

Critical Role for Kalirin in Nerve Growth Factor Signaling through TrkA

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Kalirin is a multidomain guanine nucleotide exchange factor (GEF) that activates Rho proteins, inducing cytoskeletal rearrangement in neurons. Although much is known about the effects of Kalirin on Rho GTPases and neuronal morphology, little is known about the association of Kalirin with the receptor/signaling systems that affect neuronal morphology. Our experiments demonstrate that Kalirin binds to and colocalizes with the TrkA neurotrophin receptor in neurons. In PC12 cells, inhibition of Kalirin expression using antisense RNA decreased nerve growth factor (NGF)-induced TrkA autophosphorylation and process extension. Kalirin overexpression potentiated neurotrophin-stimulated TrkA autophosphorylation and neurite outgrowth in PC12 cells at a low concentration of NGF. Furthermore, elevated Kalirin expression resulted in catalytic activation of TrkA, as demonstrated by *in vitro* kinase assays and increased NGF-stimulated cellular activation of Rac, Mek, and CREB. Domain mapping demonstrated that the N-terminal Kalirin pleckstrin homology domain mediates the interaction with TrkA. The effects of Kalirin on TrkA provide a molecular basis for the requirement of Kalirin in process extension from PC12 cells and for previously observed effects on axonal extension and dendritic maintenance. The interaction of TrkA with the pleckstrin homology domain of Kalirin may be one example of a general mechanism whereby receptor/Rho GEF pairings play an important role in receptor tyrosine kinase activation and signal transduction.

Small G proteins act as molecular switches controlling a wide array of cellular processes in organisms ranging from bacteria to humans. The Rho subgroup of G proteins is most notably involved in regulation of the actin cytoskeleton (7). Rho proteins are activated by guanine nucleotide exchange factors (GEFs), which induce the release of bound GDP and allow the binding of GTP; the Rho-GTP complexes can then bind to and activate a series of effector proteins. The activity of Rho proteins is regulated by GTPase-activating proteins, which induce the hydrolysis of bound GTP, and by guanine nucleotide dissociation inhibitors that sequester G proteins bound to GDP or GTP.

In addition to their role in cytoskeletal regulation, recent evidence has established an association of Rho proteins, GTPase-activating proteins, and GEFs with several cell surface receptors and their signaling pathways. For example, Vav links the FcεRI receptor to stress-activated protein kinase (c-Jun N-terminal kinase) activation (51). However, no RhoGEF is known to interact with the TrkA neurotrophin receptor.

Several pieces of evidence suggest that the RhoGEF Kalirin may play a role in TrkA receptor function. Antisense Kalirin oligonucleotides block initiation and outgrowth of axons from

sympathetic neuron primary cultures grown in the presence of nerve growth factor (NGF) (32). A yeast two-hybrid screen identified Kalirin as a GIPC (GIAP-interacting protein C terminus) interactor, and GIPC associates with TrkA (26, 39). Kalirin binds to Crk, an adaptor protein that links TrkA to Rap1 activation and subsequent activation of the mitogen-activated protein kinase (MAPK) pathway (57; M. R. Schiller, K. Chakrabarti, G. F. King, N. I. Schiller, B. A. Eipper, and M. Maciejewski, submitted for publication). TrkA stimulation induces activation of Rac, and Kalirin activates Rac (37, 40). Finally, Kalirin and TrkA are both localized in presynaptic and postsynaptic terminals (5, 10, 28). These experiments directed us to investigate the relationship of Kalirin with TrkA.

MATERIALS AND METHODS

Cell culture, primary olfactory bulb cultures, and transfection. PC12 and -293 cells were grown in Dulbecco's modified Eagle medium (DMEM)-F12 medium containing 120 μg/ml penicillin, 200 μg/ml streptomycin sulfate, 600 μg/ml glutamine, 25 mM HEPES, and 10% fetal bovine serum. Primary olfactory bulb cultures were prepared from dissected P1 rat olfactory bulbs. Olfactory bulbs were washed three times with DMEM-F12 containing fatty-acid-free bovine serum albumin (BSA) (1 mg/ml) and transferrin (100 μg/ml), diced, and incubated with the same DMEM-F12 medium containing 3 mg/ml trypsin and 4 mg/ml DNase for 20 min at 37°C to dissociate cells. After titration and filtration through a cell strainer (70-μm mesh), strained cells were plated on poly-L-lysine-coated dishes and grown for 5 to 9 days prior to transfection and immunostaining. All experiments reported in this paper were repeated at least three times with similar results.

Transient transfection of PC12 and 293 cells for reporter assays, Western blot

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analysis, immunostaining, and neurite outgrowth experiments was performed using Lipofectamine 2000 and Optimem as described by the manufacturer (Invitrogen). The Lipofectamine 2000/DNA ratio for all experiments was 2.5. Cells were transfected for 4 h, grown in DMEM-F12–10% fetal calf serum for 24 h, and serum starved in DMEM-F12–1% BSA supplemented with 120 μ g/ml penicillin, 200 μ g/ml streptomycin sulfate, and 25 mM HEPES for 16 h before induction with 2 or 50 ng/ml NGF (Becton Dickinson) where indicated. Olfactory bulb neurons were transfected with Fugene 6 according to the manufacturer's protocol (Roche Diagnostics). Western blot analysis was performed by a standard approach using 4 to 15% gradient gels (Bio-Rad or Invitrogen) and 1% blocking solution (Roche Diagnostics).

Coimmunoprecipitation from mouse brain and transfected cells. Embryonic mouse brains (embryonic day 17 [E17]) were isolated as previously described (11) and homogenized (10%, wt/vol) in COIP buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 10% glycerol, and 0.5% Triton X-100) with protease and phosphatase inhibitors (18). After centrifugation, lysates (200 μ g) were incubated with 2 μ g of TrkA (Santa Cruz Biotech) or a control antibody and COIP buffer (1.0 ml) for 2 h with agitation at 4°C. Samples were centrifuged at 15,000 \times g for 5 min to remove debris, and immune complexes were absorbed to protein G-Sepharose beads (40 μ l) for 1 h at 4°C with shaking. Beads were washed three times with COIP buffer, and bound proteins were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The presence of Kalirin and TrkA in the immunoprecipitated samples was revealed by Western blot analysis with a pan-Kalirin antiserum (37) and an antibody to a C-terminal peptide of TrkA (Sigma; T9566).

For coimmunoprecipitation from transfected cells, lysates from 293 cells cotransfected with His/Myc-tagged Kalirin 5 (Kal5) and TrkA (CMX-TrkA) expression constructs were prepared in COIP buffer with protease and phosphatase inhibitors. Kal5 present in lysates was immunoprecipitated with the anti-myc antibody 9E10; for a negative control, an isotype control immunoglobulin G1 (IgG1) antibody was used (23). Samples were analyzed for immunoprecipitation of Kal5 and coimmunoprecipitation of P-TrkA using a TrkA antibody.

Immunofluorescence microscopy and neurite outgrowth assays. Transfected PC12 cells and neurons were immunostained using standard approaches as previously described (44). Where indicated, cells were treated with NGF. Immunostained cells and those transfected with green fluorescent protein (GFP) expression constructs were visualized by fluorescence microscopy using an inverted Nikon fluorescence microscope. Images were obtained using a Hamamatsu digital camera and Improvision Openlabs 3.1.4 software. Where indicated, images were collected using an Improvision Orbit Z-stepmotor and deconvolved using Improvision Velocity software.

For quantitation of neurite outgrowth, images of PC12 cells transfected with pCMS.EGFP (Clontech), pCMS.EGFP/Kal-as, or pCMS.Kal5 were treated with 2 or 50 ng/ml NGF for 2 days where indicated, images were collected, and transfected cells with neurites longer or shorter than one cell body were counted (28). For quantitation of P-TrkA immunostaining in neurons, neurons expressing GFP and strongly immunostained with P-TrkA were counted for samples transfected with pCMS.EGFP, pCMS.EGFP/Kal-as, and pCMS.EGFP/Control (PAM)-as. For statistical analysis of neurite outgrowth and P-TrkA immunostaining in neurons, samples from cells transfected with different constructs or treated with NGF were blinded to the observer and rank ordered, and significant differences were determined using a nonparametric Kruskal-Wallis test (<http://faculty.vassar.edu/lowry/VassarStats.html>). At least 50 cells were counted for each category.

