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The large multidomain Kalirin and Trio proteins containing dual Rho GTPase guanine nucleotide exchange factor (GEF) domains have been implicated in the regulation of neuronal fiber extension and pathfinding during development. In mammals, Kalirin is expressed predominantly in the nervous system, whereas Trio, broadly expressed throughout the body, is expressed at a lower level in the nervous system. To evaluate the role of Kalirin in fiber initiation and outgrowth, we microinjected cultured sympathetic neurons with vectors encoding Kalirin or with Kalirin antisense oligonucleotides, and we assessed neuronal fiber growth in a serum-free, satellite cell-free environment. Kalirin antisense oligonucleotides blocked the continued extension of preexisting axons. Kalirin overexpression induced the prolific sprouting of new axonal fibers that grew at the normal rate; the activity of Kalirin was entirely dependent on the activity of the first GEF domain. KalGEF1-induced sprouting of new fibers from lamellipodial structures was accompanied by extensive actin cytoskeleton reorganization. The kalGEF1 phenotype was mimicked by constitutively active RhoG and was blocked by RhoG inhibitors. Constitutively active Rac1, RhoA, and Cdc42 were unable to initiate new axons, whereas dominant-negative Rac1, RhoA, and Cdc42 failed to block axon sprouting. Thus Kalirin, acting via RhoG in a novel manner, plays a central role in establishing the morphological phenotypic diversity that is essential to the connectivity of the developing nervous system.

Key words: Kalirin; Trio; guanine nucleotide exchange factor; Dbl-homology; Rho GTPase; neuronal fiber outgrowth

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of Kalirin-9 and joining the two fragments with a single Gly residue linker. All constructs were verified by sequencing. Transfection of Kalirin expression vectors induced lamellipodia formation and altered the phenotype of several fibroblast cell lines (Mains et al., 1999; Penzes et al., 2000). The kal9ΔGEF1 construct yielded a protein 35 kDa smaller than Kalirin-9. Fibroblasts expressing kal9ΔGEF1 had the same phenotypes as fibroblasts expressing kalGEF2. Plasmids encoding RhoA, Rac1, and Cdc42 and constitutively active Rac1-Q61L and RhoA-Q62L plasmids were kind gifts from Dr. Richard Cerione (Cornell University, Ithaca, NY). Cdc4-T17N and RhoA-T19N plasmids were generous gifts from Dr. Silvio Gutkind (National Institute of Dental Research, National Institutes of Health, Bethesda, MD). Cdc42-Q61L and Cdc42-T17N constructs were obtained from A. Ghosh (Johns Hopkins University, Baltimore, MD). Expression vectors encoding enhanced green fluorescent protein-RhoG (EGFP-RhoG (G12V)), EGFP-RhoG (F17A), and RhoGIP122 were greatly appreciated gifts from A. Blangy (Centre de Recherche en Biochimie Macromoléculaire, Centre National de la Recherche Scientifique, Montpellier, France). The EGFP plasmid pEGFP-N2 was from Clontech (Palo Alto, CA); expression plasmids for Rac2 and RhoG were from the Guthrie CDNA Resource Center (Sayre, PA). All Rho GTPase and Kalirin expression vectors were tested first for their ability to alter CHO, NIH 3T3, and pEAK Rapid cell morphology in transfected cells. Kalirin antisense oligonucleotide included TCA AAC CAT TCC GAA AAG ATC C; and TGG GAC CTC CAC GTT TAT CTC T. SCG neurons after 5–9 d of culture were viewed under an inverted phase-contrast fluorescence microscope with 20× objectives. Kalirin-1Δ and RhoA or Rho-GTPase constructs (50–200 ng/µl), pEGFP-N2 (200 ng/µl), and Texas Red-conjugated dextran (3000 MW, 2.5 mg/ml; Molecular Probes, Eugene, OR) were coinjected directly into the nuclei of SCG neurons at 95 µlHa for 400 msec via a Transfection 5264/InMan system (Eppendorf, Hamburg, Germany). After 24–72 hr the fiber structure of EGFP-coinjected neurons were viewed with GFP filters for fluorescence photomicroscopy with 20× or 40× objectives (Nikon Eclipse TE300, Nikon, Melville, NY; SPOT RT, Diagnostic Instruments, Sterling Heights, MI). GFP-positive cells were photographed 24, 48, and 72 hr after injection, and the cells were matched by an independent observer. Most cells photographed at earlier times were present at later times; the number of GFP-positive cells increased with time, presumably because of the gradual expression of GFP. Neuronal expression of Kalirin or Rho GTPases was verified by immunocytochemical staining after termination of the experiments. In neurons co-injected with Kalirin and EGFP plasmids, ~50–75% of EGFP-expressing neurons demonstrated altered phenotype, which reflected variability in cellular Kalirin expression levels as assessed by immunofluorescence staining for the myc-epitope tag. Expression from the smaller kalGEF1 expression vector was more consistent. The fourfold range of 200–900 ng kalGEF1 plasmid, EGFP-positive neurons co-injected with kalGEF1 demonstrated strong phenotypic changes. Plasmid concentrations (in moles) for microinjection were determined by using the BCA reagent (Pierce, Rockford, IL). Cdc42 and constitutively active Rac1-Q61L and RhoA-Q62L plasmids were generous gifts from Dr. Richard Cerione (Cornell University, Ithaca, NY). Cdc4-T17N and RhoA-T19N plasmids were obtained from A. Ghosh (Johns Hopkins University, Baltimore, MD). Expression vectors encoding enhanced green fluorescent protein-RhoG (EGFP-RhoG (G12V)), EGFP-RhoG (F17A), and RhoGIP122 were greatly appreciated gifts from A. Blangy (Centre de Recherche en Biochimie Macromoléculaire, Centre National de la Recherche Scientifique, Montpellier, France). The EGFP plasmid pEGFP-N2 was from Clontech (Palo Alto, CA); expression plasmids for Rac2 and RhoG were from the Guthrie CDNA Resource Center (Sayre, PA). All Rho GTPase and Kalirin expression vectors were tested first for their ability to alter CHO, NIH 3T3, and pEAK Rapid cell morphology in transfected cells. Kalirin antisense oligonucleotide included TCA AAC CAT TCC GAA AAG ATC C; and TGG GAC CTC CAC GTT TAT CTC T. SCG neurons after 5–9 d of culture were viewed under an inverted phase-contrast fluorescence microscope with 20× objectives. Kalirin-1Δ and RhoA or Rho-GTPase constructs (50–200 ng/µl), pEGFP-N2 (200 ng/µl), and Texas Red-conjugated dextran (3000 MW, 2.5 mg/ml; Molecular Probes, Eugene, OR) were coinjected directly into the nuclei of SCG neurons at 95 µlHa for 400 msec via a Transfection 5264/InMan system (Eppendorf, Hamburg, Germany). 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Expression from the smaller kalGEF1 expression vector was more consistent. The fourfold range of 200–900 ng kalGEF1 plasmid, EGFP-positive neurons co-injected with kalGEF1 demonstrated strong phenotypic changes. Plasmid concentrations (in moles) for microinjection were directly comparable in some experiments, but kalGEF1, Kalirin-9, and Kalirin-12 expression levels in individual neurons were not normalized; differences because of the relative contributions of intramolecular modulation of Kalirin-9 and Kalirin-12 GEF1 activity were not determined. Morphometric analyses were performed with Neuronlucida (MicroBrightField, Colchester, VT); statistical analyses were performed with SigmaStat (SPSS Science, Chicago, IL).

