The Rit and Rin proteins comprise a distinct and evolutionarily conserved subfamily of Ras-related small GTPases. Although we have defined a role for Rit-mediated signal transduction in the regulation of cell proliferation and transformation, the function of Rin remains largely unknown. Because we demonstrate that Rin is developmentally regulated and expressed in adult neurons, we examined its role in neuronal signaling. In this study, we show that stimulation of PC6 cells with either epidermal growth factor or nerve growth factor (NGF) results in rapid activation of Rin. This activation correlates with the onset of Ras activation, and dominant-negative Ras completely inhibits Rin activation induced by NGF. Further examination of Ras-mediated Rin activation suggests that this process is dependent upon neuronally expressed regulatory factors. Expression of mutationally activated H-Ras fails to activate Rin in non-neuronal cells, but results in potent stimulation of Rin-GTP levels in a variety of neuronal cell lines. Furthermore, although constitutively activated Rin does not induce neurite outgrowth on its own, both NGF-induced and oncogenic Ras-induced neurite outgrowth were inhibited by the expression of dominant-negative Rin. Together, these studies indicate that Rin activation is a direct downstream effect of growth factor-dependent signaling in neuronal cells and suggest that Rin may function to transduce signals within the mature nervous system.

Ras proteins function as GTP/GDP-regulated switches that cycle between an active GTP-bound and an inactive GDP-bound conformational state to regulate a wide variety of cell functions, including cell proliferation, differentiation, and apoptosis (1). Ras proteins respond to extracellular stimuli by exchanging GTP for bound GDP, thereby triggering intracellular signaling cascades through their interaction with a variety of target proteins (2). The cycle between active and inactive states is tightly controlled, being stimulated by the interaction of Ras proteins with specific guanine nucleotide exchange factors (GEFs) (3) that induce the dissociation of GDP to allow GTP.

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¶ The abbreviations used are: GEF, guanine nucleotide exchange factor; BD, Rin/Ras-binding domain; GFP, green fluorescent protein; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; GST, glutathione S-transferase; GAP, GTPase-activating protein; NGF, nerve growth factor; EGF, epidermal growth factor; ERK, extracellular regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; RT, reverse transcriptase; CM, calcium/magnesium; HEK, human embryonic kidney.
Growth Factor-mediated Regulation of the Rin GTPase

Although the expression of wild type or constitutively active Rin had no effect on neurite extension in PC6 cells, dominant-negative Rin suppressed NGF-mediated neurite outgrowth. Furthermore, constitutively active H-Ras-induced neurite outgrowth was also suppressed by dominant-negative Rin. These studies suggest that Rin may play a critical role in transducing growth factor-dependent signals that are involved in maintaining normal nervous system function.

**EXPERIMENTAL PROCEDURES**

### Tissue Culture and Transfection—PC6 is a subline of PC-12 cells that produces neurites in response to NGF but grows as well isolated cells rather than in clumps. The PC6 line used in these studies was the parental line described by Pittman et al. (20) and was the generous gift of Dr. Thomas Vanaman (University of Kentucky, Lexington, KY). PC6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% (v/v) heat-inactivated horse serum, 5% (v/v) fetal bovine serum (Invitrogen), and 50 μg/ml gentamicin at 37 °C in a humidified atmosphere of 5% CO₂. HEK293 cells were grown in DMEM supplemented with 5% (v/v) fetal bovine serum and 50 μg/ml gentamicin, whereas Vero and MCIXC cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum and 50 μg/ml gentamicin. These lines were also maintained at 37 °C in a humidified atmosphere of 5% CO₂, NGF (Becton Dickinson) and epidermal growth factor (EGF, Sigma) were administered at a dose of 100 ng/ml.

Transfection of PC6, Vero, and MCIXC cells was performed using Effectene (Qiagen). For Rin and H-Ras activation assays, PC6 cells (7.5 × 10⁴ cells/60-mm plate) were transiently transfected with 1 μg of the indicated mammalian expression plasmid mixed with 5 μl of enhancer and 10 μl of Effectene according to the manufacturer's protocol. HEK293 cells were transfected using the calcium phosphate method as described previously (21).

### Plasmid Construction and Antibodies—Mammalian expression vectors for wild type and mutant H-Ras and Rin have been described previously (17, 21). pCGN-HA-RasWT and pDCR-Ras(V12) effector domains were the kind gift of Adrienne Cooper (Department of Neurobiology, University of North Carolina, Chapel Hill, NC), whereas pCDNA-Sos1-CAAX and pCDNA-GRF1-CAAX were the gift of Dr. Lawrence Quilliam (University of Indiana School of Medicine, Indianapolis, IN). 5′-EcoRI and 3′-BamHI sites were introduced to wild type H-Ras by polymerase chain reaction (PCR)-mediated DNA amplification. The PCR product was subcloned to the corresponding sites in pEGrfP-C1 to generate the Q77L mutant H-Ras mammalian expression vector. Site-directed mutagenesis was used to generate dominant-negative H-Ras (Ras(N17)) using pEGF-RasWT as described previously (17). All PCR products were verified by DNA sequence analysis. HA epitope-tagged proteins were detected by immunoblotting using anti-HA monoclonal antibody (12CA5) followed by incubation with horse-radish peroxidase-conjugated antibody (Zymed–Lab Services Inc.). GFP fusion proteins were detected using anti-GFP Living Colors antibody (CLONTECH) and horseshadish peroxidase-conjugated goat anti-rabbit IgG (Zymed Laboratories Inc.) as described (23, 24).

### Ribonuclease Protection Assays—Total RNA was isolated using a STAT-60 kit (Tel-Test B, Friendswood, TX) according to the manufacturer's protocol. A riboprobe plasmid for mouse Rin was generated by polymerase chain reaction (PCR) amplification of a 289-bp fragment from the N terminus of Rin (alpha acids 1–76) and subcloned into pZero-2™ (Invitrogen). The plasmid containing an 89-bp fragment of the mouse ribosomal protein L32 (19) was a gift of Dr. Daniel Noonan (University of North Carolina, Chapel Hill, NC). Anti-sense radiolabeled riboprobes were prepared using linearized templates and a Maxiscript™ (Ambion) kit according to the manufacturer's protocol. RNA protection assays were performed as described (24). Protected fragments were resolved by electrophoresis in a 5% acrylamide, 8 M urea sequencing gel. The gel was dried and exposed to X-Omat AR film (Eastman Kodak Co.) for the indicated time. The gel was quantitated using a Molecular Dynamics PhosphorImager SF (model 455A). Simultaneous measurement of the rpl32 transcripts, which encode the L32 ribosomal protein (24), served as an internal control for housekeeping gene levels.