TrkA kinase assays. Kinase reactions were performed in 25 mM HEPES, pH 7.4, 10 mM MgCl₂, 2.5 mM MnCl₂, 50 μ M sodium orthovanadate, 0.5 mM dithiothreitol, 0.2% Triton X-100, 40 μ g/ml BSA, and 25 μ M ATP with 62.5 μ Ci/ml [γ -³²P]ATP (Amersham) in a total volume of 40 μ l at 25°C. TrkA autokinase reactions were performed in the presence of ATP alone, whereas 1 mM synthetic peptide corresponding to residues 309 to 326 of Shc (KKRELDPDSYVNVQNLDDK) was used as an exogenous substrate. Autokinase reactions were terminated after 10 min by boiling, products were separated in an 8% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane, and the bound radioactivity was detected by autoradiography. Peptide reactions were terminated after 10 min with 8.5% H₃PO₄, and incorporation of the radiolabel into the peptide was quantified as previously described (13). Relative amounts of TrkA in immunoprecipitates were quantified by Western blotting using a Kodak Image Station 440. Significant differences were determined using a one-tailed *t* test.

Signal transduction reporter assays. Signal transduction reporter assays were performed using the trans-reporter system (Stratagene). For these assays, 293 cells (10⁵ cells in 20-mm wells) were cotransfected with two plasmids for the reporter system (100 ng pFR.Luc and 10 ng pFA2.CREB), a TrkA expression

construct (25 ng CMX-TrkA), differing amounts of a Kal5 expression construct (0 to 400 ng pEAK10.HisMyc.Kal5), and a construct used for normalizing transfection efficiency (pCDNA. β Gal) and supplemented with an empty pEAK10 vector (Edge Biosystems) such that all samples had the same amount of DNA. Luciferase activity was measured from cell lysates by using the substrate and assay conditions described by the manufacturer (Promega). Transfection efficiency was normalized to cotransfected β -galactosidase levels using the Galacton luminescent β -galactosidase substrate (Tropix, Bedford, MA). Expression of transfected TrkA and Kalirin was confirmed by Western blot analysis. Luciferase assays were performed in triplicate, and standard deviations are reported for triplicate observations and multiple experiments. Significant differences were determined using one-way analysis of variance (ANOVA).

Several control experiments were performed to optimize conditions for the luciferase reporter assay (data not shown). Reporter plasmids were titrated to identify conditions for low basal stimulation and high induction of the reporter with the catalytic subunit of protein kinase A (pFC.PKA). The TrkA expression construct was titrated from 10 ng to 1 μ g to determine levels that produced the lowest basal versus highest NGF-stimulated reporter activation (25 ng). NGF levels were titrated to identify those that produced maximal reporter activation (25 ng/ml). A time course of NGF stimulation showed near-maximal CREB activation after 6 h of NGF treatment, which was used in subsequent experiments.

GST pulldown experiments. For mapping TrkA binding sites in Kalirin, glutathione *S*-transferase (GST) fusion proteins for fragments of Kal5 were prepared. Different Kal5 domains were spectrin 5-9 (residues M⁶²⁴ to R¹²⁷³), GST-DBL homology (DH) (residues K¹²⁷⁴ to V¹⁴⁵²), GST-pleckstrin homology (PH) (residues S¹⁴⁵³ to R¹⁵⁷⁵), also called GST-PH1, and GST DH/PH (residues K¹²⁷⁴ to R¹⁵⁷⁵). Numbering is based on the rat Kal12a sequence (AF232669). A construct for expression of a GST fusion protein with the Kal7 C terminus was described previously (39). For PH domain specificity experiments, GST-KalPH2 (residues L²⁰⁶⁵ to R²²¹⁸), GST-TrioPH1 (residues L¹³⁹⁹ to K¹⁵⁵⁰), and GST-TrioPH2 (residues C²⁰⁷⁷ to G²²³³) were prepared; numbering for Trio is based on the human Trio sequence (U42390). cDNA fragments were subcloned into pGEX6P in frame with GST.

GST fusion proteins were expressed in *Escherichia coli* BL21 or Rosetta (Novagen) and purified using glutathione Sepharose as previously described (32). Lysates containing TrkA were prepared from 293 cells transfected with the CMX-TrkA plasmid. Cells were extracted in TMT buffer [20 mM sodium-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 7.4; 10 mM mannitol, and 1% Triton X-100] containing protease inhibitors (18, 34, 55). Lysates (100 μ l) were incubated with GST or GST fusion proteins (5 μ g) in a total volume of 1.0 ml for 1 h at 4°C with agitation and then applied to glutathione Sepharose beads (40 μ l). After three washes with TMT, protein complexes were boiled in SDS-PAGE sample buffer and analyzed by Western blotting using TrkA (Sigma or Chemicon, Temecula, CA) and phospho-TrkA (Tyr⁴⁹⁹) (Cell Signaling Inc.) antibodies where indicated. Loading of each fusion protein was determined by Coomassie staining.

Rac activation in PC12 cells. Activation of Rac was analyzed using a GST-effector binding assay. Transfected PC12 cells were stimulated with 50 ng/ml NGF for 0, 2, or 10 min, and cell lysates were prepared in MLB buffer (25 mM HEPES, 150 mM sodium chloride, 1% Nonidet P-40, 10 mM magnesium chloride, 1 mM EDTA, 10% glycerol, 0.3 mg/ml phenylmethylsulfonyl fluoride, 1.0 mM sodium vanadate, and protease inhibitors [pH 7.5]) (18). Lysates were incubated with glutathione-agarose beads containing the immobilized P21-activated kinase p21 binding domain (PAK-PBD) (residues 67 to 150; 10.0 μ g), and unbound protein was removed by three washes with MLB buffer. For controls, 50 μ l of cell extract was incubated with 10 mM EDTA and 0.1 mM GTP- γ S (positive control) or 1 mM GDP (negative control) for 20 min at 30°C, cooled, and supplemented with 10 mM magnesium chloride prior to incubation with PAK-PBD-GST bound to glutathione-agarose beads. Following denaturation, bound Rac was analyzed by Western blot analysis and quantitated by densitometry using Image J (National Institutes of Health).

RESULTS

Kalirin plays an important role in NGF-induced differentiation of PC12 cells. Our previous experiments microinjecting sympathetic neurons were not amenable to biochemical analysis because neurons need to be individually microinjected. This was a limitation in assessing the mechanism by which Kalirin affects neuronal process extension. Furthermore, sym-

pathetic neurons require NGF for survival, another limitation in using this model to analyze the effect of Kalirin on neurotrophic factor-induced signaling and neurite outgrowth. The rat pheochromocytoma (PC12) cell line is a prototypical model for examining NGF-induced process extension and was tested as a model to study the role of Kalirin in this process (32). We first determined whether the levels of endogenous Kalirin in PC12 cells could be reduced using a dual-promoter construct that coexpresses GFP and Kalirin antisense RNA (GFP/Kal-as). Western blot analysis with a pan-Kalirin antiserum (37) confirms that PC12 cells contain immunoreactive bands of 188, 268, and 336 kDa corresponding to the predicted molecular masses of Kal7, Kal9, and Kal12 isoforms that arise by alternative splicing (Fig. 1A) (22). As expected, the 336-kDa band was also detected with a Kal12-specific antiserum (data not shown). Introducing GFP/Kal-as into dissociated or slice preparations of rat hippocampus reduces Kalirin immunostaining (28). Transfection of PC12 cells with this GFP/Kal-as construct exhibited a dose-dependent reduction in expression of endogenous Kal7, Kal9, and Kal12 and correspondingly increased expression of GFP, indicating that this construct can be used to assess the effect of reduced Kalirin levels in PC12 cells (Fig. 1A).

PC12 cells transfected with the GFP/Kal-as or a GFP expression construct were treated with NGF for 2 days and examined by immunofluorescence microscopy, and cells with no neurites or those with neurites longer than one cell body were scored. We also transfected cells with a control GFP/peptidyl amidating monooxygenase antisense RNA construct (GFP/Control-as) that was previously used as an antisense specificity control in hippocampal neurons (28). Seventy-two percent and 68% of the fluorescent cells transfected with the GFP or GFP/Control-as construct, respectively, had neurites more than one cell body long (Fig. 1B). In contrast, only 18% of PC12 cells expressing GFP/Kal-as had neurites more than one cell body long. These data indicate that reduced Kalirin levels significantly inhibited NGF-induced process extension from PC12 cells, and they validate this system as a model with which to investigate the effects of Kalirins on neurite outgrowth.

