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Induction of lamellipodia by Kalirin does not require its guanine nucleotide exchange factor activity

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Abstract

Guanine nucleotide exchange factor (GEF) domains of the Dbl family occur in a variety of proteins that include multiple protein–protein and protein–lipid interaction domains. We used an epithelial-derived cell line to investigate the mechanisms by which the two GEF domains of Kalirin, a neuronal Rho GEF, influence morphology. As expected, Kal-GEF1, an efficient GEF for Rac1 and RhoG, induced the formation of lamellipodia resembling those induced by active Rac1. Although Kal-GEF1 activated Rac and Pak, its ability to induce formation of lamellipodia was not blocked by dominant negative Rho GTPases or by catalytically inactive Pak. Consistent with this, a catalytically inactive mutant of Kal-GEF1 induced formation of lamellipodia and activated Pak. Active Pak was required for the GEF-activity independent effect of Kal-GEF1 and the lamellipodia produced were filled with ribs of filamentous actin. Kal-GEF1 and a GEF-dead mutant co-immunoprecipitated with Pak. The interaction of Kal-GEF1 with Pak is indirect and requires the regulatory protein binding domain of Pak. Filamin A, which is known to interact with and activate Pak, binds to both catalytically active and inactive Kal-GEF1, providing a link by which catalytically inactive Kal-GEF1 can activate Pak and induce lamellipodia. Together, our results indicate that Kal-GEF1 induces lamellipodia through activation of Pak, where GEF activity is not required. GEF-activity-independent effects on downstream targets may be a general property of RhoGEFs.

Keywords: Dbl family; UNC-73; Trio; Pak; Rac1; RhoG; Filamin

Introduction

Living organisms perform a variety of functions that require rapid rearrangement of their cytoskeleton. Actin-rich protrusions called filopodia and flattened sheets with ruffles called lamellipodia are just two of a number of structures important for cell structure and motility. RhoGTPases play a central role in modulation of these structures and several proteins and pathways are emerging as important components regulating and maintaining these structures. RhoGEFs are the upstream activators of RhoGTPase, and several RhoGEFs link receptors to downstream effects upon cell morphology and cell signaling.

Spatial precision and timing are critical to RhoGEF function. Among the Dbl family of GDP/GTP exchange factors (GEFs) for small GTP binding proteins of the Rho subfamily, Kalirin and Trio and their *Drosophila* and *C. elegans* paralogs (dTrio and Unc-73, respectively) are distinct, having dual RhoGEF domains [1,2]. The only other protein with two Dbl domains is DdracGAP [3] (by SMART analysis). Kalirin isoforms with single or dual GEF domains are generated through the tissue-specific and developmentally regulated use of several promoters and splice sites with Kalirin 12 being the longest Kalirin isoform (Fig. 1A) [4–6]. While genetic screens in worms and flies have consistently yielded mutations in the first GEF domain of these Kalirin paralogs, the fact that these proteins contain two GEF