### Neuronal Cultures and RT-PCR—Primary dissociated cultures of sympathetic neurons were prepared from the superior cervical ganglia of embryonic day 21 rats as described previously (25), except that the non-neuronal cells were minimized by incubating the dissociated ganglia for 3 h on plastic culture dishes prior to plating onto laminin-coated 35-mm dishes (~5,000 cells/dish). Cultures were maintained in culture medium containing 90% minimal essential medium (Invitrogen), 10% fetal calf serum (HyClone, Logan, UT), 2 mM t-glutamine, 20 μM uridine, and 20 μM fluorodeoxyuridine in the presence of 50 ng/ml 2.5 S NGF for either 1 (young) or 4 weeks (old).

Poly(A⁺) RNA was isolated from the cultured neurons and converted to cDNA, and specific cDNAs were amplified by subtracting 2% of the cDNA to PCR cycles, with 20–25 PCR cycles as well as the degree of PCR amplification for these specific genes and primer pairs as described previously (25–27). After amplification, cDNAs were separated by polyacrylamide gel electrophoresis on 12% gels, stained with SYBR Gold (Molecular Probes) and visualized by phosphorimaging technology (Fuji Medical Systems, Stamford, CT). Primer sequences were Rin sense primer, 5′-CTTCTTCACTGACTCAAC-3′, Rin antisense primer, 5′-CTTCTTCTGGATTTCTTCT-3′ (105 bp product); neurofilament M sense primer, 5′-AGCTGGACTCGCTGGGCAA-3′, and neurofilament M antisense primer, 5′-GGAGGCGGTCGCTGGCT-3′ (156 bp product). The identity of the amplified cDNAs was confirmed by DNA sequencing.

A similar approach was used to examine Rin expression from a series of neuronal cell lines. Poly(A⁺) RNA was isolated and converted to cDNA, and specific cDNAs were amplified by subtracting 3% of the cDNA to 20–25 PCR cycles in reactions containing 0.5 ml of [α-32P]dCTP. Reaction conditions were well within the linear range of PCR amplification for these gene/primer combinations. Following amplification, PCR products were separated on 12.5% gels and exposed to film for 2–3 days. Autoradiographs were used to examine the amplified products and the radioactivity in each band determined by scintillation counting. Measurement of the RPS16 transcripts, which encode the ribosomal protein S16, served as an internal control for housekeeping gene levels.

### Rin-GTP and Ras-GTP Precipitation Assays—A glutathione S-transferase (GST) fusion of the Rin and Ras binding domain (BD) of Raf (residues 1–140) was expressed and purified as described (21). Rin and Ras activation was assessed essentially as described previously (28) with minor modifications. Rin activation was monitored in cells transiently transfected with the mammalian expression vector pKH3-RinWT alone or in combination with pKH3-Ras(L61), pDH-Ras(V12), and the indicated pDH-Ras(V12) effector mutants, pCDNA-Sos1, pCDNA-GRF1, or pEGFP-Ras(N17), and incubated for an additional 36 h to allow maximal gene expression. Cells were then starved in serum-free DMEM for an additional 12 h and, where indicated, stimulated with growth factors for the indicated times. Cell monolayers were washed once in ice-cold PBS and lysed for 1 min on the plate with ice-cold lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 20 mM MgCl₂, 10% glycerol, 1% Nonidet P-40, and 1× protease inhibitor mix (Calbiochem)). Lysate was transferred to a 1.5 ml microcentrifuge tube and clarified for 10 min at 14,000 rpm. Protein concentration was determined by the Bradford assay (Bio-Rad) using bovine serum albumin as standard. GST-RafBD preclotted to glutathione beads (10 μg of protein/30 μl of resin beads) were added to 200 μl of total cell lysate in a final volume of 400 μl and incubated with rotation for 1 h at 4 °C to initiate the pull-down assay. Following three washes in ice-cold lysis buffer, bound proteins were eluted by incubation for 5 min at 100 °C in 2× PAGE sample buffer. Bound protein was separated by electrophoresis and Western analysis of each sample was resolved by SDS-polyacrylamide gel electrophoresis (PAGE) using a 12.5% polyacrylamide gel, transferred to nitrocellulose membranes, and subjected to immunoblotting using anti-HA or anti-GFP antibodies.

To analyze the role of MEK/ERK signaling in Ras-mediated Rin stimulation, PC6 cells were transiently transfected with pKH3-RinWT, pKH3-Ras(L61), or co-transfected with both expression vectors and incubated for an additional 36 h to allow for maximal gene expression. 24 h after transfection, cells were cultured in serum-free DMEM (± 10 μM PD-98059 as indicated) for 24 h. Fresh serum-free DMEM (± 50 μM PD-98059) was added 2 h prior to the preparation of total cell lysates in ice-cold lysis buffer containing phosphatase inhibitors (50 mM Tris (pH 7.5), 150 mM NaCl, 20 mM MgCl₂, 10% glycerol, 1% Nonidet P-40, 20 mM β-glycerol phosphate, 1 mM vanadate, 50 μM KF, and 1× protease inhibitor mix (Calbiochem)). HA-Rin-GTP was pulled down using GST-RafBD and analyzed by immunoblot analysis as described above.