Kalirin is critical for NGF-stimulated TrkA phosphorylation. Since reduced Kalirin expression levels inhibited NGF-induced neurite outgrowth, we wanted to better understand the mechanism. NGF binds the TrkA and p75 receptors. Tyr⁴⁹⁹ is one of several tyrosine residues in TrkA that are autophosphorylated upon NGF stimulation, and Western blot analysis with a phospho-TrkA/Tyr⁴⁹⁹ (P-TrkA) antibody can be used as an indirect measure of TrkA activation (19). PC12 cells transfected with expression constructs for GFP, GFP/Kal-as, or GFP/Control-as were treated with NGF, and extracts were analyzed by Western blotting with the phospho-TrkA antibody. In PC12 cells transfected with control GFP or GFP/Control-as, a rapid increase in TrkA phosphorylation was observed after treatment with NGF (Fig. 2A). The mature 140-kDa form of TrkA was phosphorylated, while no phosphorylation of the immature 110-kDa TrkA was observed, despite higher expression of the 110-kDa form; the changes in molecular mass reflect maturation of glycosyl groups of TrkA (52). Conversely, NGF-induced TrkA phosphorylation was significantly decreased in cells transfected with GFP/Kal-as. Expression of Kalirin antisense RNA did not affect the levels of total TrkA in

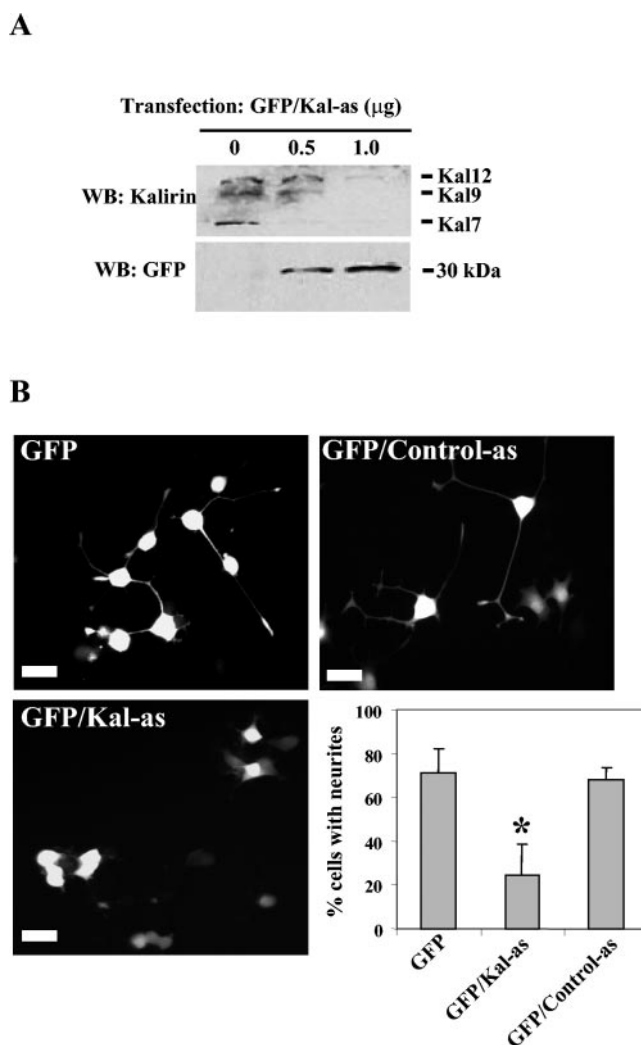


FIG. 1. Antisense Kalirin RNA inhibits NGF-induced neurite outgrowth in PC12 cells. (A) A dual-promoter GFP/Kalirin antisense construct reduces levels of endogenous Kalirin in PC12 cells. PC12 cells were transfected with a dual-promoter construct that coexpresses GFP and Kal-as. Cell extracts were analyzed by Western blotting (WB) with pan-Kalirin and GFP antibodies. (B) PC12 cells were transfected with expression constructs for GFP, a control GFP/peptidyl amidating monooxygenase antisense RNA construct (GFP/Control-as), or GFP/Kal-as and treated with 50 ng/ml NGF for 2 days. Cells expressing GFP, as visualized by immunofluorescence microscopy, were scored as cells with neurites longer than one cell body or with no neurites. Bars, 10 μ m. Standard deviations are for four experiments ($n = 50$). The asterisk indicates that cells expressing GFP/Kal-as have significantly fewer neurites than the GFP and GFP/Control-as samples ($P < 0.001$; $H = 23.1$).

cells. These results indicate that Kalirin plays an important role in NGF-induced activation of TrkA in PC12 cells and that the effects of Kalirin on process extension may be due, at least in part, to effects on TrkA.

Kalirin potentiates NGF-induced TrkA phosphorylation and increases TrkA kinase activity. To determine whether overexpression of Kalirin affects NGF-induced TrkA phosphorylation, PC12 cells were transfected with expression constructs for GFP or different amounts of Kal5, and TrkA phosphorylation was measured in cells treated with NGF for 0 or 15 min. We decided to focus on Kal5 (also called Δ Kal7 [22])

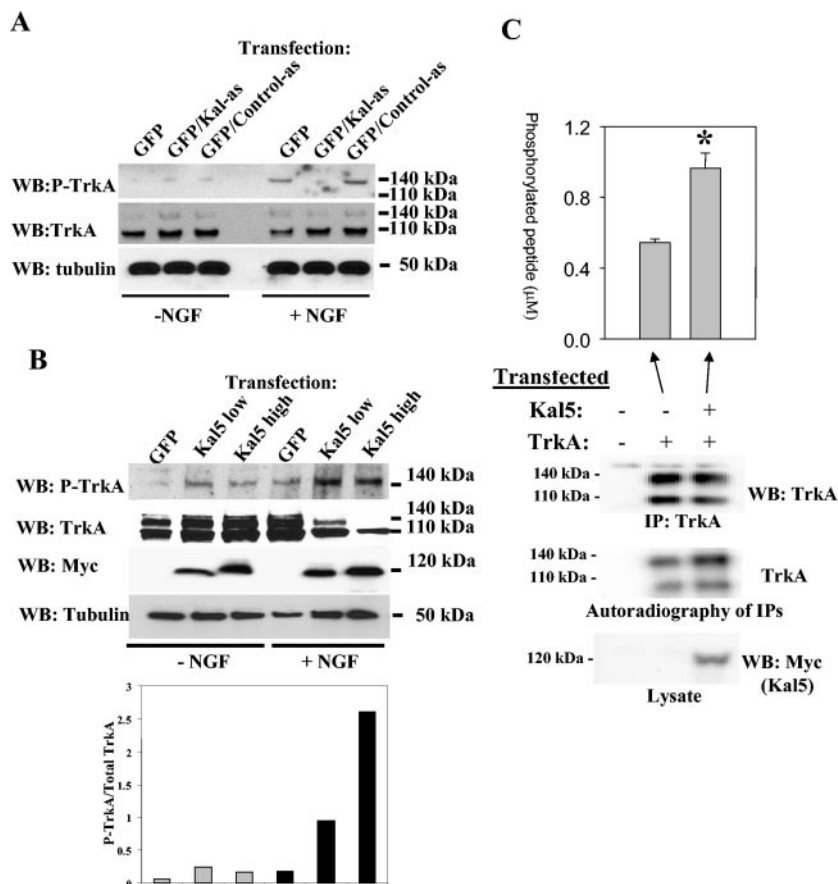


FIG. 2. Kalirin is important for NGF-induced TrkA phosphorylation. (A) PC12 cells transfected with pCMS expression constructs for GFP, GFP/Kal-as, or GFP/Control-as (1.0 µg) were treated with NGF at 25 ng/ml for 15 min and analyzed by Western blotting (WB) with P-TrkA(Tyr⁴⁹⁹), TrkA, and tubulin antibodies. Western blot analysis with a tubulin antibody shows relatively equal loading of samples. (B) Effect of Kal5 on NGF-induced TrkA phosphorylation. PC12 cells were transfected with expression constructs for GFP (2.0 µg) or Kal5 (low [0.2 µg] or high [2.0 µg]). Cells were stimulated with NGF for 0 or 15 min. Results of Western blot analyses with antibodies to P-TrkA(Tyr⁴⁹⁹), TrkA, tubulin, and myc are shown. Expression of myc-tagged Kal5 was confirmed with an anti-myc antibody. The bar graph shows levels of P-TrkA relative to those of TrkA in samples. (C) Enzymatic activation of TrkA by Kal5. 293 cells (150-mm dishes) were transfected with 1 µg of TrkA cDNA and 9 µg of either Kal5 cDNA or an empty vector. Control dishes were transfected with the empty vector alone. TrkA was immunoprecipitated from cell lysates using the anti-TrkA monoclonal antibody B-3 (Santa Cruz Biotech), and kinase assays were performed as described in Materials and Methods. Slurries containing the washed immunoprecipitates (IPs) were aliquoted, and a Shc peptide (residues 309 to 327) kinase assay, an autokinase assay (³²P]ATP only), and WB were performed. The bar graph represents incorporation of ³²P into the Shc peptide in experiments performed in triplicate (means ± standard errors of the means). The asterisk indicates significant difference (*P* < 0.001). Autoradiography represents incorporation of ³²P into TrkA (autokinase). Expression of Kal5 was confirmed by Western blotting. Control immunoprecipitates from empty-vector samples (no TrkA cDNA) did not contain significant kinase activity (data not shown).

because it is one of the shortest isoforms and lacks 12 of the 19 domains found in the longest isoform, Kalirin 12 (22). In control cells transfected with GFP, low basal TrkA phosphorylation was observed and increased phosphorylation occurred in cells treated with NGF (Fig. 2B). A bar graph shows the relative ratios of P-TrkA to TrkA. Western blot analysis with a tubulin antibody confirmed equal loading of extracts.

