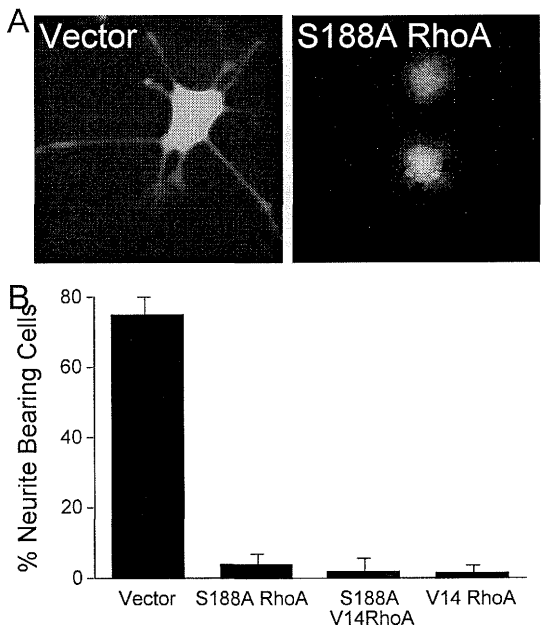


Fig. 6

Figure 6. The PKA phosphorylation-consensus site mutant RhoA^{S188A} inhibits NGF-induced neurite outgrowth in PC12 cells. PC12 cells were transfected with either pIRES-GFP, pIRES-RhoA^{S188A}-GFP, pIRES-RhoA^{V14}, or pIRES-RhoA^{S188AV14}-GFP plasmids and were exposed to 100 ng/ml NGF for 3 days. The number of neurite-bearing cells was counted and expressed as a percentage of the number of all fluorescent cells ($n > 100$). **(A)** Representative morphology for vector-GFP- and S188ARhoA-expressing cells. **(B)** The incidence of fluorescent neurite-bearing cells in PC12 cultures transfected with the different RhoA mutants (mean $\pm$ sem, $n = 3$).

Supplemental Material:

Experimental Procedures

Reagents. Forskolin, H89, PKI, db-cAMP, GDP, GTP, GTPγS, glutathione-agarose beads were from Sigma-Aldrich (St. Louis, MO, USA). Recombinant PKA catalytic subunit was obtained from Promega (Madison, WI, USA). NGF was purchased from Alamone Labs (Jerusalem, Israel).

Plasmids. pGEX-2T^{Ser1881AlaRhoA} plasmid was a kind gift of Dr. J. Bertoglio (INSERM, Unite 461, Paris, France). All other RhoA and effector constructs (His-RhoA, HA-RhoA, GST-ROK, GST-PKN, GST-mDIA, GST-rhotekin, ROK, RhoA/GFP) were generated by subcloning the respective cDNAs to either the *E. coli* expression vectors (pET15b for His-tagged expression and pGEX-KG for GST-tagged expression) or mammalian vectors (pCEFL for GST-tagged expression, pKH3 for HA-tagged expression, or pMX-IRES-GFP for bicistronic expression with GFP) have been described in our previous publications (36-38).

Cell culture. Wild-type PC12 cells and the A123.7 clone, which expresses the type 1 regulatory subunit of PKA encoding two point mutations in the B locus cAMP-binding site, were kindly provided by Drs. G. Cooper (Boston University, Boston, MA, USA) and J. Wagner (Harvard University, MA, USA). Both cell types were grown in RPMI medium supplemented with 10% horse serum, 5% fetal bovine serum, and 1% glutamine (normal medium). To initiate differentiation, 7 x 10^6 cells were plated in 100-mm dishes in normal medium and exposed to 100 ng/ml NGF. Under these conditions, more than 75% of the wild-type PC12, but not the A123.7 cells extended neurites by day 2. HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Transient transfection. Wild-type PC12 cells were transiently transfected with wild-type RhoA, constitutively active RhoA^{14Val}, RhoA^{S188A}, and RhoA^{188Ser,14Val} constructs subcloned into the pIRES-GFP vector or empty vector (pMX-IRES-GFP) using the Cytofectene reagent (Bio-Rad; Hercules, CA, USA). Plasmid (2 μg) and 20 μl of Cytofectene reagent were used for every ml of transfection solution. After exposing the cells to this mixture for 8 h, cells were grown in normal medium. Expression of GFP was detected, and pictures of cells expressing GFP were taken using fluorescent microscopy. Between days 3 and 6 after transfection, 80% of the cells expressed GFP; therefore, NGF treatment was carried out between post-transfection days 3-6. HEK293 cells were cotransfected with HA epitope-

tagged wild-type RhoA (HA-wtRhoA) or ^{188}Ser-Ala mutant RhoA (HA-RhoA^{S188A}) and with GST-ROK or GST-PKN using Cytofectene (Bio-Rad) transfection reagent as described (38).