**Antibodies and Western blot analyses.** Antisera for Kalirin have been described (Johnson et al., 2000; Penzes et al., 2000). The Trio antibody (CT35) was raised by immunizing rabbits with a 14-amino-acid peptide corresponding to the COOH terminus of Trio, Tris(O25–3038), conjugated to keyhole limpet hemocyanin with glutaraldehyde (Milgram et al., 1997). The Trio antibody was affinity purified by linking the same peptide to Affi-Gel 10 and was used at a dilution of 1:200. Western blot analyses were performed as described (Johnson et al., 2000). Tissues were extracted into radioimmunoprecipitation assay (RIPA) buffer (Johnson et al., 2000), and protein concentrations were determined by using the BCA reagent (Pierce, Rockford, IL). Commercial antisera were used to visualize RhoA, Cdc42, Rac1 (Santa Cruz Biotechnology, Santa Cruz, CA), RhoG (Santa Cruz Biotechnology, Santa Cruz, CA), and tau (Roche, Indianapolis, IN).

**Metabolic labeling.** Cultures were labeled by using [35S]Met in Met defined serum-free medium (1 mCi/ml) and chased in Met-containing media.
medium as described (Alam et al., 1997). After a 30 min pulse or an additional 120 chase, the cells were extracted in RIPA buffer with protease inhibitors. Cell extracts were immunoprecipitated with excess Kalirin or Trio antibody; after isolation on protein A-Sepharose the samples were eluted and fractionated on 5% SDS-PAGE gels. Gels were dried with Amplify for fluorography.

*Rho GTPase binding and RhoG activation assays.* Binding assays were performed by using glutathione S-transferase-GTPase (GST-GTPase) fusion proteins and extracts from cells expressing exogenous kalGFP. Rac2 and RhoG cDNAs were subcloned into pGEX6P vector for expression as GST fusion proteins. GST fusion proteins (10 μg) immobilized on glutathione-Sepharose 4B resin (25 μl) were incubated with cell extracts for 2 hr. Extracts were prepared from HEK-293 pEAK Rapid cells transiently transfected with pEAK10.HisMyc-GEF1 by using TMT buffer (20 mM Na-Hepes (hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.4, containing 10 mM mannitol, 1% Triton X-100, and protease inhibitors) and diluted 22-fold into magnesium lysis buffer [containing (in mM) 25 HEPES, pH 7.5, 150 NaCl, 10 MgCl₂, 1 EDTA plus 1% NP-40 and 2% glycerol] for binding reactions. After being washed, the beads containing bound proteins were boiled in SDS-PAGE sample buffer and analyzed by Western blot analysis with a myc-antibody.