### Nucleotide Binding Assay—In vivo guanosine nucleotide binding assays were performed essentially as described (29). Briefly, PC6 cells were transiently transfected with pKH3-RinWT or pKH3-Rin(N34) in 60-mm dishes. After 36 h, cells were incubated in phosphate-free media for 30 min followed by similar medium supplemented with 150 μCi of [32P]-labeled orthophosphate for an additional 4 h. Cells were washed once with ice-cold PBS, lysed on the plate for 1 min using ice-cold lysis buffer 2 (50 mM Tris- HCl (pH 7.5), 500 mM NaCl, 6 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, and 1× protease inhibitor mix (Calbiochem)).
centrifugation (10 min at 14,000 rpm), and added to a fresh tube containing 30 μl of glutathione beads precoupled with 10 μg of GST-RasBD. This mixture was incubated with rotation at 4 °C for 1 h, after which the beads were collected and washed three times with ice-cold lysis buffer. Bound protein was released by the addition of 20 μl of denaturing SDS buffer (0.2% SDS and 2 mM EDTA), the beads incubated at 68 °C for 15 min, and 1 mM GFP and 1 mM GAP added to serve as nucleotide standards. An aliquot of the sample (10 μl) was spotted to 20-cm poly(ethyleneimine-cellulose) TLC plates (EM Separations) for nucleotide separation. The chromatogram was developed using 1 mM LiCl, 1 mM formic acid and analyzed as described (22).

Neurite Outgrowth—PC6 cells were transiently transfected at 5×10^5 cells on coverslips placed in six-well dishes. Coverslips were precoated with 5 μg/ml laminin (Sigma) and 25 μg/ml poly-L-lysine (Sigma) in PBS for 2 h. PC6 cells were transiently transfected using Effectene (Qiagen) with one of the following plasmids: pEGFP-C1, pEGFP-Rin, pEGFP-Rin(L78), pEGFP-H-Ras(L61), pEGFP-Ras(N17), or pEGFP-Rin(N34), and examined by epifluorescence microscopy. Counting the day of transfection as day 0, cells were either not subject to growth factor stimulation or were treated with NGF (100 ng/ml) on day 2 and then fixed on day 5. Cells were then washed three times in CM-PBS (1.26 mM CaCl_2, 0.49 mM MgCl_2, 0.91 mM MgSO_4) and fixed with 3.7% formaldehyde in CM-PBS for 20 min at room temperature. Cells were permeabilized in 0.1% Triton X-100 in CM-PBS for 5 min, blocked for 30 min with 1% bovine serum albumin in CM-PBS, incubated with Texas Red-X-phalloidin (Molecular Probes) for 20 min, and washed extensively prior to mounting. For studies using HA-tagged H-Ras(L61), slips were fixed and blocked as above, incubated with 2 μg/ml anti-HA antibody for 20 min, washed with CM-PBS, and incubated with Texas Red-labeled anti-mouse (1:1000 dilution, Vector Laboratories). Fixed and stained slips were mounted on glass slides with 12 μl of Vectashield (Vector Laboratories) and examined under the appropriate illumination with a 40× objective lens on an E600 microscope (Nikon). Cells were scored positive for neurite outgrowth if one or more neurites exceeded 1 cell body diameter in length. At least 200 cells were counted per experiment with each experiment performed in triplicate.

**Western Immunoblot of ERK**—PC6 cells were transiently transfected as described above with the empty pKH3 vector control, pKH3-Rin(Y29), or pKH3-Ras(L61). Transfected cells were incubated for 48 h to allow maximal gene expression and serum-starved overnight in DMEM. Cells were then washed twice with ice-cold PBS and lysed on the plate with phosphosilic buffer (20 mM Tris (pH 7.6), 250 mM NaCl, 2.5 mM EDTA, 3 mM EGTA, 20 mM  β-glycerol phosphate, 1 mM vanadate, 50 mM KF, and 1× protease inhibitor mixture (Calbiochem)). Lysates were cleared by centrifugation, equal amounts of protein as determined by Bradford assay (Bio-Rad), and separated on 10% SDS-polyacrylamide gels, using a standard SDS-PAGE protocol. After electrophoresis, the gels were transferred to nitrocellulose membranes and probed with either polyclonal anti-ERK (New England Biolabs) or a phosphospecific ERK antibody (Promega) and developed using horse-radish peroxidase-conjugated secondary antibodies and chemiluminescence (ECL, Amersham Biosciences).

ERK activity was also monitored by immunoblot analysis following immunoprecipitation. PC6 cells were transfected as above with an expression vector encoding HA epitope-tagged ERK (the kind gift of Dr. Ginell Post, University of Kentucky, Lexington, KY) in combination with GFP-tagged Rin(L78), GFP-Ras(L61), or control plasmids. Transfected cells were incubated for 48 h to allow maximal gene expression, serum-starved overnight in DMEM, and lysates prepared in phosphosilic buffer. HA-ERK was immunoprecipitated from 500 μg of whole cell lysate using anti-HA antibody (5 μg) prebound to a slurry of protein G-Sepharose/protein A-Sepharose (80:20 mix). Bound proteins were eluted by incubation for 5 min at 100 °C in 20 ml of SDS-PAGE sample buffer. Immunoprecipitated protein and 50 μg of total cell lysate were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and subjected to immunoblot analysis using polyclonal anti-ERK (New England Biolabs), phosphospecific mitogen-activated protein kinase/ERK antibody (Promega), or polyclonal anti-GFP antibody (CLONTECH).

**RESULTS**

**Expression of Mouse Rin mRNA**—To address the tissue distribution of Rin, an exhaustive ribonuclease protection assay was performed (Fig. 1A). In contrast to the ubiquitous expression pattern seen with the closely related Rit GTPase and majority of Ras family proteins (1, 16, 18), the mouse Rin gene was expressed exclusively in neuronal tissues. Rin mRNA was most abundant in brain and spinal cord, with detectable levels found in the eye, but not in an extensive series of additional tissues. In the adult mouse brain, Rin was expressed in all of the subregions examined, with highest expression in the mid-hind region of the brain. Ribonuclease protection analysis was also used to examine Rin expression during murine development. As seen in Fig. 1B, Rin expression was initially detected in total RNA prepared from the heads of 14-day mouse embryos, and expression levels continued to steadily increase within the brain until ~20–25 days after birth, at which time Rin expression reached a plateau (Fig. 1B). This high level of expression continued in adult neuronal tissues.