In contrast to PC12 cells transfected with the GFP control, those transfected with a Kal5 expression construct exhibited four major differences. Firstly, basal TrkA phosphorylation was slightly elevated. Secondly, Kal5 expression produced a higher level of NGF-induced phosphorylation of TrkA. Thirdly, increasing Kal5 expression produced a rapid, dose-dependent decrease in total TrkA content in cells after a 15-min treatment with NGF. Finally, higher levels of Kal5 expression correlated

with an increased P-TrkA/TrkA ratio. Thus, Kal5 overexpression increased the amount of Tyr⁴⁹⁹ phosphorylation per TrkA molecule in NGF-treated PC12 cells. These results demonstrate that Kalirin induces and potentiates the activation of TrkA.

In the absence of NGF ligand, expression of Kalirin increases TrkA phosphorylation. There are several ways in which Kalirin overexpression could increase levels of TrkA phosphorylation, one being an effect on TrkA kinase activity. To determine whether Kal5 influences the kinase activity of TrkA, human embryonic kidney 293 cells were cotransfected with TrkA cDNA and either Kal5 cDNA or an empty vector, and immune complex kinase assays were performed on immunoprecipitated TrkA (Fig. 2C). The tyrosine kinase activity of TrkA was found to be stimulated by Kal5 when a synthetic

peptide derived from the TrkA substrate Shc (containing Y³¹⁷) was used as an exogenous substrate in *in vitro* kinase assays (Fig. 2C, bar graph). TrkA autokinase activity was measured by the addition of radiolabeled ATP alone and demonstrated that TrkA isolated from cells which expressed Kal5 was catalytically activated relative to TrkA from cells which did not express Kal5 (Fig. 2C, autoradiography). Thus, coexpression of TrkA and Kal5 in cells results in enzymatic activation of the TrkA tyrosine kinase.

Kalirin expression is important for TrkA phosphorylation in PC12 cells, but it is not clear whether Kalirin acts similarly in neurons. Both Kalirin and TrkA are known to be expressed in olfactory bulb neurons (6, 16, 37). Olfactory bulb neurons were transfected with GFP/Kal-as, treated with NGF for 15 min, and immunostained for phospho-TrkA (Fig. 3). As expected, untransfected olfactory bulb neurons that were stimulated with NGF had phospho-TrkA immunostaining in the cell body; however, neurons expressing GFP/Kal-as and stimulated with NGF had little phospho-TrkA immunostaining. Quantitation of these results indicates that P-TrkA immunostaining in cells expressing Kalirin antisense is significantly reduced relative to that in control cells expressing GFP or GFP/Control antisense RNA (Fig. 3, bar graph). These results indicate that Kalirin is important for phosphorylation of TrkA in olfactory bulb neurons.

Expression of Kalirin increases NGF/TrkA-mediated activation of CREB in 293 cells. Tyr⁴⁹⁹ of TrkA is phosphorylated upon NGF stimulation and recruits adaptor proteins such as Shc for signaling through the MAPK pathway to activate several transcription factors including cyclic AMP-responsive element binding protein (CREB) (8, 9, 21, 47, 54). Therefore, we would expect that Kalirin may potentiate activation of CREB by NGF, since Kalirin potentiates NGF-induced phosphorylation of TrkA on Tyr⁴⁹⁹. 293 cells were used as a model with which to study the effects of Kalirin on TrkA signaling to CREB for several reasons. These cells lack endogenous TrkA, Kalirin, and p75; p75 is a neurotrophin receptor which heterodimerizes with TrkA and is present in neurons and PC12 cells. Since Kalirin is thought to also bind the p75 receptor (S. O. Yoon, unpublished results), the presence of p75 would confound interpretation of similar experiments in neurons or PC12 cells (58). In transfected 293 cells, TrkA was previously shown to undergo NGF-induced autophosphorylation and signaling through the MAPK pathway in a manner similar to that in neurons (17). Also, the CREB luciferase reporter assay requires cotransfection of several plasmids, and 293 cells have higher transfection efficiencies than PC12 cells and neurons.

To determine whether Kalirin influences TrkA signaling, we adapted a signal transduction CREB reporter assay using 293 cells expressing transfected TrkA; several controls were performed to establish this assay (see Materials and Methods). Since overexpression of TrkA is known to induce its autoactivation, the TrkA expression plasmid was titrated to identify expression levels that produced low basal TrkA phosphorylation and activation of CREB while generating a maximal NGF-induced response (data not shown) (25).

The levels of Kal5 expression affected the extent of potentiation of NGF-induced activation of a CREB reporter (Fig. 4A). In 293 cells transfected with a TrkA expression construct, CREB was stimulated fivefold by NGF. When cotransfected

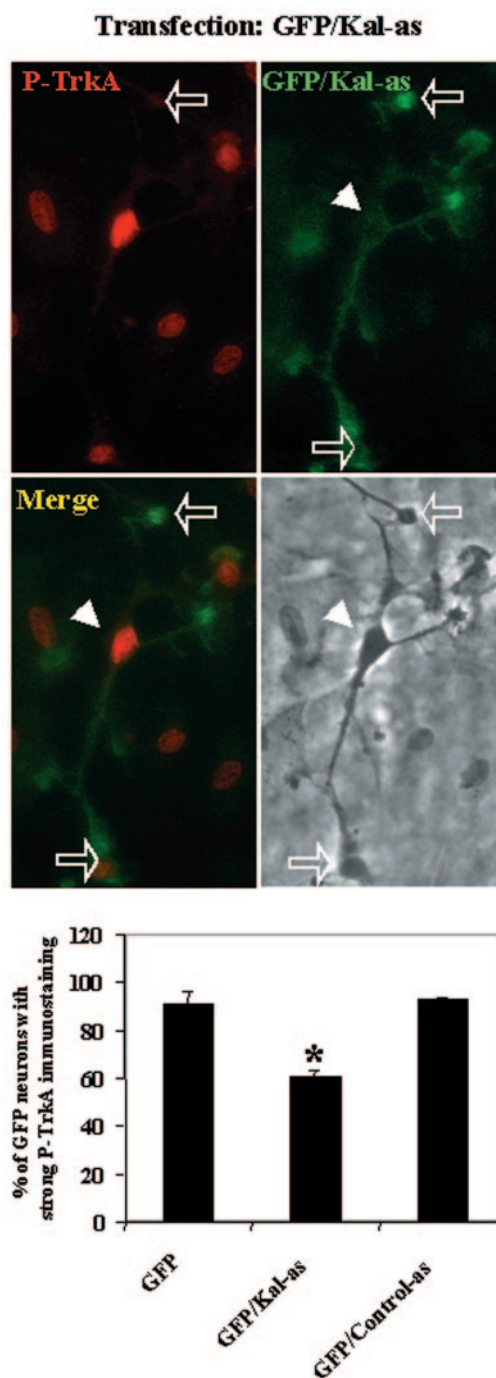


FIG. 3. Kalirin antisense RNA reduces TrkA phosphorylation in neurons. Primary olfactory bulb neurons transfected with dual-promoter GFP, GFP/Kal-as, or GFP/Control-as constructs were treated with NGF (50 ng/ml) for 15 min and immunostained with a P-TrkA antibody (red). GFP fluorescence, immunostaining, and phase-contrast pictures are shown for cells transfected with GFP/Kal-as. Unfilled arrows indicate a transfected cell (green), and filled arrowheads depict untransfected cells. The bar graph represents the percentage of neurons expressing GFP with strong P-TrkA immunostaining. Strong immunostaining is considered clearly above background staining, and a representative strongly immunostained neuron is shown (filled arrowheads). The asterisk indicates that the number of cells with strong P-TrkA immunostaining was significantly lower for cells expressing GFP/Kal-as relative to cells expressing GFP or GFP/Control-as ($P < 0.05$).