Isolation and in vitro phosphorylation of His-tagged RhoA. SF9 cells were infected by baculovirus expressing His-tagged wild-type and S188A mutant RhoA proteins, His-wtRhoA and His-RhoA$^{Ser188□□□}$, respectively. Recombinant RhoA proteins were isolated using the 6 x His Expression and Purification Kit (Pharmingen; San Diego, CA). GDP and GTPγS loading was perform as described by Zhang et al. (36). The recombinant proteins were treated with 4 U of the catalytic subunit of PKA for 10 min at 30°C in the presence of 5 μCi [^{32}P]γATP (3000 Ci/mmol; Amersham; Piscataway, NJ, USA) in 20 mM MES (pH 6.5), 100 mM NaCl, 0.1 mM EDTA, 30 μM β-mercaptoethanol, and 50% (V/V) ethylene glycol. The final reaction mixture contained 30 μl beads, 8 μl assay buffer, 4 μl PKA (1 U/μl), and 1.5 μl [^{32}P]γATP. The reaction was stopped by washing the beads with 100 μl of 300 μM imidazole. The phosphorylated proteins were separated by SDS-PAGE and detected using a Cyclone phosphorimager (Molecular Devices; Sunnyvale, CA, USA). After the phosphorylation reaction, His-tagged Rho proteins were loaded with either GTPγS or GDP (36) and then eluted from the Ni-NTA agarose beads (Sigma-Aldrich). A pull down assay was performed on these recombinant Rho proteins with GST-ROK and GST-PKN beads. To detect the amount of ^{32}P-labeled, effector-bound Rho proteins after the pull-down assay, the GST-beads were boiled and the proteins were separated by SDS-PAGE followed by detection with the phosphorimager.

Isolation of total cell lysate. Total cell lysate was isolated as described previously by Kranenburg et al. (39). Briefly, after NGF treatment, cells were rinsed with 3 ml of ice-cold PBS and lysed with 450 μl of lysis buffer A containing 20 mM TRIS (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 10% (V/V) glycerol, 0.1% (V/V) Triton X-100, and protease inhibitor cocktail from Sigma Chemical Co. (St. Louis, MO, USA). This lysate was incubated for 10 min on ice, and the crude cell debris was removed by centrifugation at 10,000 x *g* for 10 min. The supernatant was designated "total cell lysate" and used for the experiments.

Membrane fractionation. In some experiments, heavy cell membrane fractions were used for Western blotting of pull-down assays. Cells were rinsed with 3 ml of ice-cold PBS and lysed with 450 μl of lysis buffer B containing of 50 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, and protease inhibitor cocktail. The lysate was sonicated twice for

15 s; the unbroken cells and nuclei were removed by centrifugation at 1,500 x *g* for 10 min. The supernatant was further centrifuged at 15,000 x *g* for 10 min at 4°C to sediment the crude heavy membrane fraction (HMF).

Western blot analysis. Protein (20 μg applied per lane) was separated by SDS-PAGE on 12% gels and transferred to PVDF membranes (Bio-Rad). To block nonspecific binding, membranes were incubated overnight at room temperature in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 (TBST) containing 5% nonfat dry milk (Bio-Rad). After blocking, the membranes were probed for 2 h with the primary antibody diluted in TBST and washed three times with TBST before incubation for 45 min with either peroxidase-conjugated secondary anti-mouse antibody (1:5000; Sigma) or donkey anti-rabbit antibody (1:7500; Promega; Madison, WI, USA). The SuperSignal Reagent (Pierce; Rockford, IL, USA) was used to visualize antibody binding. The following primary antibodies were used in our experiments: anti-RhoA monoclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA; diluted 1:500). Band intensities were measured using digital video-densitometry by NIH Image (version 1.61) software.

RhoA pull down assays. The glutathione S-transferase fusion proteins (GST) containing the Rho-binding domain of Rho-associated kinase (GST-ROK), protein kinase N (GST-PKN), mDIA (GST-mDIA), and rhotekin (GST-rhotekin) were expressed in *E. coli* by using the pGEX-KG vector. Details of the pull-down assay were exactly as we reported previously (38). For controls, RhoA was loaded with either 10 μM GDP or GTPγS in every pull-down assay.

Statistical methods. The quantitative results represent the mean of at least three individual experiments. Student's *t*-test for paired variables was used to test for differences caused by NGF treatment or phosphorylation, and data were considered significantly different at $P < 0.05$.

Suppl. Fig. 1A.

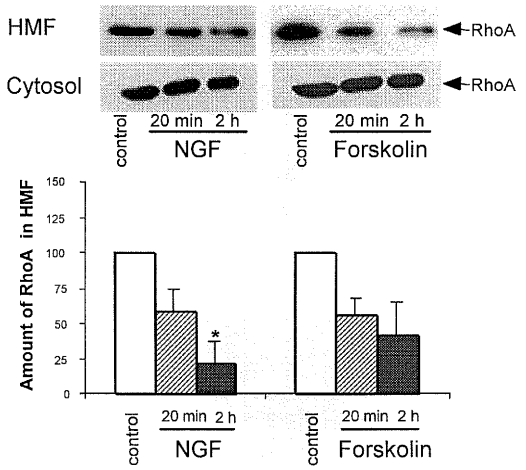
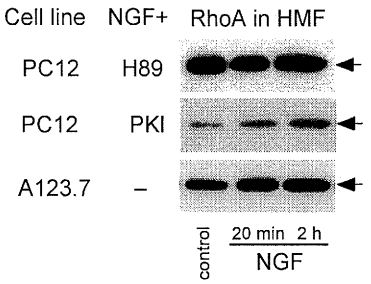


Fig. 1B.