**Expression of Rin in Premature and Mature Rat Neurons**—Although the restricted and developmentally controlled expression of Rin in neural tissues suggested that Rin was expressed in neurons, it is possible that Rin is expressed in other cell types, such as supporting cells or fibroblasts. To determine whether Rin is expressed in neurons, primary cultures of superior cervical ganglia were prepared from embryonic day 21 rats and incubated in the presence of NGF for 6 days (immature sympathetic neurons) or 30 days (mature neurons) and subjected to RT-PCR analysis. As shown in Fig. 2A (upper panel), Rin mRNA was not detected in immature neuronal cultures, but in mature superior cervical ganglia robust Rin

**FIG. 1. Tissue and developmental expression of murine Rin.** Total RNA (20 μg) extracted from a variety of mouse tissues (A) or from the indicated developmental stage (B) was subjected to ribonuclease protection assay as described under "Experimental Procedures." Radiolabeled, antisense RNA probes corresponding to Rin (228 nucleotides) or ribosomal protein L32 (89 nucleotides, internal control) were hybridized and subjected to ribonuclease treatment, and the RNA-protected probes were subjected to polycrylamide gel electrophoresis and visualized by autoradiography (12–15 h exposure). Quantitation of the protected RNA fragments was determined using a PhosphorImager, and the RNA concentrations per lane were normalized against the expression of the housekeeping gene rplL32. The data are representative of two separate assays.
expression was observed. No significant changes were seen in the mRNA level of the constitutively expressed gene neurofilament M under these same conditions (Fig. 2A, lower panel). To extend these results, and to identify a cultured cell system in which to examine the biological function of Rin, we examined Rin expression in a series of neuronal cell lines. RT-PCR analysis found Rin to be expressed in all of neuronal cell lines tested (Fig. 2B); Northern blot analysis was used to confirm Rin expression in PC6 cells (data not shown).

Use of GST-RafBD as an Activation-specific Probe for Rin—
Because the biological function of Rin is largely unknown, we wished to investigate the signaling pathways that lead to Rin activation in neuronal cells. To this end we developed a pull-down assay system to detect the levels of GTP-bound Rin in cells. This assay is based on the proven ability of Ras family effector proteins to interact specifically with the activated, GTP-bound form of their target GTPase. This type of assay has been used previously to assay for activation of a variety of Ras GTPases including Ras, Rap, and Rho family members (21, 28, 30–32). We have previously demonstrated a nucleotide-dependent interaction between Rin and the Ras interaction domains of several Raf GEFs and Raf kinase (17). To evaluate the use of these GST fusion proteins as a method to analyze in vivo levels of GTP-bound Rin, we prepared cell lysates from transfected PC6 cells, a subline of PC12 cells (44), expressing HA-tagged versions of activated (Rin(V29)) or dominant-negative (Rin(N34)) Rin mutants. These lysates were subjected to affinity precipitation assays for activated Rin using GST fusion proteins containing the minimal binding domains of Raf1, Raf, and RGL3. Although we have shown that each of these GST affinity probes interact specifically with GTP-bound recombinant Rin in vitro, preliminary studies found GST-RafBD to demonstrate the highest pull-down efficiency, allowing the most sensitive detection of GTP-bound Rin isolated from PC6 cell lysates (data not shown). Therefore, GST-RafBD was selected for use as the Rin-GTP affinity probe for the remainder of this study. Fig. 3A shows that expression of HA-tagged Rin(V29), a Rin mutant predicted to have an elevated GTP-bound state (30), yielded a robust level of Rin-GTP. Very little epitope-tagged Rin was recovered from PC6 cell lysates transiently expressing HA-Rin(N34), which is predicted to exist largely in the GDP-bound state (34). As expected, similar results were seen when these reagents were used to evaluate the guanine nucleotide state of transiently expressed activated (Ras(L61)) and dominant-negative (Ras(N17)) Ras mutants in PC6 cells (Fig. 3B). To confirm that GTP-bound Rin was selectively isolated following GST-RafBD-mediated pull-down, the nucleotide binding state of wild type Rin bound to GST-RafBD was directly assessed. Transiently transfected PC6 cells were metabolically labeled with [32P]orthophosphate, Rin proteins were precipitated using GST-RafBD, and bound nucleotides were eluted and separated by thin layer chromatography. As expected, precipitated wild type Rin contained only radiolabeled GTP, whereas dominant-negative Rin (Rin(N34)) failed to interact with the GST-RafBD and no radiolabeled guanine nucleotides were detected following chromatographic analysis (Fig. 3C). Taken together, these results demonstrate that GST-RafBD specifically interacts with Rin-GTP and can be used to monitor Rin activation.

NGF and EGF Induce Rapid Activation of Rin—
As an initial step in characterizing the regulation of Rin in vivo, the effect of polypeptide growth factors on the activation of Rin transiently expressed in pheochromocytoma cells was examined. The binding of NGF to the TrkA receptor on PC12 cells induces receptor dimerization and stimulates rapid tyrosine autophosphorylation, to elicit a well defined course of cellular signaling. To examine the ability of NGF to induce Rin activity, PC6 cells transiently transfected with HA epitope-tagged Rin were incubated in growth factor-deficient basal medium for 12 h and stimulated with NGF. Cell lysates were prepared at varying periods following NGF stimulation, and HA-Rin-GTP was
The data are representative of lysate, 100 ng/ml EGF (panel B) for the indicated periods of time. Rin-GTP and H-Ras-GTP were recovered with GST-RafBD. Each reaction contained (in a final volume of 400 µl) 200 µg of clarified cell lysate, 10 µg of GST-RafBD, and 30 µl of a 1:1 (v/v) suspension of glutathione-Sepharose. After incubation for 1 h at 4 °C, the samples were pelleted and washed as described under “Experimental Procedures.” The Rin-GTP and H-Ras-GTP precipitated by GST-RafBD precipitation was identified by immunoblot analysis with a monoclonal antibody (top panel, RafBD pellet). The level of expression of the expressed GTPases present in the lysate (bottom panel, Lysate) was also determined by immunoblot analysis. The data are representative of a typical experiment repeated five times.

Fig. 4. Growth factor-induced Rin activation. PC-6 cells were transiently transfected with expression constructs for HA-Rin or HA-H-Ras as indicated. Prior to the preparation of whole cell lysates, cells were serum-starved for 12 h and then stimulated with either 100 ng/ml NGF (panel A) or 100 ng/ml EGF (panel B) for the indicated periods of time. Rin-GTP and H-Ras-GTP were recovered with GST-RafBD. Each reaction contained (in a final volume of 400 µl) 200 µg of clarified cell lysate, 10 µg of GST-RafBD, and 30 µl of a 1:1 (v/v) suspension of glutathione-Sepharose. After incubation for 1 h at 4 °C, the samples were pelleted and washed as described under “Experimental Procedures.” The Rin-GTP and H-Ras-GTP precipitated by GST-RafBD precipitation was identified by immunoblot analysis with a monoclonal anti-HA antibody (top panel, RafBD pellet). The level of expression of the expressed GTPases present in the lysate (bottom panel, Lysate) was also determined by immunoblot analysis. The data are representative of a typical experiment repeated five times.

