Identification of a Novel, Putative Rho-specific GDP/GTP Exchange Factor and a RhoA-binding Protein: Control of Neuronal Morphology

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Abstract. The small GTP-binding protein Rho has been implicated in the control of neuronal morphology. In N1E-115 neuronal cells, the Rho-inactivating C3 toxin stimulates neurite outgrowth and prevents actomyosin-based neurite retraction and cell rounding induced by lysophosphatidic acid (LPA), sphingosine-1-phosphate, or thrombin acting on their cognate G protein–coupled receptors. We have identified a novel putative GDP/GTP exchange factor, RhoGEF (190 kD), that interacts with both wild-type and activated RhoA, but not with Rac or Cdc42. RhoGEF, like activated RhoA, mimics receptor stimulation in inducing cell rounding and in preventing neurite outgrowth. Furthermore, we have identified a 116-kD protein, $p116^{Rip}$, that interacts with both the GDP- and GTP-bound forms of RhoA in N1E-115 cells. Overexpression of $p116^{Rip}$ stimulates cell flattening and neurite outgrowth in a similar way to dominant-negative RhoA and C3 toxin. Cells overexpressing $p116^{Rip}$ fail to change their shape in response to LPA, as is observed after Rho inactivation. Our results indicate that (*a*) RhoGEF may link G protein–coupled receptors to RhoA activation and ensuing neurite retraction and cell rounding; and (*b*) $p116^{Rip}$ inhibits RhoA-stimulated contractility and promotes neurite outgrowth.

The mechanisms by which neuronal cells regulate their complex morphology are poorly understood. Extracellular agonists as diverse as growth factors, neurotrophins, matrix components, secreted proteases, and bioactive phospholipids can exert dramatic effects on neural architecture, ranging from stimulation of neurite outgrowth to induction of growth cone collapse and neurite retraction. Studies on neuronal cell lines, including N1E-115, NG108-15, and PC12, have revealed that certain G protein–coupled receptor agonists, notably lysophosphatidic acid (LPA)[1], thrombin, and sphingosine-1-phosphate (S1P), trigger rapid growth cone collapse, retraction of developing neurites, and transient rounding of the cell body (Jalink and Moolenaar, 1992; Jalink et al., 1993; Suidan et al., 1992; Tigyi and Miledi, 1992; Postma et al., 1996; see also Gurwitz and Cunningham, 1988). These dramatic shape changes are driven by actomyosin-based contraction of the cortical cytoskeleton, independently of known second messengers, rather than by a loss of adhesion (Jalink et al., 1993, 1994).

The small GTP-binding protein Rho regulates actin filament reorganization in response to extracellular stimuli (Machesky and Hall, 1996; Symons, 1996). In fibroblasts, active Rho-GTP mediates agonist-induced formation of focal adhesions and stress fibers (Ridley and Hall, 1992). In neuronal cells, Rho has been implicated in the control of neurite behavior, a notion largely based on the use of C3 toxin from *Clostridium botulinum*, which ADP-ribosylates and thereby inactivates Rho. In differentiated N1E-115 and PC12 cells, C3 toxin prevents agonist-induced cytoskeletal contraction and ensuing neurite retraction (Jalink et al., 1994; Tigyi et al., 1996). Furthermore, C3 treatment of undifferentiated N1E-115 or PC12 cells causes cell flattening followed by neurite extension and growth arrest (Nishiki et al., 1990; Jalink et al., 1994). The neurotrophic effects of C3 are not restricted to neuronal cell lines: in chick embryo sympathetic ganglia, C3 acts as effectively as nerve growth factor in promoting neurite outgrowth (Kamata et al., 1994). Together, these results support a model in which basal Rho activity is necessary to maintain cy-

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1. *Abbreviations used in this paper*: DH, Dbl homologous; GDI, GDP dissociation inhibitor; GST, glutathione-*S*-transferase; HA, hemagglutinin; LPA, lysophosphatidic acid; PH, pleckstrin homology; RIP, Rho-interacting protein; S1P, sphingosine-1-phosphate; SH3, Src homology 3.

toskeletal tension and cell shape; increased Rho activity (Rho-GTP accumulation) then drives neurite retraction, whereas Rho inactivation results in loss of contractility and induces neurite outgrowth (Jalink et al., 1994). Yet, direct evidence that Rho controls neurite behavior is still lacking, as studies with activated or dominant-negative Rho mutants have not yet been conducted in neuronal cells.

Little is known about how Rho is activated biochemically, although it is generally assumed that the mechanism is similar to Ras activation and mediated by specific GDP/GTP exchange factors (Feig, 1994; Quilliam et al., 1995; Machesky and Hall, 1996), among which are the recently identified Lbc, Lfc, and Lsc oncoproteins (Zheng et al., 1995; Glaven et al., 1996). Other proteins involved in the regulation of Rho activity include GDP dissociation inhibitors (GDIs) that keep GDP-bound Rho in a cytoplasmic, inactive form (Hancock and Hall, 1993; Machesky and Hall, 1996). Several downstream targets of Rho have recently been identified (for review see Machesky and Hall, 1996; Ridley, 1996), including a protein kinase (Rho-kinase) that may regulate actomyosin contractility (Kimura et al., 1996).

In this study we set out to explore further the role of Rho in the control of neuronal morphology, using N1E-115 cells as a model. In addition to defining the effect of activated and dominant-negative RhoA on neuronal cell shape, we have used the two-hybrid system to identify RhoA-binding proteins that control neurite behavior. We report the identification of a novel putative GDP/GTP exchange factor, RhoGEF (190 kD), and a 116-kD RhoA-binding protein, p116^{Rip}, which are highly expressed in brain and N1E-115 cells. We show that RhoGEF, like activated RhoA, induces sustained cell rounding and suppresses neurite outgrowth. In contrast, overexpressed p116^{Rip} promotes cell flattening and neurite extension in a similar way to dominant-negative RhoA or C3 toxin. Our results establish RhoGEF and p116^{Rip} as critical determinants of RhoA-mediated neuronal shape changes.

Materials and Methods

Plasmids

RhoA and V14RhoA cDNA were kindly provided by Dr. A. Hall (London, UK). These cDNAs contain a Phe-Asn mutation at position 25 (Paterson et al., 1990). This mutation was corrected by PCR; wild-type and V14RhoA cDNAs were completely sequenced. Subsequently, yeast and mammalian expression vectors were constructed with the PCR and also sequenced. Wild-type RhoA and V14RhoA cDNA were amplified by PCR using primers 5′-CGAGGTCGACAATGGCTGCCATCCGGAAGAAACTGGTGATTG-3′ and 5′-CTTAGCGGCCGCTCACAAGACAAGGCAACCAG-3′ and cloned as an SalI–NotI fragment in frame with the Gal4 DNA-binding domain (amino acids 1–147) of pPC97 (Chevray and Natans, 1992) to generate pPC97-RhoA and pPC97-V14RhoA. To prevent isoprenylation of RhoA, similar yeast vectors, pPC97-RhoA-C190R and pPC97-V14RhoA-C190R, were constructed in which the cysteine residues at position 190 in the cDNAs of RhoA and V14RhoA were replaced by arginine using PCR and backward primer 5′-CTTAGCGGCCGCTCACAAGACAAGGCGACCAGAT-3′. Subsequently, the vectors pMD4-RhoA, pMD4-V14RhoA, pMD4-RhoAC190R, and pMD4-V14RhoAC190R, in which the leucine selection marker present in pPC97 is replaced with the tryptophan selection marker present in pPC86 (Chevray and Natans, 1992), were constructed. Human RhoGDI cDNA in pGEX2T (kindly provided by J. Hancock) was cloned as a BamHI (filled in with Klenow)–EcoRI fragment in pPC86 digested with SmaI and EcoRI. Mammalian expression vectors were constructed with NH_2-terminal epitope-tagged wild-type RhoA and V14RhoA by cloning SalI–NotI fragments in pMT2SM-HA and pMT2SM-myc, modified pMT2 expression plasmids encoding the hemagglutinin (HA) epitope or myc epitope. A dominant-negative mutant of RhoA was generated by replacing threonine at position 19 for asparagine with the PCR and was cloned in pMT2SM-HA and pMT2SM-myc in a similar manner. For transient transfection studies in N1E-115 cells, the HA- and myc-tagged RhoA, V14RhoA, and N19RhoA cDNAs derived from the pMT2 vectors as PstI (filled in with Klenow)–NotI fragments were also cloned in pcDNA3 (Invitrogen, San Diego, CA) digested with EcoRI (filled in with Klenow) and NotI. In all cases, constructs generated with the PCR and nucleotide sequences of mutated cDNAs were verified by DNA sequencing.