For Rho activation assays the RhoGIP122 cDNA was subcloned into pGEX6P vector for expression as GST fusion proteins. GST fusion proteins (10 μg) were immobilized on glutathione-Sepharose 4B beads. Cell extracts were prepared from HEK-293 pEAK Rapid cells transiently cotransfected with EGFP-RhoG and PEAK10.HisMyc-GEF1 or pEGFP (negative control) or with EGFP-RhōG (G12V) (positive control), using Rho-binding lysis buffer [containing (in mM) 50 Tris, pH 7.2, 500 NaCl, and 10 MgCl₂ plus 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS]. Extracts were applied to the GST-RhoGIP resin, and the bound, activated RhoG was analyzed by Western blot analysis with the use of a Rho-G antiserum.

*Actin visualization and immunocytochemistry.* Kalirin/EGFP-expressing SCG neurons were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100, and labeled with 1:1000 TRITC-phalloidin (Sigma, St. Louis, MO) to visualize filamentous actin. Immunocytochemical staining for Kalirin forms and myc-epitope was performed by using standard methods (Braas and May, 1999; Pénzes et al., 2000).

**RESULTS**

Kalirin induces extensive new neuronal fiber outgrowth

Sympathetic postganglionic neurons have been used widely as a model to study neuronal fiber outgrowth and guidance (Giger et al., 1998; Guo et al., 1998; Francis and Landis, 1999; Tsui-Pierchala and Ginty, 1999; Xu et al., 2000). The neurons are quite homogeneous morphologically, with large acentric nuclei and homogeneous systems that respond to known guidance cues and can be maintained readily in *vitro* under defined serum-free conditions without out confounding regulatory factors. Dissociated sympathetic neurons in *vitro*, which display a simple phenotype as visualized by injecting plasmid-encoding EGFP into individual cells, provide an ideal system by which to evaluate neuronal GEF function.

Typically, within the first few days of culture one to three primary fibers emanate from the large neuronal soma (30 μm diameter), extending rapidly (5–20 μm/hr) to lengths of several millimeters (Fig. 2A). The fibers develop complexity by branching at distal regions of elongating fibers. Approximately one branch point develops per 500 μm in fiber length; no additional fibers emerge from the cell soma even after extended times in culture.

Alternate splicing events generate multiple Kalirin isoforms (Fig. 1). Because Kalirin transcripts and proteins, corresponding to Kalirin-12 and Kalirin-9, can be identified in adult and neonatal SCG as well as in cultured sympathetic neurons (see below; McPherson et al., 2002), we evaluated their effects on the growth of neuronal fibers. Constructs encoding myc-epitope-tagged Kalirin-9 and Kalirin-12 were microinjected along with a plasmid encoding EGFP. Sympathetic neuron EGFP expression within 24 hr allowed for visualization of both new and old processes, and photomicrographs of the same cells at 24, 48, and 72 hr after injection allowed for a visual record of neuronal fiber development.

Expression of Kalirin-9 (Fig. 2B,C) or Kalirin-12 (Fig. 2D,E) produced a dramatic phenotype characterized strikingly by an initiation of multiple prominent new fiber outgrowths from the soma. This Kalirin-induced outgrowth of new fibers is uniquely different from the guidance functions ascribed previously to *Drosophila* Trio (dTrio) (Awasaki et al., 2000; Bateman et al., 2000; Newsome et al., 2000; Liebl et al., 2000). Compared with control cultures that were injected with the EGFP construct alone, the expression of exogenous Kalirin-9 or Kalirin-12 in-
increased the number of somal fiber outgrowths more than sixfold in 50 and 75% of the epitope-expressing neurons, respectively (Table 1). The appearance of new fibers 24 hr after plasmid microinjection was distinguished readily by the presence of brightly fluorescent growth cones at the distal tips of fibers emerging from the soma; these were distinguished easily from the very much longer preexisting primary fibers.

The Kalirin-induced fibers were highly dynamic. After initiation, the new fibers attained 100–500 μm in length within 24 hr, which indicated an average initial extension rate that exceeded 10 μm/hr. The rate of fiber growth was not affected by the number of newly formed fibers. Fiber branching, as assessed by either fiber segment or node analyses, increased ~10-fold compared with control (Table 1), and fiber directionality changed frequently. Consistent with this observation, time-lapse photomicroscopy revealed substantial growth cone motility. As anticipated, the neuronal phenotypic profiles, observed 72 hr after injection with Kalirin-12 and Kalirin-9 expression vectors, revealed significant diversity that may reflect differences in level of Kalirin expression as well as the functional interactions of the different Kalirin domains in different neurons. No changes in the preexisting principal fibers were noted in the microinjected cells. The duration of the experimental observations was limited only by the lifetime of expression of the EGFP.