Thus, the stimulation of both NGF and EGF receptors in PC6 cells results in the rapid activation of Rin.

NGF-induced Rin Activation Is Ras-dependent—Ras serves to couple growth factor signals to a variety of cellular processes (2, 38, 39). A growing body of evidence supports the idea that this is the result, at least in part, of the ability of Ras to influence multiple downstream target proteins (2). These include the ability of Ras to promote signaling cascades that result in the activation of the Ras-related GTPases Ral and Rac (2). To investigate whether Ras was involved in mediating growth factor-induced activation of Rin, we co-transfected PC6 cells with expression vectors encoding epitope-tagged wild type Rin and GFP-tagged constitutively active Ras (GFP-H-Ras(L61)) and monitored Rin activation. As seen in Fig. 5A, the expression of Ras(L61) resulted in a potent stimulation in cellular HA-Rin-GTP levels. For quantitation, blots were scanned and evaluated using NIH Image software. The graph shows data from four independent experiments (results are mean values ± S.D.), and the Western blots are from one representative experiment.
with HA epitope-tagged Rin alone or co-transfected with expression vectors encoding Rin and dominant-negative Ras (GFP-Ras(N17)). The transfected cells were stimulated with NGF for 10 min, a time that results in maximal NGF-mediated Rin stimulation (see Fig. 4A), and HA-Rin-GTP levels determined using the GST-RafBD association assay. Although NGF activated HA-Rin in control cells, GFP-Ras(N17) expression both suppressed basal Rin-GTP levels and resulted in a complete inhibition of NGF-induced Rin activation (Fig. 5B). Taken together, these results suggest that Ras plays a role in NGF-induced activation of Rin in PC6 cells.

The structural determinates for effector interaction are provided, in part, by amino acids located in the effector domain of Ras. Certain mutations in this domain may completely inhibit Ras function, whereas other mutations have been found to selectively inhibit the binding of some effector molecules without influencing others (2). Thus, Ras(V12S35) binds Raf and not RalGDP and PI 3-kinase, whereas Ras(V12G37) selectively binds RalGEF, and Ras(V12C40) only interacts with PI 3-kinase (40-42). To determine whether Ras-dependent Rin activation relied upon one of these currently established Ras effector pathways, we co-transfected PC6 cells with expression vectors encoding epitope-tagged wild type Rin and constitutively active Ras carrying a second effector domain mutation and monitored Rin activation. As seen in Fig. 6A, the expression of activated Ras(V12) resulted in a potent stimulation in HA-Rin-GTP levels. Although not as strong as Ras(V12), Ras(V12S35) also elevated cellular Rin-GTP levels. However, co-expression of the other Ras effector domain mutants failed to activate Rin.

The experiments in Fig. 6A indicate that Ras-mediated Rin activation may rely in part on activation of the Raf kinase effector. To determine the requirement for MEK/ERK activity in this process, PC6 cells co-transfected with expression vectors encoding HA-tagged wild type Rin and H-Ras(L61) were treated with a MEK inhibitor PD-98059. Expression of activated Ras potently stimulated cellular GTP-Rin levels, and this activation was not sensitive to MEK inhibition (Fig. 6B, top panel). To confirm that the PD-98059 inhibitor suppressed the activity of the MEK pathway, the phosphorylation state of ERK was examined in PC6 cells expressing H-Ras(L61). As expected, PD-98059 inhibited Ras-induced activation of ERK (Fig. 6B, bottom panel). Taken together, these experiments suggest that Ras-mediated Rin activation relies upon an effector pathway distinct from the Raf/ERK kinase cascade.

Ras-dependent Rin Activation Requires Additional Factors—Because Rin expression is restricted to neuronal tissues, we next examined whether the regulatory pathway controlling Rin activation was ubiquitous or whether Rin activation was also restricted to neuronal cell types. To this end, the ability of EGF to induce Rin activation in human embryonic kidney (HEK293) cells was analyzed. As seen in Fig. 7A, following transient expression of HA-tagged Rin, EGF stimulation failed to elevate Rin-GTP levels. Indeed, EGF stimulation resulted in a transient decrease in GTP-bound Rin levels. The inability of EGF to stimulate Rin presumably results from a deficiency distinct from the signaling cascade that regulates Ras activity, because EGF treatment potently stimulated H-Ras in these cells (Fig. 7A). To extend this analysis, we co-transfected a series of neuronal and non-neuronal cell lines with expression vectors encoding epitope-tagged Rin and constitutively active H-Ras. As seen in Fig. 7B, expression of H-Ras(L61) stimulated Rin activation in the neuronal cell lines (PC6 and MC1XC) but not in the epithelial and fibroblast cell lines that were examined (HEK293 and Vero). Similar results were seen when using the neuronal cell lines SH-SY5Y and SN6, in which activated H-Ras was found to stimulate Rin-GTP levels (data not shown).

It has been demonstrated that residues within the switch 1 and switch 2 regions of Ras contribute to its interaction with GEFs (43). Because Rin and Ras share considerable homology within these regions, we next examined the ability of RasGEFs to regulate Rin activity. Co-expression of wild type Rin with the membrane-targeted catalytic domains of Sos1 or GRF1 in HEK293 cells failed to result in elevation of Rin-GTP levels, although, as expected, both GEFs activated H-Ras (Fig. 8A). These results indicate that Sos1 and GRF1 do not function as direct GEFs for Rin. However, co-expression of Rin with membrane-targeted Soo1 and GRF1 in PC6 cells resulted in an elevation of cellular Rin-GTP levels (Fig. 8B).