Yeast Two-Hybrid Analysis

Yeast strain Y190 (Harper et al., 1993) was transformed with plasmids encoding wild-type or mutant RhoA cDNAs fused to the Gal4 DNA-binding domain, and two-hybrid screens with a day 14.5 CD1 mouse embryo library (Chevray and Natans, 1992) and a mouse brain library (obtained from Dr. L. Van Aelst, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) were performed using the lithium acetate method (Schiestl et al., 1989). Transformants were selected for growth on plates lacking histidine and supplemented with 25 mM 3-aminotriazole. His+ colonies were subsequently tested for β-galactosidase activity as previously described (Durphee et al., 1993). Binding specificity of cDNAs obtained from positive yeast colonies was analyzed by retransformation with the different RhoA mutants and with an irrelevant protein fused with the Gal4 DNA-binding domain.

cDNA Cloning and Analysis

To obtain full-length cDNAs for Rho-interacting protein (RIP) 2 and RIP3, a day 16 mouse embryo library (Novagen, Madison, WI), a mouse brain library (Stratagene, La Jolla, CA), and a mouse lung library (Stratagene) were used initially with probes derived from the clones isolated in the two-hybrid screen. Subsequently, probes derived from these libraries were used until overlapping clones, representing full-length cDNAs, were isolated. Of each cDNA, at least two independent clones of the same region were analyzed to avoid mistakes due to possible cloning artifacts during library construction. Restriction analysis, subcloning, and other standard DNA manipulations and procedures were performed to obtain complete sequence information of the clones. DNA sequencing was done with the T7 sequencing kit (Pharmacia, Uppsala, Sweden); sequences were analyzed using Genetics Computer Group software (Devereux et al., 1984).

Truncated HA-tagged mutant RhoGEF, termed HA-ΔRhoGEF, was generated by cloning the entire two-hybrid cDNA of RIP2 (see Results) into pMT2SM-HA and subsequently in pcDNA3. Therefore, a BglII–KpnI fragment (bp 2,783–end) of RIP2 was cloned into pMT2SM, resulting in pMT2SM-RIP2a, and an EcoRI (bp 2,160–2,955) fragment was cloned into pMT2SM-HA, resulting in pMT2SM-HA-RIP2b. Subsequently, a BglII fragment from pMT2SM-HA-RIP2b was cloned into pMT2SM-RIP2a, resulting in the final expression construct, pMT2SM-HA-ΔRhoGEF, which encodes the COOH-terminal amino acids starting from amino acid 685. pcDNA3-HA-ΔRhoGEF was generated in two steps, resulting in cloning of the entire HA-ΔRhoGEF as a PstI (filled in with Klenow)–XbaI fragment in pcDNA3 digested with HindIII (filled in with Klenow) and XbaI. Full-length RhoGEF was generated by cloning an EcoRI–SphI fragment (bp 1–1,343), derived from pBSKML4 and an SphI–NotI fragment (1,343–end) from pBSK-ML3 into pcDNA3 digested with EcoRi and NotI. Both pBSK-ML3 and pBSK-ML4 are clones obtained from the mouse cDNA libraries.

We constructed two NH_2-terminal deletion mutants of p116^{Rip}, termed ΔI-p116^{Rip} and ΔII-p116^{Rip}, both with an NH_2-terminal HA tag. For ΔI-p116^{Rip}, the EcoRI fragment containing almost the entire insert, including the RhoA-binding region, of the two-hybrid clone was cloned into pMT2SMHA, resulting in pMT2SM-HA-ΔIp116^{Rip}. Subsequently, the entire fragment, including the NH_2-terminal HA tag, was subcloned in two steps into the HindIII–EcoRI sites of pcDNA3, resulting in pcDNA3A-ΔIp116^{Rip}. Plasmid pcDNA3-ΔIp116^{Rip} was obtained by cloning an EcoRI–HindIII (filled in with Klenow) fragment (bp 1,824–end) from clone RP20 in pcDNA3-ΔIp116^{Rip} digested with EcoRI and EcoRV. pcDNA3-ΔIp116Rip encodes amino acids 545–824, and pcDNA3-ΔIIp116^{Rip} encodes amino acids 545–1,024 (end). A full-length p116^{Rip} construct, termed pcDNA3-p116^{Rip}, was generated by cloning the EcoRI–HindIII (filled in

with Klenow) from RP20 in pcDNA3 digested with EcoRI and EcoRV, followed by insertion of an EcoRI fragment from ML27 that contains the 5′ end. Clones RP20 and ML27 were obtained from the mouse cDNA libraries.

RNA Isolation and Northern Blot Analysis

Total RNA was prepared using TRIzol Reagent (GIBCO BRL, Gaithersburg, MD) according to the instructions of the manufacturer. RNA was separated on a 1.5% agarose-formaldehyde gel and blotted onto Hybond-N membrane (Amersham Intl., Little Chalfont, UK). The membrane was hybridized with α[^{32}P]dCTP-labeled (Amersham Intl.) probes generated with the Prime-It RmT random primer labeling kit (Stratagene).

Antibodies

A polyclonal anti-RhoGEF serum was made by immunizing rabbits with a synthetic peptide corresponding to amino acids 1,529–1,547 (sequence NKHSRQRSLPAVFSPGSKEV) coupled to a lysine backbone (Posnett et al., 1988). A polyclonal anti-RIP3 serum was raised by immunizing rabbits with a glutathione-*S*-transferase (GST) fusion protein containing amino acid residues 545–824 of RIP3 (EcoRI fragment derived from two-hybrid screen).

Cos Cell Transfections

Cos-7 cells were transfected using the DEAE-dextran method as described (Zondag et al., 1996). After 48 h, the cells were washed and lysed in SDS sample buffer followed by boiling for 5 min. Samples were analyzed on 10% SDS-PAGE gels followed by immunoblotting. Immunoblots were blocked with 5% nonfat dry milk in TBST (50 mM Tris, pH 8.8, 150 mM NaCl, 0.05% Tween-20) and incubated with anti-RhoGEF or anti-p116^{Rip} polyclonal antibodies followed by chemiluminescence detection (ECL; Amersham Intl.) using HRP-conjugated secondary antibodies.

Transfection of N1E-115 Cells

N1E-115 cells were transiently transfected with pcDNA3-p116^{Rip}, pcDNA-Rho, pcDNA-GEF, together with pCMVlacZ in a 1:5 ratio, using the calcium phosphate precipitation method as described (Kranenburg et al., 1995). After 14–16 h, the cells were washed and either incubated for 24–36 h in DME containing 10% FCS or in serum-free DME to induce morphological differentiation. Cells were fixed in 3.5% formaldehyde/PBS and assayed for β-galactosidase activity. For each transfection, the percentage of undifferentiated (rounded without neurites) and neurite-bearing cells (flattened) was calculated from at least 300 positive cells counted. An average percentage was calculated from three dishes per transfection and three independent transfections.