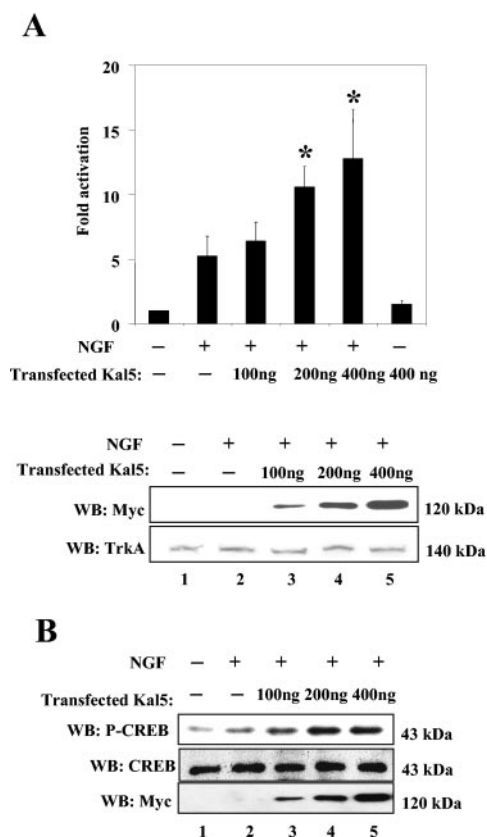


FIG. 4. Kalirin expression increases NGF-induced activation of CREB. (A) Signal transduction reporter assay for assessing the effect of Kal5 on TrkA signaling. 293 cells were cotransfected with expression constructs for TrkA (25 ng), a Gal-dbd/CREB-TD chimera (40 ng), β -galactosidase (10 ng), varying amounts of Kal5, and a Gal4-luciferase reporter plasmid (400 ng). An empty pEAK10 vector was added such that each sample had the same amount of DNA. Cells were treated with NGF (25 ng/ml) for 6 h where indicated. Luciferase activities of cell extracts were normalized to β -galactosidase activity, and fold activation was relative to samples not cotransfected with Kal5 or treated with NGF. Standard deviations were calculated from triplicate observations from four experiments. Asterisks indicate that the 200- and 400-ng samples were significantly different from the samples with no Kal5 and with or without NGF in a one-way ANOVA ($P < 0.05$). Western blot analysis (WB) confirmed that TrkA levels are constant and that increasing amounts of transfected Kal5 cDNA produced increased expression of Kal5 protein (bottom panels). (B) WB of 293 cells cotransfected with expression constructs for TrkA and varying amounts of Kal5, or with an empty pEAK10 vector (lanes 1 and 2). Cells were treated with 25 ng/ml NGF for 15 min, harvested in SDS-PAGE lysis buffer, and probed with antibodies for phospho-Ser¹³³ CREB to assess CREB activation, CREB (total CREB), and Myc (Kal5).

with TrkA, increasing levels of Kal5 cDNA produced a dose-dependent potentiation of NGF-induced reporter activation, with a maximal 12-fold activation. The levels of CREB reporter activation in cells transfected with 200 and 400 ng of the Kal5 expression construct were significantly higher than basal levels and levels in NGF-stimulated cells not transfected with Kal5 cDNA. Minimal activation of the CREB reporter was observed in cells transfected with Kal5 alone. These results demonstrate that Kal5 expression increased NGF/TrkA-induced activation of a CREB reporter; they are consistent with

the observation that Kalirin potentiates NGF-induced TrkA phosphorylation (Fig. 2B); and they strongly suggest that Kalirin enhances NGF-induced transcriptional activation.

We next examined whether Kal5 affected NGF-induced phosphorylation of endogenous CREB on Ser¹³³, a residue that, when phosphorylated, activates CREB-mediated transcription. 293 cells cotransfected with TrkA and varying levels of a Kal5 plasmid were treated with NGF for 15 min. CREB phosphorylation was measured indirectly by Western blot analysis comparing blots probed with phospho-Ser¹³³ CREB and pan-CREB antibodies. Control cells possessed low basal CREB phosphorylation that was enhanced by treatment with NGF for 15 min (Fig. 4B). As in the CREB reporter assay, dose-dependent increases in NGF-induced CREB phosphorylation were observed with increasing levels of Kal5 expression, as assessed with a myc antibody. These experiments indicate that expression of Kal5 enhances TrkA signaling to CREB.

Kalirin potentiates NGF-stimulated Mek phosphorylation. Activation of CREB can be mediated through the MAPK, protein kinase C, or other signaling pathways (46). Kalirin increased the NGF-induced activation of CREB, and TrkA signals through the MAPK pathway to activate CREB, but we did not know whether Kalirin influences NGF-induced activation of the MAPK pathway (8, 9, 21, 47, 54). We next examined the effects of Kalirin overexpression on MAPK pathway activation in PC12 cells. PC12 cells transfected with expression constructs for Kal5 or an empty vector were stimulated with NGF for 15 min and analyzed for Mek phosphorylation as a measure of MAPK pathway activation. Cell lysates were analyzed by Western blotting with an activation-specific Mek1/2 (P-Mek) antibody directed against phospho-Ser^{217/221} (Cell Signaling). As expected, P-Mek was detected in cells stimulated with NGF (Fig. 5A). No activated Mek was observed in lysates derived from cells transfected with the Kal5 expression construct alone (no NGF). However, cells expressing Kal5 and stimulated with NGF possessed significantly higher activated P-Mek levels than control cells (no Kal5) stimulated in the same manner, indicating a potentiated response. These data support the previously observed increases in NGF-induced CREB activation by Kal5 in 293 cells and provide evidence that Kal5 potentiates TrkA signaling through the MAPK pathway.

Overexpression of Kalirin enhances the responsiveness of PC12 cells to TrkA-mediated neurite outgrowth. Since Kal5 potentiated TrkA autophosphorylation and activation of Mek in PC12 cells, we wanted to know whether Kalirin overexpression could sensitize PC12 cells to neurite outgrowth elicited by NGF. In the absence of NGF, PC12 cells transfected with a Kal5 expression construct or the dual-promoter Kal5/GFP expression construct did not produce neurite outgrowth that was significantly different from that in nontransfected cells or cells transfected with an empty vector (data not shown). This result was not surprising, considering that Kal5 overexpression did not increase P-Mek levels in unstimulated cells. We next examined whether Kal5 might potentiate neurite outgrowth in PC12 cells treated with a low dose of NGF (2 ng/ml). PC12 cells transfected with a Kal5/GFP expression plasmid and stimulated with 2 ng/ml of NGF produced neurites longer than one cell body in 17% of the cells, significantly more than control cells expressing GFP and treated with NGF (7.1% [Fig. 5B]). Without NGF, cells transfected with the GFP or Kal5/GFP

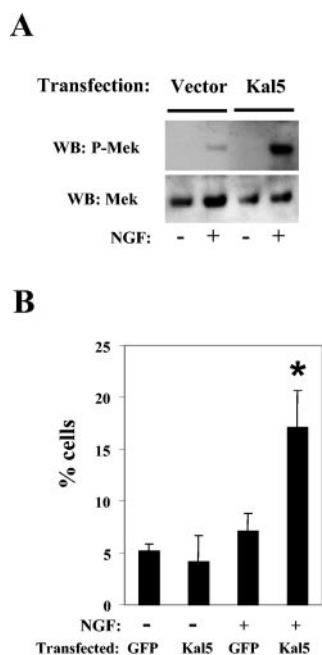


FIG. 5. Effect of Kalirin overexpression on NGF-induced Mek phosphorylation and neurite outgrowth in PC12 cells. (A) Kal5 potentiates NGF activation of the MAPK pathway. PC12 cells transfected with the indicated constructs under basal conditions or stimulated with 50 ng/ml NGF for 15 min were analyzed by Western blot (WB) analysis with phospho-Mek1/2 and pan-Mek1/2 antibodies. (B) PC12 cells transfected with a dual-promoter GFP/Kal5 expression construct (Kal5) or an empty vector for GFP expression were treated with 2 ng/ml NGF in serum-free medium for 2 days and scored for neurite outgrowth. The bar graph shows the percentage of transfected cells with neurites ($n > 50$), with standard deviations from triplicate observations. The asterisk indicates that cells transfected with the Kal5/GFP expression construct and treated with NGF had significantly more neurites than cells under other conditions ($P < 0.05$; $H = 7.88$).

expression construct yielded only 4 to 5% of cells with neurites. These experiments demonstrate that overexpression of Kal5 increases the sensitivity of PC12 cells to NGF-induced process extension.

TrkA and Kalirin colocalize in neurons. The effects of Kalirin on neurite outgrowth and TrkA signaling imply that Kalirin and TrkA colocalize in neurons. Primary neuronal cultures prepared from P1 dorsal root ganglia (DRG) and olfactory bulbs were immunostained with pan-Kalirin and TrkA antibodies and examined by deconvolution fluorescence microscopy. Small puncta could be resolved in axons of DRG neurons (Fig. 6A). Several of these puncta were immunostained with antibodies to Kalirin, TrkA, or both proteins. While these results indicate that Kalirin and TrkA are colocalized in axons, little colocalization was observed in the neuronal cell soma (data not shown). Similar colocalization of Kalirin and TrkA was observed in processes of olfactory bulb neurons (Fig. 6B). In contrast to DRG neurons, Kalirin and TrkA were also colocalized in the perinuclear region of the neuronal cell soma of olfactory bulb neurons (Fig. 6C).