### Kalirin-9 and Kalirin-12 are expressed in sympathetic SCG neurons

We used an antiserum to the spectrin-like region of Kalirin to identify the forms and levels of Kalirin in extracts of postnatal day 2 (P2) SCG or cultured SCG neurons (Fig. 3); extracts of adult rat cerebral cortex were analyzed for comparison. Western blot analysis identified Kalirin-12 (470 kDa) and Kalirin-9 (370 kDa) (Fig. 3A). Kalirin-9 is slightly more abundant than Kalirin-12, and Kalirin levels in the P2 ganglia are approximately one-half of the levels in adult cortex. Western blot analysis of the same extracts with an antisemir specific for the C terminus of Kalirin-12 confirmed the identity of the 470 kDa protein (Fig. 3A, right). Kalirin-7 (190 kDa), the most abundant Kalirin isoform in adult cortex and hippocampus, is not prevalent in the cultured SCG neurons.

To evaluate the contribution of Kalirin to SCG function, we developed an antiserum to the C terminus of Trio and used recombinant Kalirin and Trio to ensure that our Kalirin-12 and Trio antisera had similar sensitivities. Both Kalirin-12 and Trio could be detected in extracts of P1 SCG, with levels of Kalirin-12 slightly greater than levels of Trio (Fig. 3A); taking into account the existence of multiple Kalirin isoforms, it is clear that SCG Kalirin protein levels exceed those of Trio. We also used metabolic labeling followed by immunoprecipitation and fluorography to compare expression of the two proteins. Although synthesis of Kalirin-12 and Kalirin-9 was readily apparent, synthesis of Trio was not detectable in the same extracts (Fig. 3C). From these biochemical assessments Kalirin appeared to be the more physiologically relevant protein in sympathetic neurons.

### Kalirin GEF1 domain is responsible for the induction of fiber sprouting

To evaluate whether one particular catalytic region of Kalirin was able to drive the observed fiber initiation phenotype, we microinjected expression constructs for kalGEF1, kalGEF2, or Kalirin kinase (kalKinase) into the sympathetic neurons along with the EGFP expression vector (Fig. 4). The kalGEF1 domain alone consistently produced the dramatic neuronal fiber outgrowth of
Figure 4. KalGFEF1 domain expression is key to the Kalirin-induced fiber outgrowth phenotype. A, Microinjection of sympathetic neuron with 200 ng/μl kalGFEF1 expression vector induced robust fiber outgrowth and branching (compare with Fig. 2A). Growth cones at distal fiber terminals appeared as broad lamellipodial sheets (arrowheads). B, C, A neuron injected with kalGFEF1 and EGF (B) and processed immunocytochemically to visualize the myc-epitope of the kalGFEF1 construct (C) demonstrated kalGFEF1 expression in newly formed fibers and terminals. D, E, KalGFEF1-induced fiber initiation resulted from extensive actin cytoskeleton reorganization. Micrographs of two different kalGFEF1/EGFP-injected neurons (green) were merged with micrographs of the same neurons visualized with TRITC-phalloidin (red). The noninjected sympathetic neurons (D, asterisk) displayed relatively uniform staining for filamentous actin. Expression of kalGFEF1 caused a redistribution of filamentous actin to emerging fiber outgrowths (D; arrowheads mark red lamellipodial filigree emerging from green microinjected neuron) and to large aggregates in the perinuclear region of the cell soma (yellow represents actin aggregates from green and red fluorescence overlay). At later stages of fiber development the kalGFEF1-injected neurons typically displayed prominent staining for filamentous actin (red) at growth cones (E, arrowheads); aggregates of filamentous actin were still apparent in neuronal soma (asterisk).

Figure 5. Kalirin GEF2 and kinase domains do not induce fiber outgrowth. Microinjection of kalGFEF2, kalKinase, or kal9ΔGFEF1 did not induce changes in sympathetic neuronal phenotype. A, A neuron injected with kalGFEF2 and EGF was processed immunocytochemically to visualize the myc-epitope of the kalGFEF2 construct; the EGF image (green) was merged with the Cy3 image (red) for the epitope (yellow; from green and red Cy3 overlay). No changes in phenotype were noted in kalGFEF2-expressing neurons when compared with control despite high levels of myc-expression. B, Two kalKinase/EGFP-injected neurons (green) with high levels of myc-epitope expression (yellow) failed to demonstrate an outgrowth phenotype. C, Similarly, expression of a Kalrin-9 GEF1 deletion construct in sympathetic neurons did not elicit the fiber initiation phenotype despite high myc-expression levels (yellow). The patchy staining for kalKinase in the processes was a reproducible observation. Scale bars, 25 μm.

the Kalirin phenotype in >90% of the injected neurons (Fig. 4A). Neither kalGFEF2 nor kalKinase expression organized new somal fiber outgrowths despite the demonstrated high expression of each protein (Fig. 5A,B). Microinjection of SCG neurons with Kalirin constructs with a kalGFEF1 deletion (Fig. 5C; kal9ΔGFEF1) also failed to elicit the characteristic outgrowth phenotype, indicating that the neuronal fiber initiation response was dependent on the GEF1 domain.