GST-RhoA Fishing Experiments

Purified GST–RhoA (wild-type) fusion protein (45 μg) was incubated with either GDPβS or GTPγS (40 μM) for 30 min at 30°C in 500 μl reaction mixture containing 20 mm Tris/HCl (pH 7.5), 10 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$. N1E-115 cell lysates were prepared in a buffer containing 50 mM Tris, 250 mM NaCl, 1 mM EDTA, 0.5% NP-40, supplemented with protease and phosphatase inhibitors. Preloaded GST–RhoA proteins, or GST alone, were then added to the lysates and incubated for 1 h on ice. Glutathione-Sepharose beads were used to collect the GST fusion proteins. The beads were washed three times in lysis buffer and subjected to 8% SDS-PAGE followed by immunoblot analysis using anti-p116^{Rip} antibody.

Results

Isolation of Novel RIPs

We set out to identify and characterize RhoA-binding proteins using the yeast two-hybrid system. cDNAs encoding wild-type RhoA and a constitutively active mutant (V14RhoA) were fused to the DNA-binding domain of the GAL4 transcription factor and used as bait to screen a mouse brain cDNA library fused to the GAL4 activation

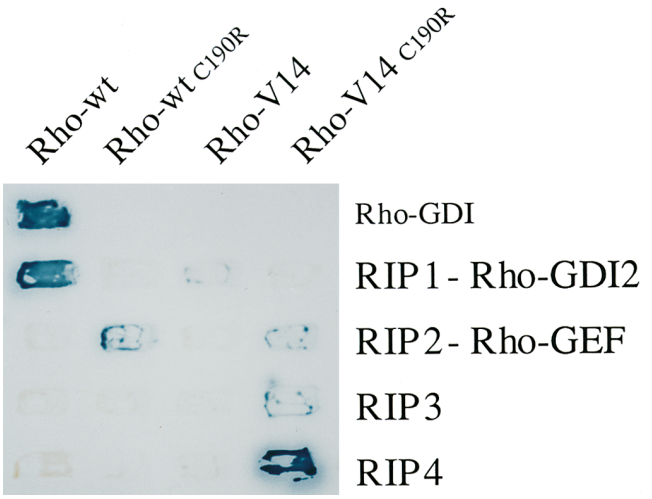


Figure 1. Two-hybrid interaction between RIPs and RhoA. Binding of RIPs, identified in two-hybrid screens, to wild-type (*wt*) RhoA and activated V14RhoA mutants was analyzed using the two-hybrid system. The C190R mutation in RhoA removes the isoprenylation site to prevent membrane targeting. *Rho-GDI*, guanine nucleotide dissociation inhibitor (Hancock and Hall, 1993); RIP1 encodes a novel Rho-GDI, termed Rho-GDI2; full-length RIP2 encodes RhoGEF and RIP3 represents p116^{Rip}. The RIP4 isolate encodes a novel protein that remains to be characterized. For further details see text.

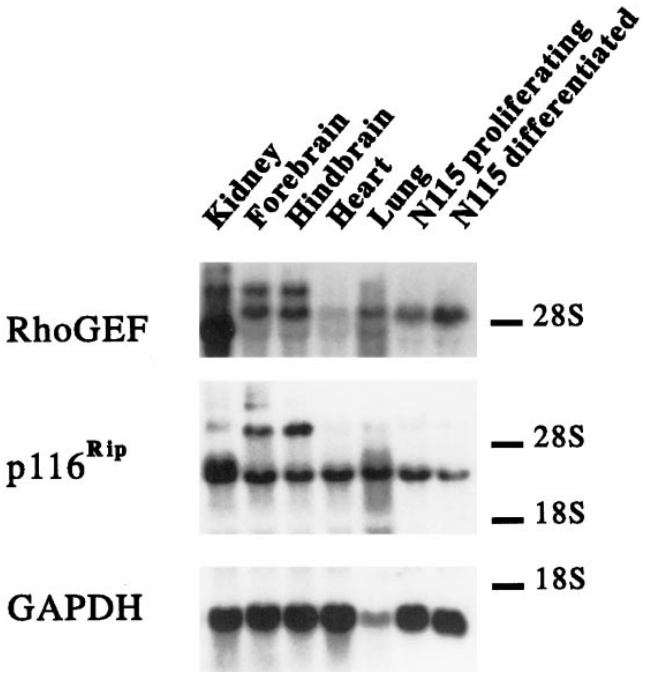


Figure 2. Expression pattern of RhoGEF and p116^{Rip} analyzed by Northern blotting. Total RNA (15 μg) of the indicated mouse tissues was analyzed for expression of RhoGEF and p116^{Rip}. In addition, total RNA from proliferating and differentiated (serum-deprived) N1E-115 cells was analyzed. The smaller, ubiquitously expressed transcripts correspond to the cDNAs cloned. As a control for the amount of RNA loaded, the blot was reprobed with a GAPDH probe.