Expression of Rin Does Not Induce Neurite Outgrowth in Pheochromocytoma Cells—The expression of Rin in the mature central nervous system suggests that Rin might function to maintain neural differentiation. To explore this possibility, we next determined whether, like oncogenic Ras, activated Rin could induce neurite outgrowth in pheochromocytoma cells (13). In PC12 cells, expression of activated Ras is characterized by the cessation of mitosis and extension of neuron-like pro-
cesses in a program of events that is similar to those induced by NGF treatment (7, 13, 44, 45). PC6 cells transiently transfected with expression constructs for HA-tagged Rin or H-Ras, serum-starved for 12 h, and then stimulated with 100 ng/ml EGF for the indicated periods of time. Rin-GTP and H-Ras-GTP were recovered with GST-RafBD and identified by immunoblot analysis with a monoclonal anti-HA antibody (top panel, RafBD pellet). The level of expression of the expressed GTPases present in the lysate (bottom panel, Lysate) was also determined by immunoblot analysis using anti-HA antibody. B, PC6, MCIXC, HEK293, and Vero cells were co-transfected with plasmids encoding HA-tagged wild type Rin and pEGFP-C1, pEGFP-Ras(L61), pKH3, or pKH3-Ras(L61) as indicated. After 48 h, cells were serum-starved, Rin-GTP was precipitated by GST-RafBD, and both pellet (upper panels) and whole cell lysates (lower panels) were analyzed by immunoblotting with the indicated antibodies. Data are representative of a typical experiment repeated three times.

Fig. 7. EGF and Ras-mediated Rin activation requires additional factors. A, HEK293 cells were transiently transfected with expression constructs for HA-tagged Rin or H-Ras, serum-starved for 12 h, and then stimulated with 100 ng/ml EGF for the indicated periods of time. Rin-GTP and H-Ras-GTP were recovered with GST-RafBD and identified by immunoblot analysis with a monoclonal anti-HA antibody (top panel, RafBD pellet). The level of expression of the expressed GTPases present in the lysate (bottom panel, Lysate) was also determined by immunoblot analysis using anti-HA antibody. B, PC6, MCIXC, HEK293, and Vero cells were co-transfected with plasmids encoding HA-tagged wild type Rin and pEGFP-C1, pEGFP-Ras(L61), pKH3, or pKH3-Ras(L61) as indicated. After 48 h, cells were serum-starved, Rin-GTP was precipitated by GST-RafBD, and both pellet (upper panels) and whole cell lysates (lower panels) were analyzed by immunoblotting with the indicated antibodies. Data are representative of a typical experiment repeated three times.

Fig. 8. RasGEFs do not directly regulate Rin activation in HEK293 cells. Rin is activated by RasGEFs in PC6 but not HEK293 cells. HEK293 (A) or PC6 cells (B) were co-transfected with plasmids encoding HA-tagged wild type Rin or HA-tagged wild type H-Ras and pCDNA3, pCDNA3-RasGRF1/CDC25-CAAX, or pCDNA3-Sos1-CAAX as indicated. After 48 h, cells were serum-starved for 12 h, and Rin-GTP and H-Ras-GTP precipitated by GST-RafBD and both pellet (upper panels) and cell lysate (lower panels) were analyzed by immunoblotting with the indicated antibodies. Data are representative of a typical experiment repeated three times.

Growth Factor-mediated Regulation of the Rin GTPase

Because the expression of activated Raf and MEK kinases can cause terminal differentiation of PC12 cells, it is believed that Ras activation of the Raf/ERK pathway plays a central role in neuritogenesis (11, 46). Because Rin-transformed NIH3T3 cells did not show up-regulated ERK kinase activity (19), we rationalized that the inability to stimulate this pathway might explain, at least in part, the different biological properties of these GTPases. To address this possibility, we introduced expression plasmids that encoded activated versions of Rin or Ras into cultures of PC6 cells. After 48 h, the transfected cells were serum-starved for 12 h, and cell lysates prepared. To determine the consequences of activated G-protein expression on ERK activity, we measured phospho-ERK using an antibody specific for the active phosphotyrosine and phosphothreonine ERK. We found that activated Rin failed to increase ERK phosphorylation, whereas activated Ras potently stimulates ERK activation (Fig. 10). Thus, although Rin is a Ras subfamily member expressed within neurons, it does not activate the cellular signaling pathways necessary to induce PC6 cell differentiation.
Involvement of Rin in NGF-induced Neurite Formation—Although activation of Rin alone is not sufficient to induce neurite outgrowth, NGF-dependent signaling pathways are capable of both stimulating Rin activation and the process of neuritogenesis in PC6 cells. Therefore, we next examined the influence of Rin function on neurite outgrowth by altering the activity of endogenous Rin by expression of dominant-negative Rin (Rin(N28)). By analogy with other Ras-related GTPases, Rin(N28) is expected to be maintained in a conformational state in which it cannot bind to downstream targets, but to retain high affinity for its guanine nucleotide exchange factor. Cognate mutants have been used extensively because of their ability to sequester specific GEFs and thus to prevent the activation of their endogenous wild type counterparts (34). After transient transfection with vectors expressing GFP-tagged wild type or dominant-negative Rin proteins, PC6 cells were treated with NGF, and 72 h later the morphologies of the transfected cells were examined. Expression of wild type Rin did not alter NGF-induced neurite outgrowth (data not shown); the population of the cells with long neurites (exceeding 2 times the length of the cell body) was equal to that of control cells transfected with empty vector. On the other hand, expression of dominant-negative Rin potently inhibited NGF-stimulated neurite outgrowth (Fig. 11A and B). However, Rin(N28) had no significant effect on cell morphology or the viability of the cells compared with that of control cells (data not shown), indicating that overexpression of dominant-negative Rin did not have any significant toxic effect. In these experiments, Rin(N28) could not completely suppress NGF-induced neurite outgrowth, although it was as effective as dominant-negative H-Ras (H-Ras(N17)), a proven inhibitor of this process (14).

Involvement of Rin in Ras(V12)-induced Neurite Outgrowth—In PC12 cells, the activity of Ras is known to be required for NGF-induced neurite outgrowth and the expression of constitutively active Ras is alone sufficient to induce neurite outgrowth (13, 45). Because Rin is a downstream target of Ras-dependent signaling and dominant-negative Rin inhibits NGF-mediated neurite outgrowth in PC6 cells, we examined whether Rin was also involved in Ras-induced neurite outgrowth. Expression of the constitutively active Ras mutant, Ras(V12), potently induced neurite outgrowth in PC6 cells. When cells were co-transfected with expression vectors encoding HA-tagged Rin(V29), HA-Ras(L61), or empty vector as indicated. After 48 h, cells were serum-starved for 12 h, cell lysates were prepared, and HA-ERK collected by immunoprecipitation as described under “Experimental Procedures”; both bound fractions and whole cell lysates were evaluated by immunoblot analysis with the indicated antibodies. Data are representative of a typical experiment repeated three times.