The kalGFEF1 phenotypic response was robust but not identical to that for Kalirin-9 or Kalirin-12. Unlike the Kalirin-induced fiber outgrowths, which exhibited tapered growth cones at their terminals, the growth cones at the terminals of kalGFEF1-induced processes terminated in broad lamellipodial sheets (Fig. 4A). These large lamellipodial structures at the advancing fiber terminals resisted condensation during fiber elongation and retained their broad lamellipodial features until the fibers migrated significant distances from the soma. Expression of the kalGFEF1 domain induced a ninefold increase in somal fiber outgrowth compared with control (Table 1). Staining for the myc-epitope revealed kalGFEF1 expression not only in the soma but also in the newly formed fibers and growth cones, suggesting that kalGFEF1 participates in local cytoskeletal restructuring for the outgrowth process (Fig. 4B,C). Consistent with these ideas, the kalGFEF1-induced changes were accompanied by striking cellular actin cytoskeleton reorganization; the bulk of the cellular and cortical actin adjacent to the plasma membrane was redistributed to the newly formed lamellipodial fiber structures (Fig. 4D,E). This redistribution was so extensive that the filamentous actin staining in the soma proper was almost eliminated. These studies demonstrate that kalGFEF1 activity is coupled to actin reassembly processes that are necessary for neuronal fiber initiation and outgrowth.

By collecting sequential photomicrographs to evaluate fiber growth, we deduced that the kalGFEF1-induced fibers presented a structured delayed mode of branching, characterized by the protrusion of collateral fibers and growth cones at specific regions of cytoskeleton instability along the advancing fiber. The sites of branching were identified first by the appearance of lamellipodial and filopodial activity. This is one preferential mode described for axon branching and is distinct from growth cone splitting mechanisms more characteristic of dendrites (Acebes and Ferrus, 2000). The initiation of new fiber formation from the soma was
often continuous; as newly formed fibers extended in length, new lamellipodial structures appeared around the soma with the subsequent appearance of neurites (Fig. 6A–C).

**The new fibers induced by kalGEF1 are axons**

We wanted to determine whether the newly initiated fibers exhibited axonal or dendritic characteristics. The kalGEF1-induced processes were uniform in diameter and exhibited both tau and filament protein immunoreactivity, suggesting that the fibers were axonal (data not shown). However, as a more rigorous demonstration of their functionality and axonal properties, the fibers and terminal growth cones of kalGEF1-injected neurons were examined for their abilities to transport and store neuropeptides. For this purpose the kalGEF1 expression vector was coinjected into sympathetic neurons with a neuropeptide Y-EGFP fusion protein that is stored in regulated secretory vesicles (El Meskini et al., 2001). Under these experimental conditions the soma and fibers of the kalGEF1-injected neurons were visualized solely by localizing NPY-EGFP. Like before, the microinjection of vectors encoding constitutively active forms of each Rho GTPase to mimic the kalGEF1-induced neuronal outgrowth, and then we examined the ability of the corresponding dominant-negative variant to block or attenuate the kalGEF1-phenotypic response.

![Figure 6](image)

**Figure 6.** KalGEF1-induced fiber extensions are axons. A–C. Two sympathetic neurons microinjected with kalGEF1 and EGFP were photographed 24, 48, and 72 hr after injection. Fibers initiated after kalGEF1 expression extended rapidly over time. Note the continued emergence of some new fibers even 48 and 72 hr after injection. D, KalGEF1-induced fibers contained neuropeptide vesicles. Neurons were coinjected with kalGEF1 and NPY-EGFP constructs. Secretory granules containing the NPY-EGFP fusion protein were visualized on the basis of the localization of EGFP; proteins were routed from cellular sites of biosynthesis to growth cones of newly formed fibers (arrowheads; punctate green fluorescent endings). E, Sholl analysis of Kalirin-initiated fibers and kalGEF1-initiated fibers 2–3 d after microinjection. Control EGFP-injected neurons (CTL; n = 24) demonstrated a slight increase in fiber crossings at distant intervals from the branching of principal fibers. Fiber outgrowths from kalGEF1-injected neurons (n = 14) produced a ninefold increase in fiber crossings (50 μm distance) that diminished to control levels as the Sholl radii exceeded the length of the newly formed fibers. Post hoc Student–Newman–Keuls analyses revealed significant differences from CTL at 25–175 μm distances (p < 0.001). Kalirin-injected neurons produced a sixfold increase in intersections (n = 9). Fiber lengths were longer than kalGEF1-injected neurons but variable; differences in the number of fiber crossings compared with control were significant for all radial points (p < 0.001). Data are mean ± SEM, from counting fiber intersections for many neurons. Scale bars, 50 μm.