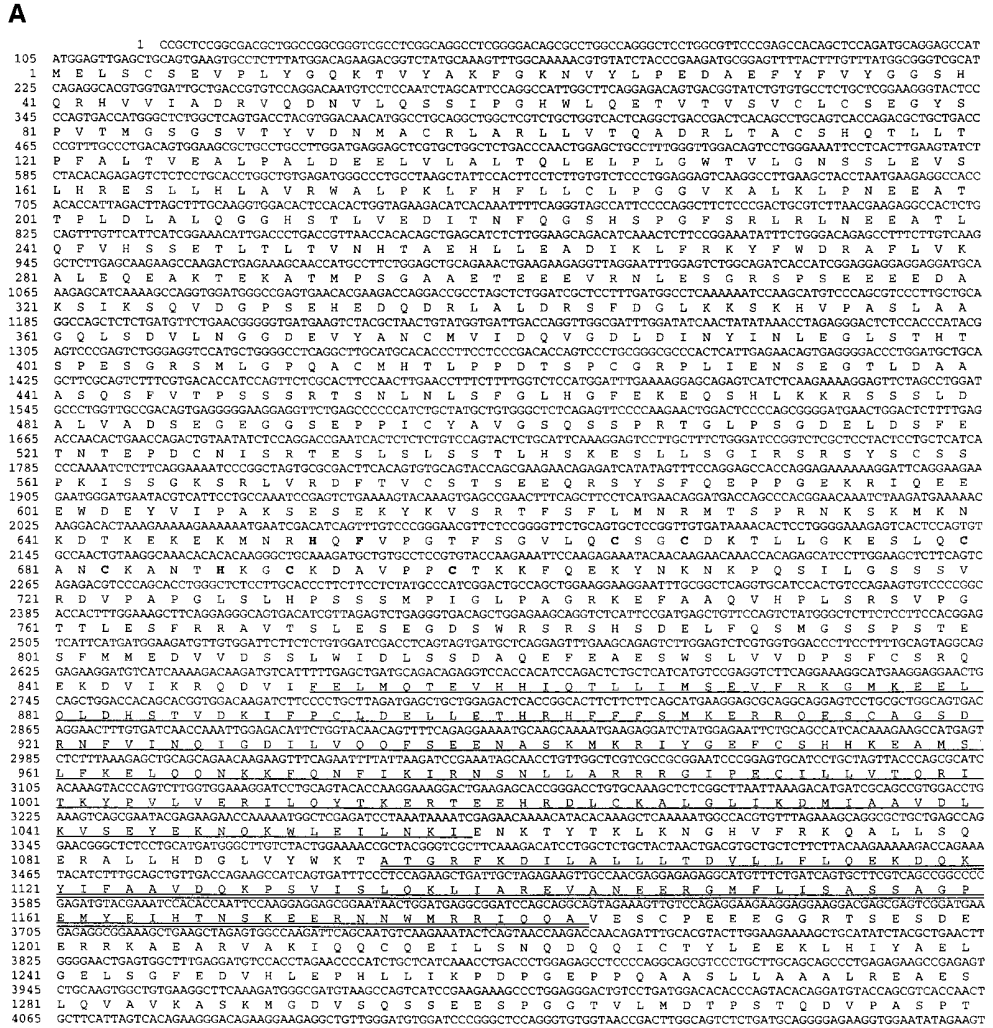
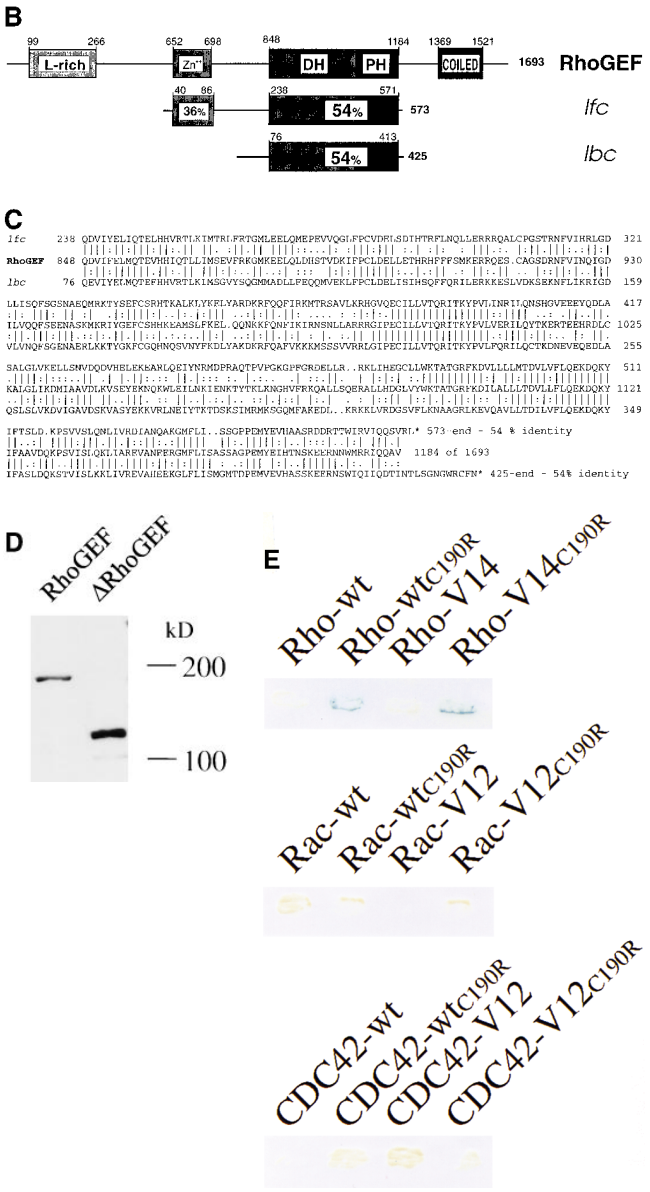


Figure 3. RhoGEF cDNA and predicted amino acid sequence. (*A*) RhoGEF nucleotide and polypeptide sequence. The DH domain is underlined; the PH domain is doubly underlined. The conserved residues of the zinc finger–like motif are in bold. These sequence data are available from EMBL/GenBank/DDBJ under accession number U73199. (*B*) Schematic representation of the RhoGEF protein and analogous domains in Lfc and Lbc, with relevent amino acid numbering indicated. Relevant residues are numbered. *DH*, Dbl-homologous domain; *PH*, pleckstrin homology domain; *COILED*, coiled-coil region; *L-rich*, leucine-rich region; Zn^{2+}, zinc finger motif. Percentages indicate relative identity of DH/PH or Zn finger domains in pairwise comparison with the corresponding domains in RhoGEF. (*C*) Alignment of the tandem DH/PH domains of RhoGEF with those of lbc and lfc (GenBank accession numbers U03634 and U28495, respectively). (*D*) Immunoblot analysis of RhoGEF expression. Lysates of transiently transfected COS cells were analyzed on a 10% SDS-PAGE gel followed by immunoblotting with polyclonal anti-RhoGEF antiserum. Both full-length RhoGEF and an NH_2-terminally truncated version of RhoGEF (ΔRhoGEF, lacking amino acids 1–684) were expressed. (*E*) Lack of two-hybrid interactions between RhoGEF (RIP2) and Rho-family members Rac and Cdc42. Experimental details as in Fig. 1.

domain. We obtained six positive clones from a screen with wild-type RhoA. Sequence analysis showed that the clones were identical and encoded a novel protein with >55% identity to known GDIs for Rho-family GTPases. We termed this clone RIP1-RhoGDI2 (RIP for Rho-Interacting Protein). We next screened mouse cDNA libraries with constitutively active V14RhoA (containing the C190R mutation; see below) to isolate three additional clones, termed RIP2, RIP3, and RIP4, that encode previously unknown proteins. As will be detailed below, RIP2 represents a novel GDP/GTP exchange factor (RhoGEF), while RIP3 and RIP4 encode putative structural proteins.

Binding to Wild-Type and Mutant RhoA Proteins

We tested the various RIP clones for their ability to interact with wild-type and activated RhoA. Since the two-hybrid assay detects interactions only when both proteins translocate to the nucleus, we also used mutant versions of RhoA and V14RhoA in which the cysteine at codon 190 in



the RhoA CAAX lipid modification motif was replaced by an arginine to prevent membrane localization (Katayama et al., 1991; Adamson et al., 1992*a*). The results of the two-hybrid screens are shown in Fig. 1. RIP1, encoding the novel RhoGDI, binds strongly to wild-type RhoA, but only poorly to V14RhoA; further details of RIP1 will be reported elsewhere. RIP2, encoding RhoGEF (see below), binds equally well to both wild-type and activated RhoA, suggesting similar affinities for the GDP- and GTP-bound forms of RhoA. In contrast, RIP3 and RIP4 interact only with activated RhoA, at least under two-hybrid conditions, and thus represent potential targets of RhoA-GTP. We note that, in the case of RIP2, RIP3, and RIP4, the interaction with RhoA was critically dependent on the C190R mutation that disrupts the membrane localization signal (Fig. 1). In the absence of this mutation, lacZ staining with RIP2, RIP3, and RIP4 was only observed after very long incubations. In contrast, binding between RhoA and RIP1 (RhoGDI2) apparently requires the CAAX lipid modification motif (Fig. 1), as one would expect for Rho–GDI interactions (Hancock and Hall, 1993).

Northern Blot Analysis

We investigated *RIP* gene expression by Northern blot analysis (Fig. 2). We found that RIP1 (not shown), RIP2, and RIP3 are highly expressed in brain (both forebrain and hindbrain); furthermore, RIP2 and RIP3 are equally expressed in both proliferating and differentiated (serum-deprived) N1E-115 cells. As can be seen from Fig. 2, RIP2 and RIP3 transcripts are also prominent in lung and kidney, while RIP3 shows additional expression in heart. Although isolated from a brain cDNA library, RIP4 appears to be specific for lymphoid organs, with no message detectable in brain or N1E-115 cells (total RNA blot; not shown). Therefore, RIP4 was not further characterized in the present study.

RhoGEF, a Novel Putative GDP/GTP Exchange Factor

Sequence analysis of the RIP2 cDNA insert (3.25 kb) revealed an open reading frame encoding a predicted polypeptide (1,009 amino acids) with a Dbl-homologous (DH) domain in tandem with a pleckstrin homology (PH) domain. Such tandem DH/PH domains are conserved among the GDP/GTP exchange factors for Rho-family GTPases (Quilliam et al., 1995). We found that RIP2 fails to undergo two-hybrid interactions with either of two other Rho-family members, Rac1 and CDC42Hs (Fig. 3 *E*), strongly suggesting that RIP2 is a Rho-specific exchange factor. Therefore, we called this protein RhoGEF.