Legend to Supplemental Fig.1.

Panel A. PC12 cells were treated with NGF (100 ng/ml) or forskolin (1 μM) for the times indicated, and the heavy membrane fraction (20 μg/lane) and cytosol (10 μg/lane) were isolated and analyzed by Western blotting for the amount of RhoA. Bars represent the mean of three independent experiments. Note that the amount of membrane-associated RhoA decreases rapidly after NGF and forskolin treatment. **Panel B.** Inhibitors of PKA, H89 (10 μM, 30 min) and PKI (5μM, 30 min) reverse the translocation of RhoA after NGF treatment (100 ng). In PKA deficient A123.7 cells NGF fails to elicit RhoA translocation.

Abbreviation	Explanation
ATP	adenosine triphosphate
C3 toxin	C. botulinum C3 Rho-ADP-ribosylating exoenzyme
cAMP	cyclic adenosine monophosphate
citron-K	citron kinase
CLM	caveolae-like membranes
CNS	central nerve system
CREB	cAMP response element binding protein
CRMP-2	collapsin response mediator protein-2
dbcAMP	dibutyryl cAMP
DH	Dbl homology
DNA	dezoxy-ribonucleic acid
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
ERM	Ezrin-Radixin-Moesin family of proteins
FBS	fetal bovine serum
FGF	fibroblast growth factor
FH	formin homology
G-protein	GTP-binding protein
GAP	GTPase-activating protein
GDI	guanosine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GDS	GDP dissociation stimulator (GEF)
GEF	guanine nucleotide exchange factor (GDS)
GFP	green fluorescent protein
GST	glutathion S-transferase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
GTPgS	guanosine 5'-3-O-(thio)triphosphate
HMF	heavy membrane fraction
HR1	homology region 1
JNK	c-jun N-terminal kinase
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MBS	myosin binding subunit
MEK	MAP kinase/ERK-activating kinase
MLC	myosin light chain
NGF	nerve growth factor
NGFR	nerve growth factor receptor
p75NTR	p75 neutrophin receptor
PAGE	polyacrylamide gel electrophoresis
PAK	p21 (Sdc42/Rac)-activated kinase
PBD	p21-binding domain

Abbreviation	Explanation
PDA	pull down assay
PDK1	3-phosphoinositide-dependent protein kinase
PH	pleckstrin homology
PI	phosphatidylinositol
PI3-K	phosphatidylinositol 3-kinase
PKA	cAMP-dependent protein kinase / protein kinase A
PKC	protein kinase C
PKI	protein kinase inhibitor
PKN	protein kinase N (p120PKN / PRK1)
PLCd	phospholipase-C-d-1
PRK	protein kinase C-related protein kinase
RDB	Rho-binding domain
rho	ras homologue gene
Rho-kinase / ROK	Rho-binding kinase (ROKα / ROCK-II)
ROCK	p160 Rho-associated coiled coil-forming protein kinase (ROKβ / ROCK-I)
ROCK-I	p160 Rho-associated coiled coil-forming protein kinase (p160ROCK / ROKβ)
ROCK-II	Rho-binding kinase (ROKα / Rho-kinase)
ROK	Rho-kinase (ROKα / ROCK-II)
ROKα	Rho-binding kinase (Rho-kinase / ROCK-II)
ROKβ	p160 Rho-associated coiled coil-forming protein kinase (p160ROCK / ROCK-I)
S188A-RhoA	Ser188Ala mutant of RhoA
SAPK	stress-activated prtoein kinase
SH2 domain	scr-homology 2 domain
SH3 domain	scr-homology 3 domain
SRE	serum response element
SRF	serum response factor
TBST	Tris buffered saline with Tween 20
TNF	tumor necrosis factor
Trk	tyrosine kinase neutrophin receptor
wt	wild type

Cell lines:	
Abbreviation	**Explanation**
A123.7	a derivatives of PC12 cell line, which is defective in cAMP-induced pathways
B5	a derivatives of nnr5 cell line, which stably overexpresses TrkA protein
HEK293	transformed human embryo kidney cell line
M-M17-26	a derivatives of PC12 cell line, which stably expresses high amounts of dominant negative mutant Asn17-Ras protein
N1E-115	neuroblastoma cell line
NIH3T3	mouse fibroblast cell line
nnr5	variant of PC12 cell line, which lacks functional TrkA protein
PC12	rat pheochromocytoma cell line
Rat-1	rat fibroblast cell line
SF9	insect cell line
Swiss 3T3	mouse fibroblast cell line