The formation of lamellipodia, filopodia, and stress fibers regulated by the Rho small GT Pase family members Rac1, Cdc42, and RhoA in fibroblasts bears significant similarities to advancing growth cone structures. The same Rho GTPases clearly participate in neuronal morphogenesis (Hall, 1998; Luo, 2000); Rac1, RhoA, Cdc42, and RhoG are known to affect neuronal process development, and all four Rho GTPases are expressed in the SCG (see below). To determine whether any of these Rho GT Pase mediated the dramatic fiber initiation phenotype that was observed, we first tested the ability of constitutively active forms of each Rho GT Pase to mimic the kalGEF1-induced neuronal outgrowth, and then we examined the ability of the corresponding dominant-negative variant to block or attenuate the kalGEF1-phenotypic response.

Unexpectedly, the microinjection of vectors encoding constitu-
tively active variants of Rac1, RhoA, or Cdc42 GTPases had no apparent modulatory effects on fiber initiation or on the formation of lamellipodia and/or filopodia from the soma of sympathetic neurons (Fig. 7). The soma of a very small fraction of the constitutively active Rac1-injected neurons (~10%) displayed a modest spreading phenotype reminiscent of Rac1-induced lamellipodia expansion in fibroblast lines; the few principal extensions were never observed despite high levels of myc-expression. Scale bar, 50 μm.

Figure 7. Kalirin-induced neuronal fiber outgrowth is mediated by RhoG. A–D, Constitutively active Rho GTPases (Rac1-Q61L, RhoA-Q62L, Cdc42-Q61L, and Rho G12V; all at 200 ng/μl) were microinjected, and the neurons were examined 48 hr later. Only the microinjection of constitutively active variants of Rac1, RhoA, or Cdc42 GTPases had no apparent modulatory effects on fiber initiation or on the formation of lamellipodia and/or filopodia from the soma of sympathetic neurons (Fig. 7). The soma of a very small fraction of the constitutively active Rac1-injected neurons (~10%) displayed a modest spreading phenotype reminiscent of Rac1-induced lamellipodia expansion in fibroblast lines; the few principal extensions were never observed despite high levels of myc-expression. Scale bar, 50 μm.

RhoG is present in SCG and binds to KalGEF1

These results implicate RhoG or a close relative in Kalirin-induced axonal initiation and growth. The ability for kalGEF1 to activate RhoG was evaluated directly in Rho GTPase binding and activity assays. Rho GTPase binding to kalGEF1 was sensitive to magnesium concentrations, and, under optimal conditions, kalGEF1 demonstrated significant binding to Rac1 and RhoG (Fig. 8A); binding to Cdc42, RhoA, and Ras was extremely weak, as reported for TriolGEF1 (Debant et al., 1996). When the data were normalized to Rho GTPase protein levels, kalGEF1 binding to RhoG was threefold greater than to Rac1 (Fig. 8A, bottom panel).

We used the fact that RhoGIP122 interacts specifically with activated RhoG to assess the ability of kalGEF1 to activate RhoG (Fig. 8B). Fibroblasts (HEK-293), which do not contain high levels of RhoG, were transiently transfected with RhoG in the absence or presence of kalGEF1. RhoGIP122 resin was incubated with extract, and cellular-activated RhoG bound to RhoGIP122 was visualized by Western blot analysis via the antibody to RhoG. As shown, kalGEF1 is a potent activator of RhoG (Fig. 8B).

Because the mixture of Rho proteins expressed in a given cell type is a critical determinant of its ability to respond to a particular GEF, we evaluated the expression of Rac1, RhoA, Cdc42, and RhoG in SCG, adult cortex, and liver. Sympathetic neurons express all four of these Rho GTPases (Fig. 8C). Although Rac1, RhoA, and Cdc42 are expressed at similar levels in adult cortex and SCG, it is notable that RhoG is not present at high levels in adult cortex. The tissue-specific expression of RhoG and other
Rho family members may account for some of the differences observed in Kalirin-induced responses in central versus peripheral neurons. More recent experiments also have demonstrated that Kalirin-9 and Kalirin-12 bind to RhoG (data not shown). Together, the expression of Kalirin and Rho GTPases, especially RhoG, in peripheral sympathetic neurons and the ability of Kalirin to stimulate RhoG activity provide a physiological mechanism for Kalirin-mediated neuronal fiber outgrowth.