The complete cDNA sequence of RhoGEF was obtained from various overlapping clones isolated from a mouse brain cDNA library. The full-length RhoGEF cDNA encodes a protein of 1,693 amino acids with a predicted molecular mass of 190.3 kD (Fig. 3 *A*). A schematic representation of the RhoGEF protein and a comparison with the structures of the most closely related GDP/GTP exchange factors are shown in Fig. 3 *B*. The sequence of the tandem DH/PH domain of RhoGEF is 54% identical to that of two other exchange factors, notably the Rho-specific exchanger Lbc and its close relative Lfc (Toksoz and Williams, 1994; Zheng et al., 1995; Whitehead et al., 1995). An alignment of these DH/PH regions is shown in Fig. 3 *C*. Markedly less similarity exists with the DH/PH domains of other exchanger factors, such as Tiam-1 (21%; Habets et al., 1994).

Upstream of its conserved DH/PH domain, RhoGEF contains a leucine-rich region and a cysteine-rich zinc finger–like motif, which is also found in the Lfc exchangers (Fig. 3 *B*); such a motif is thought to be involved in protein–lipid interactions (Ahmed et al., 1991). Downstream of the DH/PH domain, RhoGEF contains a region with weak homology to cytoskeleton-associated proteins such as plectin and myosin heavy chain. This highly charged region is supposed to form an α-helical coiled-coil structure mediating protein–protein interactions (Lupas et al., 1991).

Transfection of an expression vector for RhoGEF into COS cells yields a protein with an apparent size of ~200 kD in Western blot (Fig. 3 *D*, which also shows expression of a truncated RhoGEF protein, ΔRhoGEF). That RhoGEF

migrates somewhat slower on SDS-PAGE than predicted is presumably due to the charged coiled-coil region.

RhoGEF, Like V14RhoA, Induces Cytoskeletal Contraction and Inhibits Neurite Outgrowth

To examine the possible role of RhoGEF and RhoA in controlling neurite behavior, we determined their effect on the morphology of N1E-115 cells. These cells acquire a flattened morphology and begin to extend neurites after serum removal. Readdition of serum, LPA (the active ingredient of serum; Moolenaar, 1995), a thrombin receptor agonist peptide, or S1P triggers rapid retraction of developing neurites and transient rounding of the cell body (Jalink and Moolenaar, 1992; Jalink et al., 1993, 1994; Postma et al., 1996). We used a transient transfection protocol, in which transfected N1E-115 cells are identified by blue staining (Kranenburg et al., 1995; see Materials and Methods).

The majority of the V14RhoA-expressing cells are fully rounded and fail to extend neurites upon serum removal, whereas untransfected or control transfected cells remain flattened and undergo morphological differentiation (Fig. 4 *A* and Table I). Correct protein expression of the transfected cDNAs was verified by immunoblot analysis (Fig. 4 *B*). Thus, activation of RhoA is sufficient to prevent neurite outgrowth, consistent with active RhoA maintaining the cortical actomyosin system in a fully contracted state (Jalink et al., 1993, 1994). As shown in Fig. 4 *A* and Table I, the effect of RhoGEF expression on cell morphology is indistinguishable from that observed with V14RhoA. These results strongly suggest that RhoGEF activates RhoA in vivo to promote cytoskeletal contraction and to inhibit neurite outgrowth. Expression of NH_2-terminally truncated ΔRhoGEF (Figs. 3 *D* and 4 *B*), lacking amino acids 1–684, yielded the same phenotype (Fig. 4 *A* and Table 1). This implies that the leucine-rich region and the zinc finger motif of RhoGEF are dispensable for its activity.

Table I. Morphology of Transfected N1E-115 Cells

	Rounded	Neurite-bearing*
	%	%
Serum-free:		
Control	11 ± 1	30 ± 4
V14RhoA	33 ± 3	13 ± 2
RhoGEF	33 ± 2	17 ± 2
ΔRhoDEF‡	34 ± 6	14 ± 2
8% serum:		
Control	54 ± 4	5 ± 2
N19RhoA	24 ± 5	34 ± 2
$p116^{Rip}$§	38 ± 5	20 ± 2
$\Delta p116^{Rip}$	56 ± 10	9 ± 3

N1E-115 cells were transiently transfected with the indicated plasmids together with the *LacZ* gene to identify transfected cells, as described in Materials and Methods. Cell morphology was assessed in both serum-free medium (which promotes neurite outgrowth) and serum-containing medium (which contains LPA and prevents neurite outgrowth), as indicated. The percentage of undifferentiated (rounded without neurites) and neurite-bearing cells (flattened) was calculated from at least 300 positive cells counted. An average percentage was calculated from three dishes per transfection and three independent transfections. Numbers are percentages of total number of blue-stained cells (mean ± SEM). Differences with control cells are statistically significant ($P < 0.001$) according to *t* test. For details see Materials and Methods.

*Neurites longer than twice the soma diameter were scored positive.

‡ΔRhoGEF lacks amino acids 1–684.

§$\Delta p116^{Rip}$ lacks amino acids 1–544.

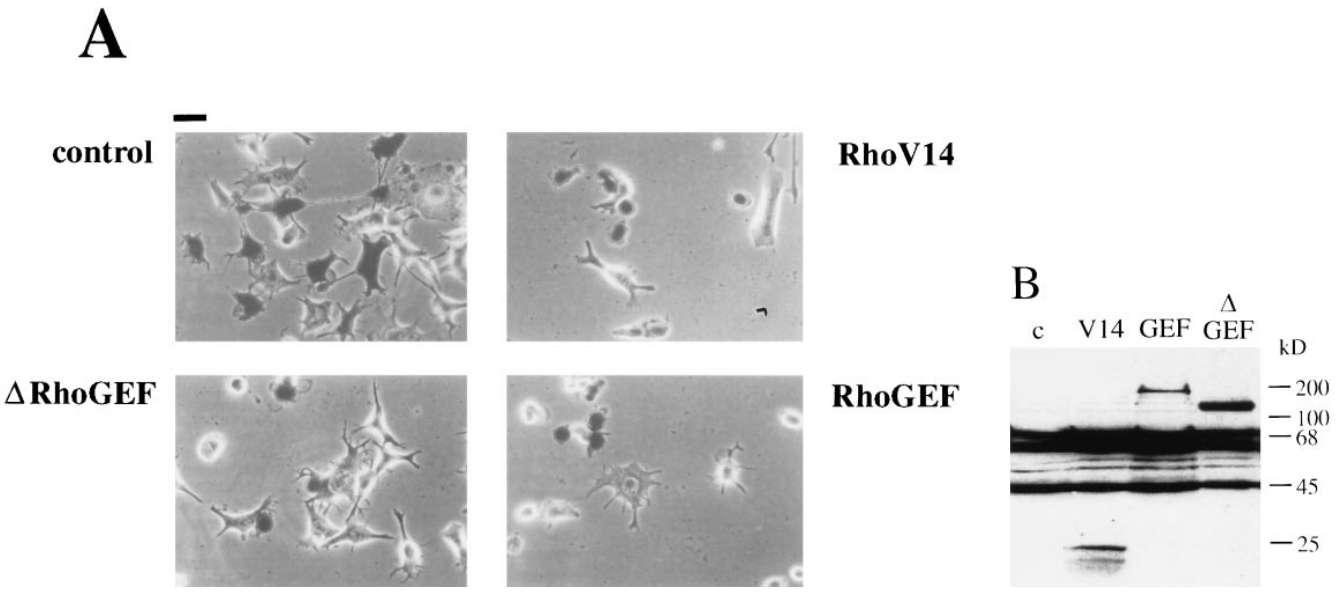


Figure 4. Regulation of neuronal cell shape by RhoGEF expressed in N1E-115 cells. N1E-115 cells were transiently transfected with the indicated cDNAs, all containing an NH_2-terminal epitope tag (HA) and cotransfected with a plasmid containing the gene for β-galactosidase (*control*, empty vector plasmid). Truncated RhoGEF (*ΔRhoGEF*) lacks amino acids 1–684. After 48 h, the cells were fixed and stained for β-galactosidase activity. (*A*) Phenotype of transfected cells (*dark cells*). Both V14RhoA- and RhoGEF-transfected cells have a rounded morphology without neurites; see also Table I. (*B*) Analysis of protein expression in immunoblot. Total lysates were prepared from a duplicate transfected culture and analyzed on a 12% SDS-PAGE gel. The blot was probed with anti-HA antibody 12CA5. Bar, 30 μm.