Kalirin binds in a complex with TrkA and phospho-TrkA. To determine whether Kalirin and TrkA are present in the same complex in vivo, coimmunoprecipitation experiments were performed. Large projection neurons in adult rodents

express low levels of both Kalirin and TrkA, making it difficult to determine whether endogenous Kalirin and TrkA colocalize (4, 15). During development, when neurons are establishing connections, both TrkA and Kalirin are expressed at higher levels, so we used lysates prepared from an E17 embryonic mouse brain for coimmunoprecipitation analysis. Kal12 (336 kDa), Kal9 (268 kDa), and Kal7 (188 kDa) isoforms present in embryonic mouse brain were coimmunoprecipitated with TrkA but not with an isotype-matched control antibody (Fig. 7A) (22). Probing of blots with a TrkA antibody confirms immunoprecipitation of TrkA by the TrkA antibody. Approximately 5% of the input Kalirin was coimmunoprecipitated with TrkA. This result demonstrates that neuronal Kalirin and TrkA exist in the same protein complex in vivo.

To determine if the phosphorylated, activated form of TrkA binds in a complex with Kalirin, we used 293 cells transfected with a large amount of TrkA cDNA (1 μ g) that induces constitutive TrkA autophosphorylation and abundant phospho-TrkA levels. Lysates from 293 cells cotransfected with a TrkA expression construct and a plasmid encoding the Kal5 cDNA were immunoprecipitated with myc or control antibodies and analyzed by Western blotting with phospho-TrkA and myc antibodies. The mature 140-kDa phospho-TrkA coimmunoprecipitated with myc-tagged Kal5 (myc antibody) but not with a control IgG1 antibody, confirming the specificity of the anti-myc immunoprecipitation (Fig. 7B). Furthermore, no detectable P-TrkA was immunoprecipitated with the myc antibody when extracts expressing TrkA alone were used. These results indicate that Kalirin can form a complex with the phosphorylated TrkA receptor.

TrkA binds specifically to the N-terminal PH domains of Kalirin and Trio. To identify a domain(s) in Kal5 important for interaction with TrkA, different fragments of Kal5 were expressed in *E. coli* as GST fusion proteins, purified, and used in GST pulldown experiments. Extracts prepared from 293 cells transfected with a large amount of a TrkA expression plasmid (1 μ g) were incubated with purified GST fusion proteins, and complexes were pulled down with glutathione Sepharose and analyzed by Western blotting with a TrkA antibody. TrkA bound to the PH domain of Kal5, whereas no detectable binding was observed for GST alone or for other parts of Kal5 (Fig. 7C, top panel). In addition, phospho-TrkA present in these extracts specifically bound to the PH domain of Kal5 (Fig. 7C, center panel). Approximately 77 and 100% of the TrkA and phospho-TrkA present in extracts bound to Kal5-PH, respectively. Analysis of a Coomassie-stained gel confirmed relatively equal loading of GST and GST fusion proteins in this experiment (Fig. 7C, bottom panel). Thus, Kal5-PH binds both TrkA and phospho-TrkA.

While Kal5 has only one PH domain, several alternatively spliced Kalirin isoforms and a Kalirin homologue, Trio, contain two PH domains. To determine whether the interaction of TrkA with the Kalirin N-terminal PH domain (PH1, present in Kal5) was specific, binding of TrkA to GST fusion proteins containing each of the PH domains of Kalirin and Trio was assessed using a GST pulldown assay. TrkA bound to both Kalirin and Trio N-terminal PH domains, whereas no substantial binding to either C-terminal PH domain (PH2) or GST alone was observed (Fig. 7D). The Kalirin and Trio PH1 domains that bind TrkA have 85% amino acid identity. This

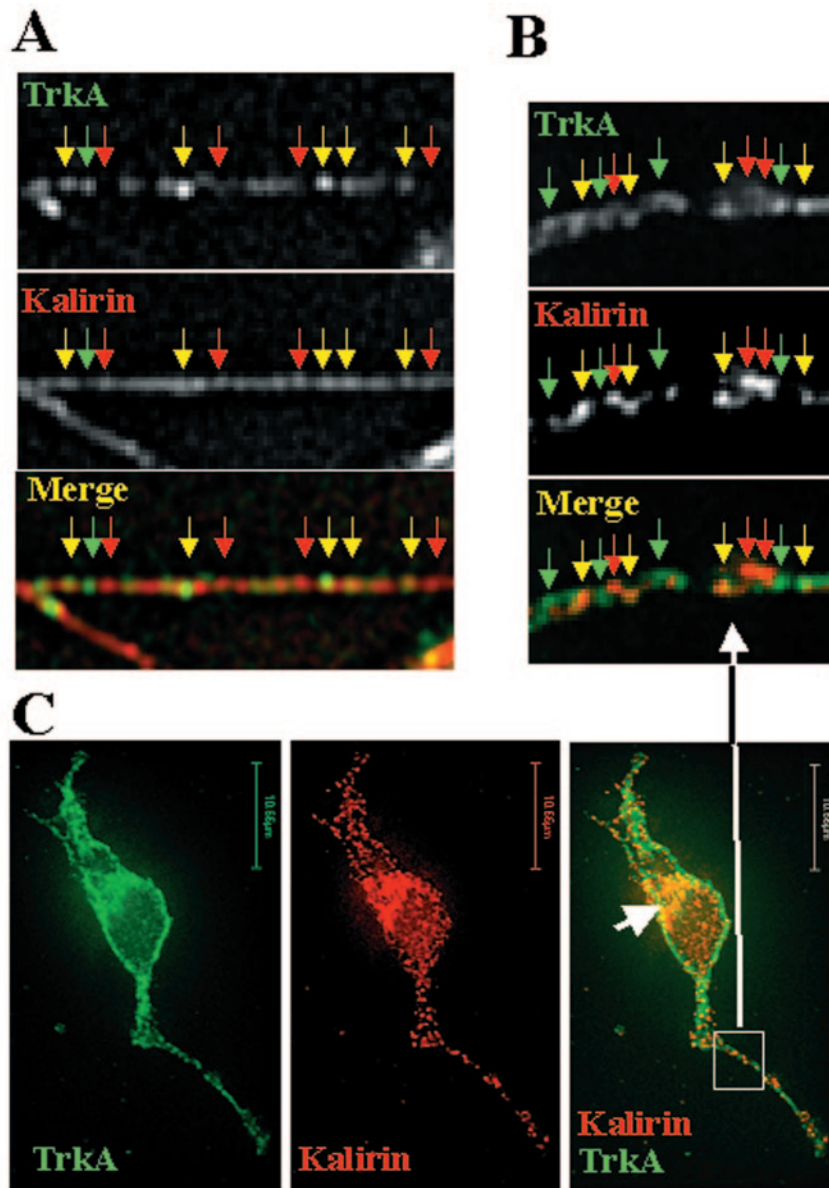


FIG. 6. Kalirin colocalizes with TrkA in neurons. (A) Immunostaining of a neuronal axon from a primary dorsal root ganglion culture with a pan-Kalirin antiserum and a TrkA antibody. Colored arrows indicate whether puncta are stained for endogenous Kalirin (red), endogenous TrkA (green), or both (yellow). (B and C) Primary olfactory bulb cultures immunostained with TrkA (green) and pan-Kalirin (red) antibodies, indicated by colored arrows; yellow arrows indicate colocalization. A higher magnification of the process in the white box in panel C is shown in panel B. For all microscopy experiments, Z-stacks were collected and images were deconvolved.

experiment indicates that not all RhoGEF PH domains have the ability to bind TrkA and demonstrates a degree of specificity in the Kal5-PH—TrkA interaction.

Overexpression of Kalirin enhances NGF-induced activation of Rac. The PH and DH domains of Kalirin comprise the Kalirin N-terminal GEF domain, which catalyzes guanine nucleotide exchange and activation of Rac1 and RhoG (32, 38). Since PH domains are often involved in regulation of GEF activity and TrkA binds the Kalirin PH domain, Kalirin may be a RhoGEF that mediates the effects of NGF on Rac (59). Stimulation of PC12 cells with NGF induces rapid activation of Rac, and Kalirin can activate Rac in vitro and in cells (38, 40).