Kalirin antisense oligonucleotides block normal neuronal fiber development

The data establish the ability of exogenous Kalirin to initiate axonal outgrowth mediated by RhoG. To determine whether endogenous Kalirin plays a role in fiber outgrowth, we microinjected a series of antisense oligonucleotides into primary sympathetic neurons shortly after initial plating. In several independent experiments the antisense oligonucleotides caused significant alterations in normal fiber outgrowth. Sympathetic neuronal fibers typically demonstrate rapid outgrowth rates; however, in neurons that were microinjected with the Kalirin antisense oligonucleotides, the growth cones became static, resulting in a failure of the axonal processes to extend over time (Fig. 9A,B). The example shown is one of 21 cases of axon growth arrest or slight axonal retraction observed after the Kalirin antisense oligonucleotides had been injected; 63 cells in total were followed after antisense oligonucleotide injection. By contrast, 12 cells were followed for 3 d after the injection of scrambled control oligonucleotides, and none showed any axon growth arrest, exhibiting instead continuous growth like the control GFP-injected neurons shown in Figures 2A and 7A–C. As another control, Kalirin antisense oligonucleotides were injected along with the constitutively active RhoG and did not abrogate the GTPase-initiated fiber outgrowth (Fig. 9C).

A small number of sympathetic neurons under the culture conditions that were used displayed dendrites as identified by microtubule-associated protein (MAP2) staining (Fig. 10, inset). Introduction of Kalirin antisense oligonucleotides into these neurons not only arrested dendritic development but caused a partial retraction of existing dendrites (Fig. 10A–D). The examples shown are two of nine examples of dendritic retraction seen after injection of the Kalirin antisense oligonucleotide. The mean ratio of dendritic length for 48 to 24 hr or 72 to 48 hr was 0.21 ± 0.08 (SD), documenting a dramatic collapse of dendrites after Kalirin antisense oligonucleotide injection. The 12 cells injected with the scrambled oligonucleotides showed no dendritic collapse and continued growth as in Figures 2 and 7.

Because neuronal growth cone motility and fiber extension require cytoskeletal actin reorganization, these results suggest that sustained Kalirin expression may be essential to initiate and maintain neuronal fiber development.

DISCUSSION

The development and geometry of neuronal fibers are main determinants of the functional connectivity and complexity of the nervous system. Axonal and dendritic outgrowth, elongation, and guidance require receptor translation of diverse extracellular cues to affect both cytoskeleton reorganization and membrane biogenesis. GEF-facilitated GDP/GTP exchange on Rho GTPases is a critical component of the complex interplay of regulatory mechanisms that guide these processes. The human genome encodes at least 46 GEFs for 18 Rho GTPases, and the specific Rho GTPases, GEFs, and regulatory mechanisms that drive neuronal outgrowth responses in a physiological context are not well understood. For example, ephexins are Rho GEFs that bind EphA4 receptors; interaction of EphA4 receptors with their cell surface ligands then modulates the ability of ephexin to act as an exchange factor (Shamah et al., 2001). GEFs that are components of large multifunctional proteins may accommodate more complex regulation and coordinate the expression of more elaborate phenotypic responses to specific demands at localized cellular sites.

Kalirin and Trio are large multidomain proteins that appear to fulfill a number of important criteria for these functions. They not only contain two distinct GEF domains that activate key Rho GTPases but also possess multiple protein/protein and protein/lipid binding motifs that could integrate the necessary signaling elements for local actin assembly at precise cellular sites. It is not yet clear whether the various isoforms of Kalirin and Trio have complementary or parallel functions during nervous system development. Unlike Trio, which is expressed in both nervous and peripheral tissues, Kalirin is expressed preferentially in the nervous system. Mice unable to express Trio display only modest alterations in nervous system organization (Ó’Brien et al., 2000). In contrast, Drosophila and C. elegans lacking their only Trio/
Kalirin family member (dTrio and UNC-73A, respectively) have a severely affected nervous system. Our antisense studies indicate that endogenous Kalirin is important in the formation of both axons and dendrites. Disruption of Kalirin expression at an early stage of fiber outgrowth compromised the development of both axons and dendrites.

Our current studies emphasize a new and different neuronal fiber outgrowth activity associated with Kalirin function. Post-ganglionic sympathetic neurons possess the necessary cellular elements to generate simple, long projection fibers to appropriate targets and have been well studied both in vivo and in vitro with respect to receptor-mediated mechanisms in fiber production. SCG neurons express both Kalirin and Trio, with levels of Kalirin protein in excess of levels of Trio protein. Based on these properties, these neurons afford a tenable means for evaluating Kalirin function in a physiologically relevant context. Distinct from the neuronal guidance functions described previously for dTrio, Kalirin orchestrates the genesis of new somal and axonal elements to generate simple, long projection fibers to appropriate targets.

Expression of kalGEF1 resulted in the production of large lamellipodial formations and multiple fiber sprouts. Cellular filamentous actin labeled with phalloidin was translocated to aggregates in the cell body and to the leading edges of emerging lamellipodia and growth cone structures. Even after fiber elongation, the most prominent areas of filamentous actin staining were identified at distal growth cones. Although the number of newly initiated fibers and branch nodes in the kalGEF1-injected neurons was 50% greater than in neurons overexpressing Kalirin-12 or Kalirin-9, the overall length of the fibers 72 hr after injection appeared shorter than the Kalirin-9-overexpressing neurons and Kalirin-12-overexpressing neurons (Fig. 6E).