p116^{Rip}, a Novel RhoA-binding Protein

The full-length RIP3 cDNA was obtained from several overlapping clones. The cDNA sequence encodes a protein of 1,024 amino acids with a predicted molecular mass of 116.4 kD, which we termed p116^{Rip} (Fig. 5 *A*). While the sequence of p116^{Rip} is not closely related to that of known proteins, it contains several motifs typical for signaling molecules, including a PH domain and two proline-rich regions that are putative binding sites for Src homology 3 (SH3) domains. In addition, p116^{Rip} contains a COOH-terminal coiled-coil region showing distant relationship with that of myosin heavy chain, plectin, and Rho-kinase (Fig. 5 *B*). This coiled-coil region overlaps with the RhoA-binding region (residues 545–823), as inferred from the two-hybrid binding experiments.

When expressed in COS cells, p116^{Rip} has an apparent molecular mass of ~125 kD and exactly comigrates with the endogenous protein in N1E-115 cells (Fig. 5 *C*). As with RhoGEF, the discrepancy between the apparent and predicted size of p116^{Rip} may be due to the charged coiled-coil region.

The Interaction of p116^{Rip} with RhoA in N1E-115 Cells Is Nucleotide Independent

Although RIP3 binds only to activated RhoA under yeast two-hybrid conditions (Fig. 1), it remains to be examined whether the interaction between p116^{Rip} and RhoA is GTP specific under physiological conditions. To this end, N1E-115 cell lysates were incubated with either the GDPβS- or GTPγS-bound forms of GST–RhoA, or with GST alone. The fusion proteins were pulled down with glutathione beads and analyzed for interaction with endogenous p116^{Rip} by immunoblotting. As shown in Fig. 5 *E*, both the GTP- and the GDP-bound forms of RhoA are able to bind p116^{Rip}. Thus, whereas p116^{Rip} binds only to activated RhoA in yeast, the interaction of p116^{Rip} with RhoA is nucleotide independent in N1E-115 cells. One explanation for this discrepancy would be that the p116–RhoA interaction requires an additional protein not present in the yeast system.

Overexpressed p116^{Rip} Induces Cell Flattening and Neurite Outgrowth Similar to Dominant-Negative N19RhoA and C3 Toxin

We next examined the effects of p116^{Rip} on N1E-115 cell morphology. As can be seen from Fig. 6 *A* and Table I, overexpression of p116^{Rip} (Fig. 6 *B*) induces cell flattening and neurite outgrowth in the presence of serum, similar to the action of C3 (Jalink et al., 1994). The same morphological effects were observed after transfection of dominant-negative RhoA, N19RhoA (Fig. 6, *A* and *B*, and Table I). By analogy to dominant-negative Ras mutants (Feig, 1994; Quilliam et al., 1995), N19RhoA is thought to form stable, inactive complexes with Rho-specific exchange factors, thus titrating out RhoGEF activity. An NH_2-terminally truncated form of p116^{Rip} (Δp116^{Rip}), which lacks amino acids 1–545 encompassing the PH domain and both proline-rich regions, had no detectable effect on N1E-115 cell morphology, even when it was expressed at much higher levels than full-length p116^{Rip} (Fig. 6, *A* and *B*, and Table I). This suggests that the PH domain and/or the SH3-binding sites are essential for p116^{Rip} to exert its effect.

When p116^{Rip}-overexpressing cells were serum deprived and subsequently exposed to LPA (1 μM) or serum (which contains LPA; Moolenaar, 1995), no detectable cell rounding or neurite retraction ensued (as observed in three independent transfections; data not shown). Resistance of newly formed neurites to LPA and serum was also observed after transfection of dominant-negative N19RhoA (Table I, and results not shown), as was shown earlier for C3-treated cells (Jalink et al., 1994). Thus, overexpressed p116^{Rip} counteracts RhoA-mediated signaling.

Discussion

Previous studies using the Rho-inactivating C3 toxin suggested that Rho plays an important role in the control of neurite retraction as well as neurite outgrowth in both neuronal cell lines and primary sympathetic ganglion cells (for references see Introduction). In the present study we have established the physiological importance of RhoA in controlling neurite behavior in N1E-115 cells. By using the yeast two-hybrid system, we have isolated a novel, putative Rho-specific exchange factor (RhoGEF) and a RhoA-binding protein (p116^{Rip}) that affect neuronal morphology. Of note, successful two-hybrid interaction between RhoGEF and RhoA and between p116^{Rip} and RhoA was only observed after disrupting the membrane localization signal in RhoA (C190R mutation) to facilitate entry of RhoA into the nucleus. We find that RhoGEF mimics activated RhoA (V14RhoA) in causing sustained neurite retraction and cell rounding, whereas overexpressed p116^{Rip} mimics dominant-negative RhoA and C3 toxin in stimulating cell flattening and neurite extension. Our results establish RhoA, RhoGEF, and p116^{Rip} as critical determinants of neuronal cell shape.

RhoGEF

RhoGEF is a large protein (190 kD), whose catalytic DH/PH region is 54% identical to those of the Rho-specific exchangers Lbc and Lfc (Whitehead et al., 1995; Glaven et al., 1996). RhoGEF mRNA is abundant in brain and N1E-115 cells, whereas Lbc transcript is prominent in skeletal muscle and hematopoietic cells but not detectable in brain (Tokosz and Williams, 1995). Therefore, Lbc is unlikely to participate in Rho-dependent neuronal morphogenesis. RhoGEF expression is not restricted to neuronal tissue and cell lines but is also found in kidney, lung, and, to a much lesser extent, heart (Fig. 2).