To determine if Kalirin plays a role in the NGF-induced activation of Rac, PC12 cells were transfected with a GFP or Kal5 expression construct and assayed for levels of activated Rac/GTP. Cells were treated with NGF as indicated, and Rac activation was assessed using an established effector binding assay with a PAK-PBD—GST fusion protein (3). Control cells transfected with GFP showed low basal Rac activation that increased after 2 and 10 min of NGF stimulation (Fig. 8), consistent with previously reported rapid NGF-induced Rac activation (40). Overexpression of Kal5 led to Rac activation, and NGF stimulation increased Rac activation to levels higher than those observed in cells expressing the GFP control. The

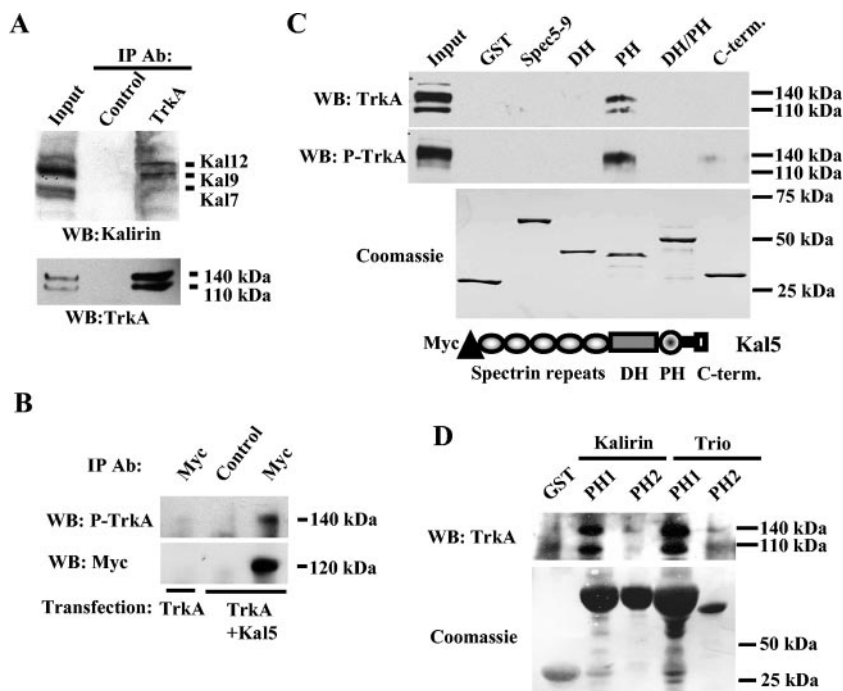


FIG. 7. TrkA interacts with the Kalirin PH domain. (A) Cell lysates prepared from a mouse E17 brain homogenate were immunoprecipitated with TrkA or control antibodies (IP Ab). The immune complexes were captured on protein G-Sepharose, washed, and boiled in SDS-PAGE sample buffer. Immunoprecipitated TrkA and coimmunoprecipitated Kalirin isoforms were detected by Western blotting (WB) with TrkA and pan-Kalirin antibodies. The input sample represents 13% of the extract that was immunoprecipitated. (B) Cell lysates prepared from 293 cells cotransfected with an expression construct for TrkA (1 μ g) and either a construct expressing Kal5 or an empty vector (1 μ g) were immunoprecipitated with myc or isotype control IgG1 antibodies. Immunoprecipitated myc-tagged Kal5 and coimmunoprecipitated P-TrkA were detected by WB with myc and P-TrkA (phospho-Tyr⁴⁹⁹) antibodies, respectively. (C) Mapping of Kalirin domains that bind TrkA by a GST pull-down assay. Extracts prepared from 293 cells transfected with a TrkA expression construct (1 μ g) were incubated with GST or a GST-Kalirin domain chimera. Protein complexes were applied to glutathione Sepharose beads, which were washed, and bound proteins were analyzed by WB with TrkA and P-TrkA antibodies. Bottom panel shows a Coomassie-stained gel of GST and the GST-Kalirin chimera. Input represents 67% of cell lysates applied to GST fusion proteins in binding assays. A schematic diagram of Kal5 with domains labeled is provided. (D) TrkA binds specifically to the N-terminal PH domains of Kalirin and Trio. GST pull-down assays were performed as for panel C by using the GST or GST-PH fusion proteins indicated and WB analysis with a TrkA antibody (Chemicon). A Coomassie-stained gel shows fusion proteins used in the GST pull-down experiment.

specificity of the assay for GTP-bound Rac was demonstrated by binding of Rac to the PAK-PBD-GST fusion protein in the presence of excess GTP γ S, whereas no binding was detected in the presence of excess GDP. These results demonstrate that Kalirin overexpression in PC12 cells can increase Rac activation in response to NGF.

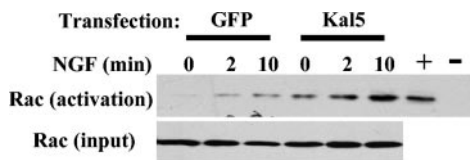


FIG. 8. Kalirin overexpression increases NGF-induced activation of Rac in PC12 cells. PC12 cells transfected with GFP or Kal5 expression constructs were stimulated with 50 ng/ml NGF for the times indicated. Cell extracts were assessed for activation of Rac by a pull-down assay with a PAK-PBD GST fusion protein that recognizes Rac with GTP bound and Western blot analysis with a Rac antibody. Western blot analysis of input samples is shown. + indicates a positive control where extracts are incubated with GTP γ S, and - indicates a negative control where extracts are incubated with GDP.

DISCUSSION

Initiation and outgrowth of axons from sympathetic neurons are known to be induced by Kalirin expression and blocked by antisense Kalirin oligonucleotides (32). We found that endogenous Kalirin in PC12 cells is important for neurite outgrowth, with reduced levels inhibiting and overexpression potentiating neurite outgrowth at a low dose of NGF (Fig. 1 and 5). These experiments indicate that PC12 cells are a good model with which to investigate the mechanism by which Kalirin induces axonal growth. The experiments and results discussed below support the model shown in Fig. 9 and provide a mechanistic explanation for Kalirin's effects on neurite outgrowth.

Kalirin plays an important role in NGF-induced activation of TrkA. Several pieces of evidence suggested that Kalirin might affect one of the NGF receptors or downstream signal transduction pathways. We first examined whether Kalirin affected activation of TrkA, a receptor that transmits the NGF signal across the plasma membrane in cells. Reduced Kalirin expression decreased NGF-induced TrkA phosphorylation on Tyr⁴⁹⁹, while Kal5 overexpression increased the level of NGF-induced TrkA phosphorylation.

There are several possible mechanisms by which Kalirin

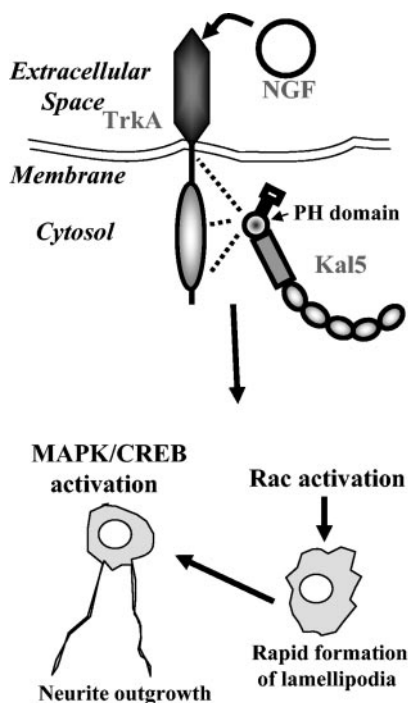


FIG. 9. Model of Kalirin's effects on TrkA signaling and neurite outgrowth. Kalirin is critical to NGF-induced TrkA phosphorylation and neurite outgrowth. Kalirin overexpression also enhanced NGF-stimulated activation of MAPK, CREB, and Rac. Rac activation induces formation of lamellipodia, the first morphological change induced by NGF in PC12 cells and a precursor of neurites (40).

could affect the levels of NGF-induced TrkA phosphorylation in cells. Kal5 may alter the localization of TrkA in cells. While Kal5 expression does not affect the levels of TrkA expression in PC12 cells (Fig. 2B and 4A), NGF stimulation of cells overexpressing Kal5 shows rapid disappearance of TrkA compared to control cells (Fig. 2B). This result could be explained by Kal5 affecting increased activation, internalization, and/or routing of TrkA to lysosomes. This will need to be investigated further. We also cannot rule out the possibility that, in the presence of Kal5, a higher proportion of TrkA could be available on the cell surface for ligand stimulation. If TrkA were maintained inactive by a phosphorylation/dephosphorylation turnover in unstimulated cells, then inhibition of tyrosine phosphatases or protection of phospho-TrkA sites by Kalirin would also result in increased TrkA activation.

Some of these mechanisms may contribute to increased TrkA phosphorylation in cells. However, immunoprecipitated TrkA had increased tyrosine kinase activity when isolated from cells overexpressing Kal5, supporting a mechanism whereby Kalirin enhances TrkA catalytic function and thus autophosphorylation. Consistent with this hypothesis, in the absence of NGF, PC12 cells transfected with Kalirin exhibit increased levels of phospho-TrkA compared to transfected control cells.