**FIG. 9.** Constitutively active Rin does not induce neurite outgrowth. PC6 cells were transiently transfected with an expression vector encoding GFP or with GFP-tagged Rin, Rin(L78), or Ras(L61) and allowed to grow for 5 days. Cells were fixed and examined by epifluorescence microscopy to identify transfected, GFP-expressing cells (right panels). The morphology of the cells visualized by filamentous actin staining with Texas Red-phalloidin (left panels).

**FIG. 10.** Effect of activated Rin on ERK activation in PC6 cells. Immunoblots for phospho-ERK demonstrate no increase in ERK activity in PC6 cells transiently transfected with constitutively active Rin(V29) or Rin(L78). A, PC6 cells were transfected with expression vectors encoding HA-tagged Rin(V29), HA-Ras(L61), or empty vector as indicated. After 48 h, cells were serum-starved for 12 h, and cell lysates were prepared and evaluated by Western blotting using antibody specific for phospho-ERK (upper panel). Expression of constitutively active H-Ras(L61) was used as a positive control for activated ERK. The expression of activated Rin and Ras proteins were identified with anti-HA monoclonal antibody (bottom panel), and equal sample loading was confirmed using anti-ERK antibody (middle panel). B, PC6 cells were co-transfected with expression vectors encoding HA-tagged Ras(L61) and GFP-Rin(L78), GFP-Ras(L61), or empty vector as indicated. After 48 h, cells were serum-starved for 12 h, cell lysates prepared, and HA-ERK collected by immunoprecipitation as described under “Experimental Procedures”; both bound fractions and whole cell lysates were evaluated by immunoblot analysis with the indicated antibodies. Data are representative of a typical experiment repeated three times.
Inhibition of NGF-induced and RasL61-induced neurite outgrowth by RinN34. A, PC6 cells were transfected with expression vectors encoding GFP, GFP-tagged RinN34, or GFP-RasN17, allowed to recover for 48 h, and then treated with 100 ng/ml NGF for 72 h. Cells were fixed and stained as described above. The results shown are representative of three independent experiments. Cells expressing GFP-RinN34 are indicated (arrows). Similar results were seen with GFP-RasN17 (data not shown). B, right panel, quantification of the effect of Rin(N34) and RasN17) on NGF-induced neurite outgrowth. Transfected cells were treated as described above, fixed and stained with Texas Red-phalloidin, and analyzed by epifluorescence, and GFP-positive cells were assessed. Cells with neurites exceeding 1 cell body diameter in length were scored as a percentage of the total number of transfected cells. At least 200 cells were assessed in each experiment, and data are the means ± standard errors of triplicate experiments.

DISCUSSION

In this study, we examined the endogenous expression pattern and in vitro regulation of mammalian Rin. From these studies we have characterized Rin as a developmentally regulated gene expressed within the mature nervous system. To begin to elucidate the biological function of Rin, we investigated its in vitro regulation in neuronal cells. The major findings of these studies are as follows. First, we have demonstrated for the first time that Rin activation is a direct downstream effect of growth factor receptor activation. Second, we provide evidence that the activation of Rin requires cellular factors that are expressed in a variety of neuronal cell lines but not in several unrelated cell lines. Finally, we demonstrate that the expression of dominant-negative Rin inhibits both NGF-induced and oncogenic Ras-induced neurite outgrowth in PC6 cells, but that activated Rin fails to activate ERK or induce neuritogenesis. These studies provide new insight into Rin function and indicate that Rin is a novel regulator of growth factor-mediated signaling, likely regulating signal transduction events within the mature nervous system.

An important clue to the cellular function of Rin may be provided by its developmentally regulated and neural specific expression pattern. In contrast to the majority of Ras family members, Rin mRNA was detected exclusively in neuronal tissues and in a series of neuronal cell lines (Figs. 1A and 2B). Expression of Rin mRNA is also developmentally regulated. Rin mRNA is first detected in mice at embryonic day 14, and expression continues to increase rapidly within brain tissue during subsequent development until ~3 weeks after birth, at which point maximum Rin expression remains essentially stable (Fig. 1B). Developmental regulation was also found in primary superior cervical ganglia cell cultures, in which Rin expression was very low in immature neurons but up.

regulated robustly in NGF-aged superior cervical ganglia cultures (Fig. 2A). These studies suggest that Rin regulates signal transduction cascades specific for the physiology of mature neurons, but is not likely to be involved in regulating the proliferation and survival of neuronal cells during early embryonic development.