In addition to its catalytic DH/PH region, RhoGEF contains a zinc finger motif, a leucine-rich domain, and a coiled-coil region; the latter two domains may be involved in mediating protein–protein interactions. Experiments with an NH_2-terminally truncated version of RhoGEF reveal that the leucine-rich region and the zinc finger motif are dispensable for the catalytic activity of RhoGEF in vivo. We find that RhoGEF binds equally well to wild-type and activated RhoA, at least under two-hybrid conditions. This finding supports a model in which RhoGEF not only stimulates GDP release but also actively participates in promoting GTP accumulation. In contrast, Ras-specific

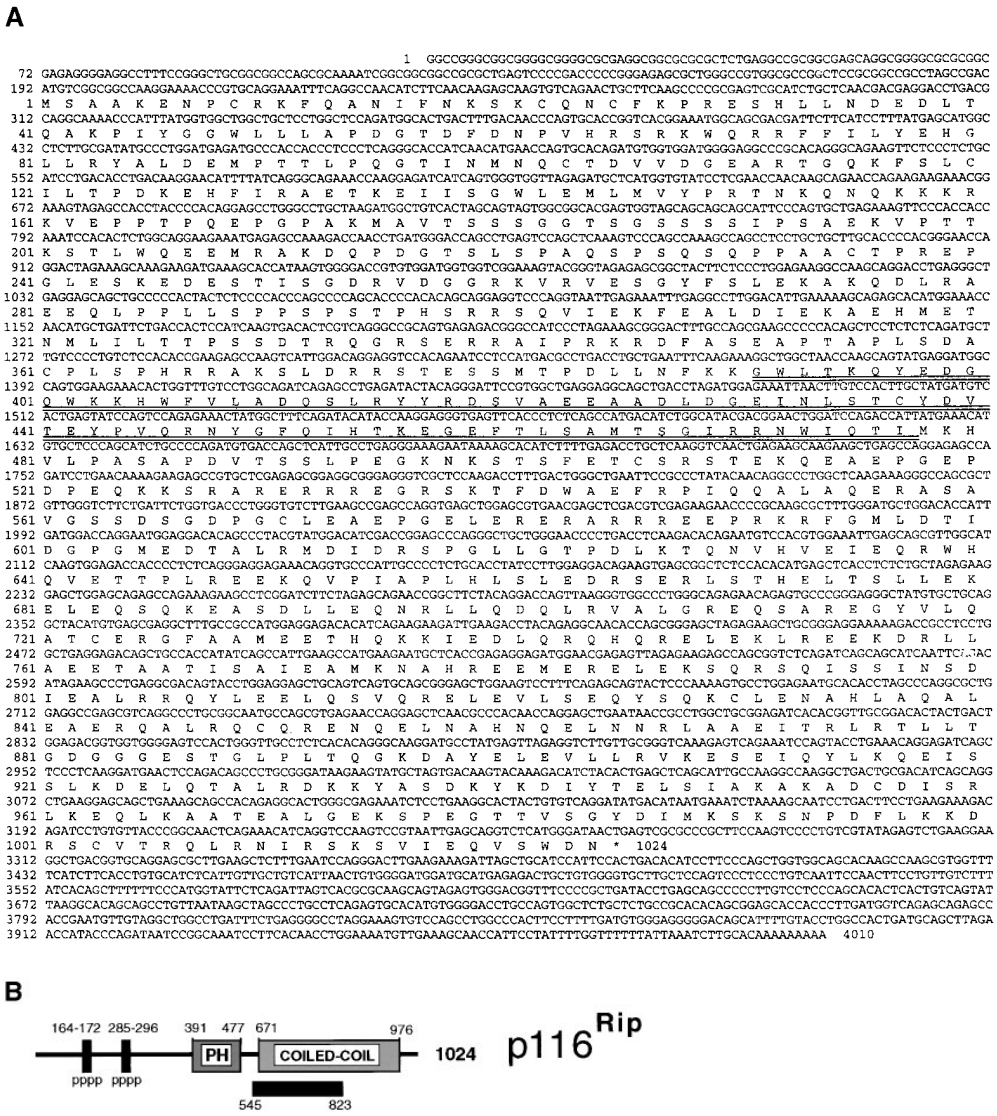


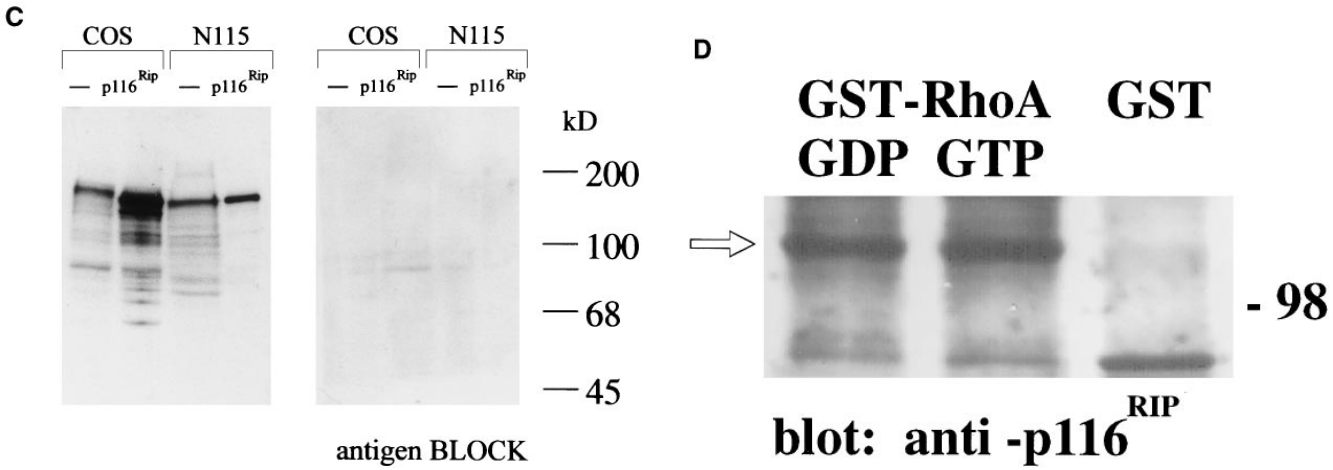
Figure 5. p116^{Rip} cDNA, predicted amino acid sequence, and interaction with RhoA in N1E-115 cells. (*A*) p116^{Rip} nucleotide and polypeptide sequence. The PH domain is underlined. The sequence contains two proline-rich regions (amino acids 164–172: PPTPQEPGP, and amino acids 285–296: PPLLSPPSP) that are putative SH3-binding sites (*B*). These sequence data for p116^{Rip} are available from EMBL/GenBank/DDBJ under accession number U73200. (*B*) Schematic representation of domains in p116^{Rip}; *pppp*, proline-rich putative SH3-binding regions (see above); *PH*, pleckstrin homology domain; *COILED-COIL*, coiled-coil domain. The RhoA-interacting region is indicated (amino acids 545–823, *black bar*). (*C*) Immunoblot analysis of p116^{Rip} transiently expressed in COS and N1E-115 cells. Cell lysates were analyzed on a 10% SDS-PAGE gel followed by immunoblotting with a polyclonal anti-p116^{Rip} antibody. (*Right*) Analysis of identical samples, in which the blot was incubated with primary antiserum in the presence of the synthetic peptide used for immunization (see Materials and Methods). (*D*) Interaction of p116^{Rip} with the GDP- and GTP-bound forms of RhoA in N1E-115 cells. Purified GST–RhoA fusion proteins, preloaded with either GDPβS or GTPγS, were used to fish in lysates of serum-starved N1E-115 cells, as described in Materials and Methods. GST alone served as a control. The GST proteins were isolated on glutathione beads and analyzed by immunoblotting using anti-p116^{Rip} antibody. (*Open arrow*) Position of p116^{Rip}.

GDP/GTP exhanger factors, such as yeast CDC25 and mammalian SOS, predominantly recognize the inactive, GDP-bound form of their target GTPase (e.g., Munder and Fuerst, 1992; Quilliam et al., 1995). That RhoGEF acts specifically on Rho is further supported by the finding that overexpression of Rac or Cdc42 fails to mimic RhoGEF action in N1E-115 cells: both activated Rac and Cdc42 promote cell flattening instead of cell rounding (van Leeuwen, F., and J. Collard, unpublished observations).