Kalirin could influence TrkA activation by several mechanisms. NGF induces TrkA dimerization, so Kalirin could influence TrkA dimerization by increasing the rate of dimerization or altering the stability of homodimers. Alternatively, Kalirin could influence the conformation of TrkA, rendering the receptor more prone to autophosphorylation or increasing

the accessibility to the substrate. Although effects of cytosolic proteins on TrkA autokinase activity have not been documented, a monovalent NGF ligand that does not induce receptor dimerization induces enough, albeit attenuated, TrkA phosphorylation to drive neurite outgrowth in PC12 cells (30). This study indicates that, in addition to receptor dimerization, there are other mechanistic components of TrkA activation.

Kalirin enhances activation of the MAPK pathway by TrkA. Upon engaging ligand, Tyr⁴⁹⁹ is one of the TrkA residues that is autophosphorylated. Elevated Kalirin expression potentiated NGF-induced phosphorylation of Tyr⁴⁹⁹ in intact cells and increased TrkA kinase activity toward an Shc peptide substrate in immune complex kinase assays. Consistent with its effect on TrkA kinase activity, elevated Kal5 also potentiated NGF-induced phosphorylation of Mek, a downstream target in the MAPK pathway. Like Kalirin's effect on TrkA, other cytosolic proteins are known to influence the activation of receptor tyrosine kinases and the MAPK pathway. APS and SH2B adaptor proteins potentiate insulin-induced autophosphorylation of the insulin receptor and TrkA and also potentiate MAPK activation (1, 2, 41). There is an interesting correlation with these proteins having PH domains, the domain in Kalirin that interacts with TrkA.

Our experiments provide a mechanistic explanation as to why process outgrowth in PC12 cells and sympathetic neurons is blocked by antisense Kalirin RNA. Kalirin is important for NGF-induced TrkA kinase activation (Fig. 2), TrkA phosphorylation is required for signaling to the MAPK pathway, and the MAPK pathway is required for neurite extension (19, 56). While our experiments with Kalirin focused on TrkA, which is expressed mostly in neurons of the forebrain and periphery, Kalirin may similarly affect TrkB and TrkC, which are more widely expressed in cortical and hippocampal neurons. Kalirin overexpression in cortical neurons increases dendritic spine density (36, 39). Kalirin-induced TrkA phosphorylation may play a role in spine formation, since Trk receptors are involved in the maintenance of dendrites, and activation of the MAPK pathway is critical for dendritic filopodia and spine extensions (28, 53). Likewise, our observation that reduced Kalirin levels inhibit NGF-induced TrkA phosphorylation might explain why expression of antisense Kalirin RNA reduces dendrite length in dissociated and slice preparations of hippocampal neurons (28). Since Kalirin appears to play a role in NGF-induced activation of the MAPK pathway and CREB, its localization in spines may also be involved in long-term potentiation and memory, which requires sustained MAPK activation (53).

Kalirin binds to and colocalizes with TrkA. Several experiments support an interaction of Kalirin with TrkA. Kalirin and TrkA coimmunoprecipitate from the brain, the Kalirin PH domain binds to TrkA *in vitro*, and these proteins colocalize with each other in neurons. While these experiments indicate that Kalirin is in the same complex with TrkA, a GST pulldown assay demonstrated that purified Kal5 binds to the purified GST-TrkA cytosolic domain, suggesting that this interaction is direct; however, this GST-TrkA produced in bacteria had no detectable kinase activity (data not shown). Nevertheless, the TrkA receptor is the first receptor found to associate with Kalirin, and this represents a major step in understanding the function of Kalirin.

Kalirin binds to TrkA through its PH domain. The interaction of TrkA with Kalirin is also supported by experiments mapping the Kalirin domains mediating this interaction. Domain-mapping experiments indicate that TrkA binds to the PH domain of Kalirin. While Kal-PH bound TrkA, the DH/PH fragment containing this PH domain did not. The DH/PH is enzymatically active as a RhoGEF, indicating that DH/PH folds properly (38, 43). Therefore, the inability of the Kalirin DH/PH to bind the receptor is consistent with a sequestered TrkA binding site in its PH domain. Other protein-protein interactions or binding of phosphoinositide lipids to the PH domain may be required to liberate the TrkA binding site. Phosphoinositide 3-kinase, an enzyme that binds the TrkA receptor, is activated in response to NGF stimulation, producing phosphatidylinositol(3,4,5)trisphosphate, and is required for neurite outgrowth (20). Binding of phosphoinositides or other molecules to the Kalirin PH domain may increase GEF activity toward Rac. Although we do not know if the Kalirin PH domain is accessible to phosphoinositides when bound to TrkA, it is likely that TrkA-bound Kalirin is localized at the cytoplasmic side of the plasma membrane in close proximity to phosphoinositides. The potential coupling of the TrkA receptor to Rac activation through Kalirin is interesting because within the first few minutes of NGF stimulation, PC12 cells flatten into lamellipodia in a Rac-dependent manner (40). Consistent with the potential linkage of TrkA/Kalirin to Rac activation, overexpression of Kal5 enhances the NGF activation of Rac during the first 10 min of NGF stimulation.

Receptor-RhoGEF pairings may play an important role in receptor activation and signal transduction. The interaction of the Kalirin RhoGEF with a receptor is interesting because several other RhoGEFs are known to associate with receptors or receptor signaling pathways: the MER receptor interacts with Vav1; the Ephrin A receptor interacts with ephexin; platelet-derived growth factor and epidermal growth factor receptors interact with Vav1, Vav2, and Vav3; Tiam interacts with Ephrin-B1 and EphA2; and Plexin B1 and IGF-1 receptors interact with Larg (29, 33, 45, 48-50). One possibility is that these receptor-RhoGEF pairings may be mediated through the PH domains of the RhoGEFs, as is the case for TrkA/Kalirin. In support of this hypothesis, mutation of the PH domain in the RhoGEF Dbs inhibits its effects on signal transduction to a serum response factor reporter (14). The interaction of Kalirin with TrkA may be one example in an emerging theme of receptor-RhoGEF pairings. In addition, $G_{\beta\gamma}$ subunits bind to the Sec14 domains of Kalirin, Trio, and Dbs RhoGEFs, thus providing another way in which Kalirin may link to another class of receptors, G-protein-coupled receptors (35).

The interaction of Kalirin with TrkA is mediated through its N-terminal PH domain. PH domains have little amino acid conservation and are present in nearly all RhoGEFs, making them attractive targets for specific RhoGEF-receptor pairings. Consistent with this hypothesis, the N-terminal PH domains of Kalirin and its homologue Trio bind the TrkA receptor, whereas the C-terminal PH domains do not. The N-terminal PH domains have 85% amino acid identity, suggesting that Kalirin and Trio bind to a common site(s) in TrkA. While many tyrosine kinase receptor-associated proteins have PH domains (e.g., PLC γ , Gab1, APS, SH2B, phosphoinositide 3-kinase, and IRS1-4), and the PH domain of PLC γ 1 binds to

the insulin receptor (1, 2, 24, 27, 31, 42), this is the first report of a PH domain from a RhoGEF interacting with a receptor tyrosine kinase. It is noteworthy that the three-dimensional fold of PH domains is very similar to phosphotyrosine binding domains, such as that in Shc, which binds to a phosphotyrosine in the juxtamembrane region of TrkA.

The interaction of the Trio PH domain with TrkA is also interesting because Trio induces neurite outgrowth in PC12 cells in the absence of NGF (12). Unlike Trio, Kal5 did not induce neurite outgrowth in PC12 cells; rather, it potentiated the response of PC12 cells to NGF. This may be because induction of neurites by Trio required its SH3 domain, which is not present in the Kal5 isoform that we used in this study. Nevertheless, the interaction of the Trio PH domain with TrkA links the results of this paper to the studies of Trio in neurite outgrowth.

In conclusion, we have found that Kalirin is in a complex with TrkA, colocalizes with TrkA in neurons, and affects the kinase activity of TrkA. The effects of Kalirin on TrkA and NGF signaling provide a mechanistic explanation for Kalirin's effects on process extension in PC12 cells and sympathetic neurons. The influence of Kalirin on TrkA activation also provides an explanation for previously observed effects of Kalirin on dendritic maintenance and spine formation. Domain-mapping experiments indicate that the Kalirin PH domain mediates the binding to TrkA. Although several RhoGEFs are known to interact with receptor tyrosine kinases, Kalirin is the first RhoGEF shown to bind to a receptor tyrosine kinase through its PH domain. Since most RhoGEFs have PH domains, this interaction may be an example of a more general interaction scheme where the PH domains of RhoGEFs mediate interactions with specific receptor tyrosine kinases.

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