Fiber length may be driven by microtubule formation and the ability of microtubules to protrude into the actin meshwork at the growth cones (Bradke and Dotti, 1999). Microtubule entry, fiber elongation, and axon formation are facilitated by growth cones with highly dynamic actin processes that produce loose meshwork structures; in contrast, growth cones with stable polymerized actin may be destined to bear dendritic characteristics. Although KalGEF2 alone had no apparent phenotype, when present in the context of Kalirin-12 or Kalirin-9, it might coordinate the regulation of actin stability and facilitate fiber elongation. Consistent with this suggestion, when expressed in neonatal cortical neurons, kalGEF2 alone promoted the extension of neurites (Penzes et al., 2001b). TrioGEF2 demonstrates selectivity for RhoA (Debant et al., 1996), and kalGEF2 or post-translationally modified forms of kalGEF2 also may exhibit differential Rho GTPase specificity.

Despite differences in overall fiber length and branching, both the kalGEF1-induced fibers and Kalirin-induced fibers were axon-like in character. The fibers demonstrated immunoreactivity for axonal markers, and time-lapse microscopy revealed an axonal mode of branching. As a more direct means of elucidating the functional character of these fibers, we expressed a chimeric...
uncleavable NPY-EGFP fusion protein in neurons expressing kalGEF1 (El Meskini et al., 2001). Sympathetic neurons express high levels of neuropeptide Y and, like the endogenous neuropeptide, NPY-EGFP is routed to dense core vesicles via the regulated secretory pathway (El Meskini et al., 2001). When the kalGEF1 and NPY-EGFP constructs were coinjected, the soma

When the uncleavable NPY-EGFP fusion protein in neurons expressing May et al., 2002a,b). When the neuron expressed RhoA, Cdc42, or Rac1, NPY-EGFP was transported and stored at the new fiber terminals for potential release. These are key properties of functional fibers and suggest that the majority of the Kalirin-initiated fibers and kalGEF1-initiated fibers is axonal in character.

We demonstrated previously that kalGEF1 is an exchange factor for Rac1 (Penzes et al., 2001a). However, the initiation of neuronal outgrowth by Kalirin or kalGEF1 is not mediated directly by Rac1, RhoA, or Cdc42; instead, RhoG plays an essential role. Among the constitutively active Rho GTPases that were examined, only the activated RhoG mimicked the kalGEF1 phenotype. Conversely, dominant-negative forms of Rac1, RhoA, or Cdc42 failed to abrogate kalGEF1-induced neuronal outgrowth. In contrast, both a RhoG competitive inhibitor and a protein that binds activated RhoG were able to block the kal-GEF1 and Kalirin-12 phenotypes. RhoG also was shown to be a neurite outgrowth regulator in pheochromocytoma cells (Katoh et al., 2000). Expression of RhoG in sympathetic neurons, but not in cortical neurons, may contribute to their distinctly different responses to kalGEF1.

KalGEF1 binds to and activates multiple Rho GTPases, as described previously for Vav and TrioGEF1 (Scheubel et al., 1998; Bellanger et al., 2000; Blangy et al., 2000). Although kal-GEF1 binds to several Rho GTVPases in in vitro assays, the ability of the RhoG competitive inhibitor and binding protein to abrogate completely any kalGEF1-mediated phenotype in sympathetic neurons supports recent suggestions that RhoG functions upstream of other Rho GTPases. Studies in fibroblasts and PC12 pheochromocytoma cells indicate that Cdc42, Rac1, and RhoA can form an interactive cascade (Nobes and Hall, 1995). Similarly, activated RhoG can result in the independent activation of Rac1 and Cdc42 (Gauthier-Rouvière et al., 1998; Blangy et al., 2000). In the physiological setting that was examined, our studies suggest that kalGEF1 does not activate multiple Rho GTPases directly; the Kalirin neuronal outgrowth phenotype appears to be initiated instead by RhoG-mediated signal transduction that coordinates multiple downstream pathways.

The distinct and differential roles of Kalirin and Trio in the nervous system are still unclear. There is a progression in Kalirin expression during neuronal development such that Kalirin becomes predominant compared with Trio at neuronal system maturation (McPherson et al., 2002), yet the functional implications of that transition with respect to dendritic structure or axonal outgrowth have not been determined. Whereas the GEF1/GEF2 domains between the two proteins may display preferences for specific Rho GTPases, recent studies demonstrating TrioGEF1 binding to RhoG suggest that the GEF domains may be functionally similar. The associated domains in Kalirin and Trio may harbor differential intracellular-targeting information for each protein to organize specific fiber patterns; alternatively, Kalirin and Trio may be regulated differentially under various physiological contexts. More detailed studies may elucidate the functional distinctions.

With migration to the appropriate destination, developing neurons extend dendritic and axonal processes to adopt a character-