In experiments designed to further address the biological function of Rin, we investigated the in vitro regulation of Rin in PC6 cells. When PC6 cells are stimulated with NGF or EGF, Rin is rapidly activated (Fig. 4). These studies provide the first demonstration of ligand-induced Rin activation and indicate that Rin activation is a downstream effect of growth factor-induced receptor tyrosine kinase activation in PC6 cells. How activation of these receptor tyrosine kinases subsequently activate Rin is unclear, although several possibilities can be envisioned. Growth factor stimulation of both NGF and EGF receptors is known to result in the activation of Ras in PC12 cells (2). The ability of Rin to be regulated downstream of these receptor tyrosine kinases, in a manner that was temporally coordinated with the onset of Ras activation, suggested that Rin activation might be Ras-dependent. Evidence for this is provided by the observation that dominant-negative Ras completely inhibits Rin activation induced by NGF (Fig. 5), whereas the expression of constitutively activated Ras (RasL61) is alone sufficient to potently stimulate cellular GTP levels in PC6 cells (Fig. 5A). However, because a RinGEF has not been identified, it is possible that expression of dominant-negative Ras might block the activity of both RasGEF and RinGEF proteins. Thus, additional studies will be necessary to determine whether Ras-mediated Rin activation results from a direct regulatory cascade as seen for Ras-mediated control of Ral activation (19), or via a more complex signaling cascade.
To more thoroughly investigate the potential contribution of Ras to growth factor-induced Rin activation, we examined the ability of Ras effector mutants to stimulate Rin-GTP levels. Our observation that only a mutant of oncogenic Ras (Ras(V12S35)) capable of activating the Raf/ERK cascade is competent to activate Rin suggested that either ERK signaling, or a novel effector protein that shares binding determinates with Raf, is involved in coupling Ras activity to Rin stimulation (Fig. 6A). However, Ras(L61)-mediated Rin activation was not blocked by pharmacological inhibition of MEK activity, suggesting that MEK/ERK activity is not involved in the signaling pathway connecting Ras to Rin. Interestingly, the kinetics of Rin activation in PC6 cells reveals a good correlation with the activation of ERK but a less clear correlation with Ras. Thus, although NGF- and EGF-induced activation of Rin, Ras, and ERK are all quite rapid (Fig. 4; Refs. 35, 47, and 48; data not shown), Rin and ERK remain activated for an extended period, whereas the activation of Ras is more transient (Fig. 4; Refs. 35, 47, and 48). Because the duration of Ras activation is determined by its interaction with GTPases, these results suggest that Rin and Ras are likely to be regulated by distinct regulatory GAPs. Furthermore, it is likely that Rin activation may occur via additional signaling mechanisms that are not growth factor-dependent. This is based on the observation that the basal levels of GTP-bound Rin remained quite high, whereas those of Ras were barely detectable, following prolonged serum deprivation (Figs. 4–8). The ability of Rin to interact directly with calmodulin in a Ca\(^2\+\)-dependent manner (16, 18) suggest that intracellular calcium may play a role in the regulation of Rin activity. However, further studies will be needed to investigate the role of additional signaling pathways in the regulation of this small G protein.

Although members of the Ras superfamily display distinct cellular functions, they also participate in complex and interconnected signaling networks (1, 2). Ras subfamily-specific GEFs play a central role in the regulation of these complicated signaling cascades (43). Regulation of GEFs is quite complex, involving their specific subcellular localization, as well as direct activation by protein-protein or protein-ligand interactions (13, 45). For example, the RalGDS family of Raf GEFs rely on interaction with Ras-GTP (30, 49–52) or Rit-GTP (21) for membrane translocation and Raf activation, whereas the PDZ domains of some other Raf proteins might function as both membrane localization modules and to potentiate GEF activity through phosphoinositide binding (53). Such mechanisms allow a signal to diverge into multiple downstream pathways (2, 53, 54). The mechanism by which NGF and EGF signaling cascades activate Rin is unclear, although the studies presented here suggest that Rin activation may require distinct regulatory molecules. Thus, although the expression of constitutively active RasGEFs or EGF treatment potently stimulate H-Ras in HEK293 cells, we found that these same stimuli failed to elevate Rin-GTP levels in these cells (Figs. 7 and 8). These findings indicate that EGFR- and Ras-mediated Rin activation relies on an additional regulatory protein(s) that is not universally expressed. Indeed, although expression of H-Ras(L61) in a variety of neuronal cell lines resulted in Rin activation, Ras(L61) failed to stimulate Rin when expressed in either HEK293 or Vero cells (Fig. 7B). Moreover, these studies strongly suggest that Rin is not directly regulated by the RasGEFs, Sos1 or GRF1 (Fig. 8). In light of these observations, it is interesting to speculate that Rin activation relies on a neurally expressed regulatory factor(s), perhaps a specific RinGEF. However, we cannot exclude the possibility that the lack of Ras-mediated Rin activation results from the absence of an adapter protein or another regulatory protein in these cells. Whether there are specific GEFs and GAPs that regulate Rin function and if additional Ras-independent signaling pathways regulate Rin-GTP levels will be topics of future investigation.

In PC12 cells, constitutive Ras activity promotes cell cycle arrest and differentiation into a neuronal phenotype. Differentiation appears to require Ras-induced Raf activation, because constitutively activated versions of Raf can mimic, at least in part, the effects of activated Ras (46). Moreover, inhibition of the Ras-Raf-ERK pathway blocks stimulus-induced neurite outgrowth (14, 15, 55). This is consistent with a model that has emerged in which Ras-mediated neurite outgrowth and cell survival are dependent upon activation of both Raf and PI 3-kinase signal transduction (56–58). Our earlier studies have shown, at least in COS cells, that Rin does not activate either of these effector pathways, and indicate that Rin regulates cellular processes distinct from those controlled by Ras (19). This is supported by the observation that activated Rin mutants, when expressed in PC6 cells, fail to induce a program of neurite extension characteristic of neural development (9) and are unable to activate ERK (Fig. 10).

Although activated Rin alone was not sufficient to induce neurite outgrowth, we found that expression of dominant-negative Rin potently inhibited NGF-induced neurite outgrowth in PC6 cells (Fig. 11). Moreover, dominant-negative Rin also suppressed neurite outgrowth induced by activated Ras (Fig. 11B). Thus, Rin function may play a role in the process of neurite outgrowth in PC6 cells. The molecular mechanisms regulating the cytoskeletal changes necessary for neurite outgrowth are only beginning to be understood (59). NGF is known to induce neurite outgrowth through the activation of Ras, and a study in N1E-115 neuroblastoma cells indicates that Rac and Cdc42 act downstream of Ras during neurite outgrowth (60–62). Whether Rin modulates the activity of Rac1 and Cdc42, or the activity of another protein involved in neurite outgrowth, remains to be addressed. However, we cannot exclude the possibility that Rin(N34) may act to sequester a GEF that regulates the activity of additional Ras-related proteins, preventing the activation of these collateral GTPases in addition to endogenous Rin.

In conclusion, Rin expression in mature neurons, together with its regulation by a Ras-dependent signaling pathway in neuronal cell lines, indicates that Rin functions in Ras-dependent signaling pathways that are distinct from those involved in the control of cellular proliferation during embryonic development. In particular, Rin may play a role in the regulation of signal transduction cascades that are required for maintaining the mature nervous system. Because recent studies have implicated Ras signaling pathways in the modulation of synaptic transmission and neuronal plasticity required for learning and memory, an intriguing possibility exists that Rin may participate in the regulation of these crucial cellular signaling cascades (63–65). Further investigations are necessary to understand the role of Rin function in the nervous system.

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