A major challenge for future studies is to delineate how receptor stimulation may couple to the RhoGEF-RhoA pathway. We previously showed that the Src tyrosine kinase is involved in LPA- and thrombin-induced neurite retraction, but cause–effect relationships are not clear (Jalink et al., 1993). We are currently investigating whether RhoGEF may be activated, either directly or indirectly, via a protein tyrosine kinase.

p116^{Rip}

The amino acid sequence of p116^{Rip} shows little homology to known proteins. While the protein specifically interacts with activated RhoA under two-hybrid conditions, our GST fishing experiments indicate that in N1E-115 cells p116^{Rip} interacts with both RhoA–GDP and RhoA–GTP. Although such a nucleotide-independent interaction seems unusual for molecular "switches" like RhoA, there is a precedent for proteins to interact with Rho in a nucleotide-independent manner. For example, both the GDP- and GTP-bound forms of RhoA are capable of binding phosphatidylinositol-5 kinase type 1 (Ren et al., 1996). Furthermore, the interaction between the p140 PRK2 kinase and RhoA is nucleotide independent, at least in vitro (Vincent and Settleman, 1997). Our failure to detect an association between p116^{Rip} and wild-type RhoA under two-hybrid con-



ditions may suggest that this association requires an additional protein (possibly RhoGDI) that is not present in yeast. We are currently investigating this possibility.

Although their primary sequences are highly divergent, p116^{Rip} shares some characteristics with the Rho-binding protein *citron* (Madaule et al., 1995), particularly a PH domain and a large coiled-coil region. In p116^{Rip}, as in *citron*, the coiled-coil region largely overlaps with the putative Rho-binding domain. While the biochemical function of p116^{Rip} is as yet unknown, it is likely that p116^{Rip} partici-

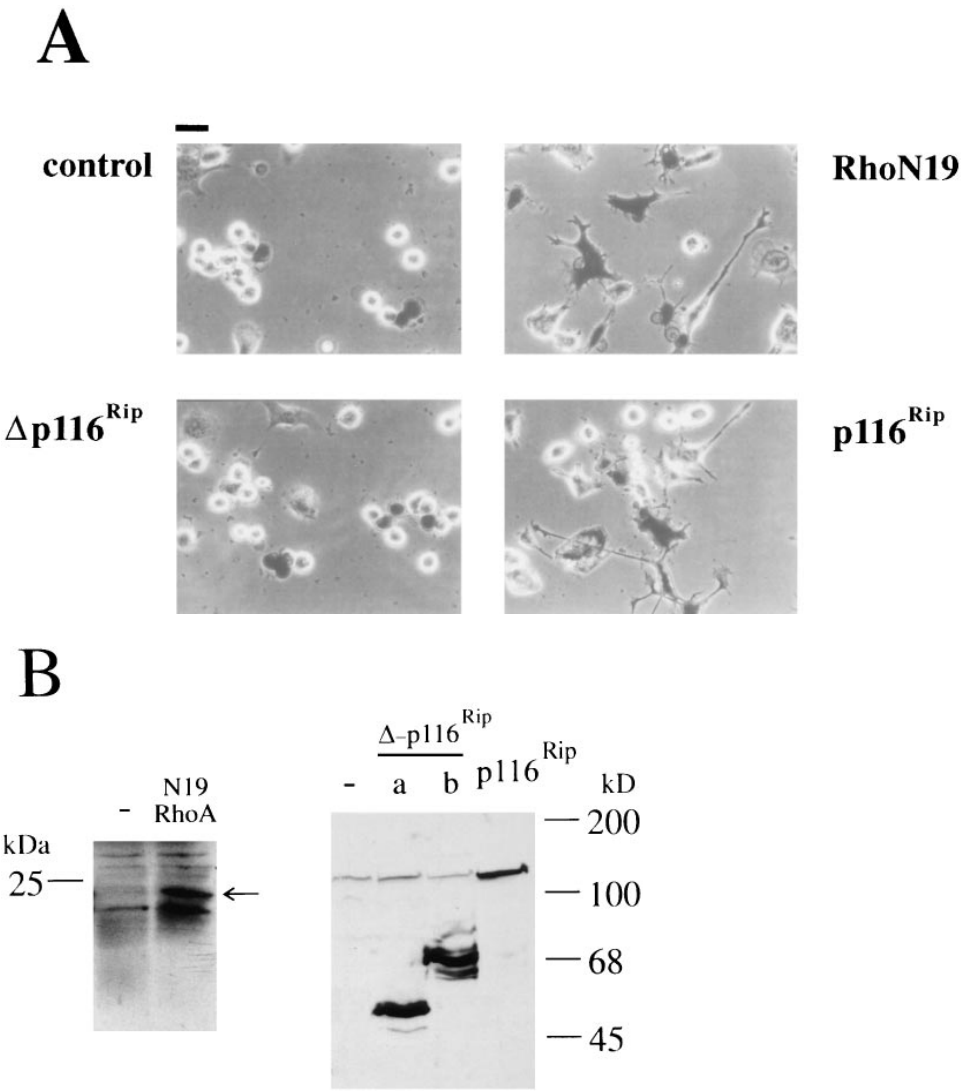


Figure 6. Induction of neurite outgrowth by p116^{Rip} and dominant-negative N19RhoA expressed in N1E-115 cells. (*A*) N1E-115 cells were transiently transfected with the indicated cDNAs, as in Fig. 4. (*B*) Analysis of protein expression in immunoblot, as in Fig. 4 *B*. (*Left*) HA-N19RhoA expression visualized in an anti-HA blot; (*right*) expression of p116^{Rip} and Δp116^{Rip} (anti-p116^{Rip} blot); *a* and *b* refer to two distinct truncation mutants (amino acids 545–1,024 and 545–823, respectively; see Materials and Methods). Note the presence of endogenous p116^{Rip} in all lanes. This signal is markedly increased in the cells transfected with full-length p116^{Rip} (lane *4*). Bar, 30 μm.

pates in a multimolecular signaling complex that regulates Rho action. Overexpression of $p116^{Rip}$ stimulates neurite formation and, furthermore, renders the newly formed neurites resistant to receptor stimulation, similar to what is observed with dominant-negative N19RhoA or C3 toxin. This suggests that $p116^{Rip}$ is a negative regulator of RhoA signaling that inhibits, either directly or indirectly, RhoA-stimulated actomyosin contractility. Importantly, overexpression of truncated $\Delta p116^{Rip}$ (to higher levels than the full-length protein) has no morphological effects, while $\Delta p116^{Rip}$ does bind to activated RhoA (Fig. 6 *A* and Table I). This rules out the formal possibility that overexpressed $p116^{Rip}$ would inhibit RhoA signaling by sequestering active RhoA–GTP. Studies to elucidate the normal biochemical function and protein–protein interactions of $p116^{Rip}$ are underway.

Concluding Remarks

The present study indicates that RhoA, RhoGEF, and $p116^{Rip}$ are critical determinants of neuronal cell shape. The available evidence supports a model in which certain G protein–coupled receptors stimulate RhoGEF-RhoA "activation" and consequent actomyosin contraction mediated by Rho-kinase (Kimura et al., 1996). Rho inactivation would then abrogate RhoGEF/RhoA/Rho-kinase signaling, thereby inducing cytoskeletal relaxation followed by neurite formation (see also Jalink et al., 1994). This model is probably not unique for neuronal cells, as Rho-stimulated contractility is also observed in serum-deprived fibroblasts (Chrzanowska-Wodnicka and Burridge, 1996), albeit to a much lesser extent than in N1E-115 cells. While our present work focuses on the role RhoA, there can be little doubt that other members of the Rho family, particularly Rac and Cdc42, also play a critical role in several aspects of neuronal morphogenesis, such as growth cone formation and axonal pathfinding; in fact, genetic evidence points to a role for both Rac and Cdc42 in neurite formation during *Drosophila* embryogenesis (see Mackay et al., 1995, and references therein).

Finally, at the molecular level, a number of key questions are outstanding. What is the nature of the G protein subunit(s) that couple(s) the receptors for LPA, S1P, and thrombin to RhoGEF activation? What are the intermediate biochemical steps? And, what is the normal function of $p116^{Rip}$ in RhoA signaling? Future experiments should provide answers to these questions.

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