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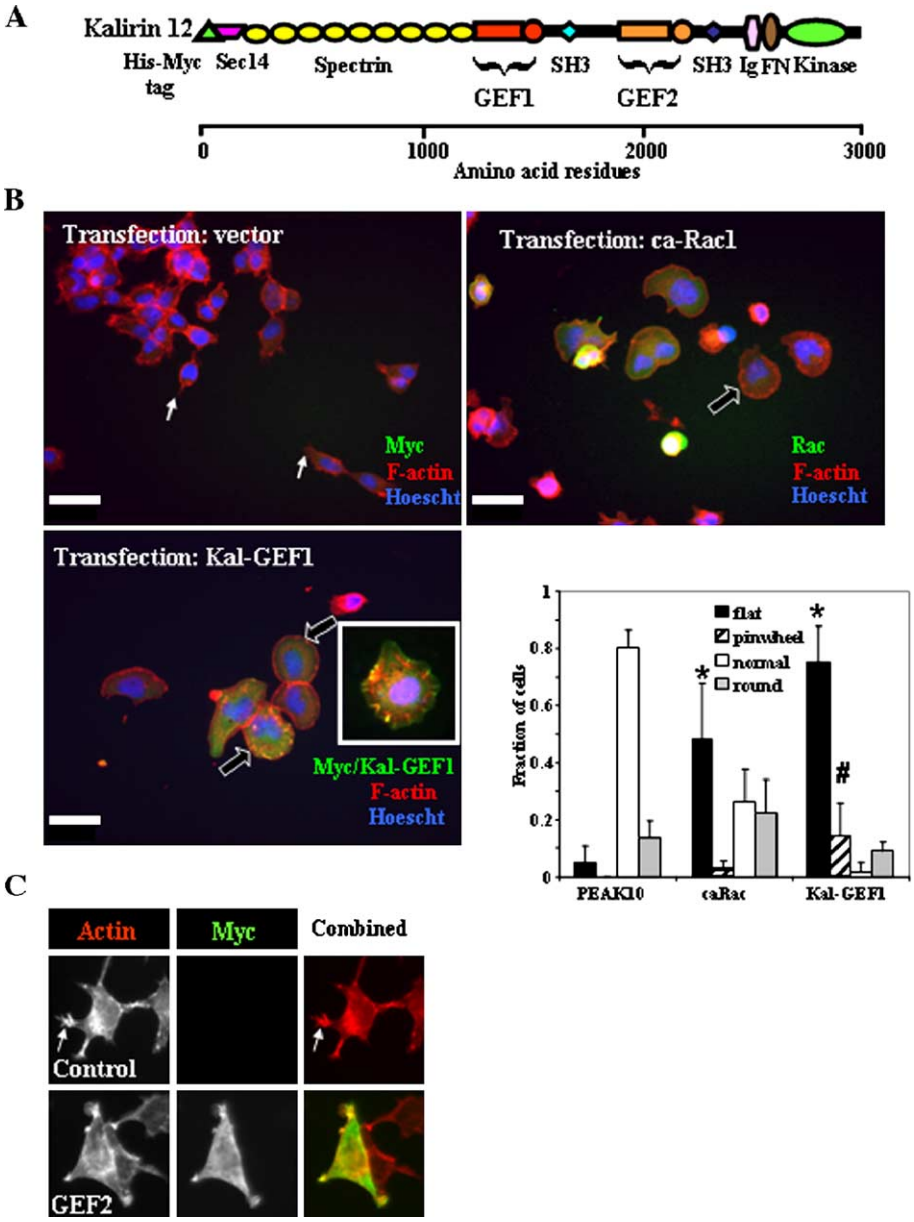

Fig. 1. Kal-GEF1, but not GEF2, induces lamellipodia. (A) Schematic diagram of Kal12, the longest Kalirin isoform. Kal12 contains putative Sec14p, spectrin, Dbl homology (DH), pleckstrin homology (PH), Src homology 3 (SH3), immunoglobulin (Ig), fibronectin III (FN), and kinase domains as labeled. All Kalirin expression vectors encode an N-terminal His-myc tag (triangle). (B) pEAK Rapid cells transfected with vectors encoding pEAK10.βGal (vector), caRac(Q61L), or myc-Kal-GEF1 were stained simultaneously with a monoclonal antibody specific for Rac or the myc epitope (green), TRITC-phalloidin to visualize filamentous actin (red), and Hoechst to visualize nuclei (blue). Representative images are shown. Arrows indicate processes in control cells with filopodial projections and open arrows indicate filamentous actin spokes in lamellipodial sheets. A pinwheel lamellipodium is shown in the inset (white box). Scale bar = 20 μm. Bar graph shows quantification of morphological changes. Asterisks indicate significant differences in the fraction of cells with flattened lamellipodia compared to control ($P < 0.001$). The fraction of pinwheel lamellipodia induced by Kal-GEF1 is significantly different compared to control or caRac ($^{\#}P < 0.005$). (C) Control cells or cells transfected with myc-Kal-GEF2 were visualized as in panel B.

domains is likely critical to their function, allowing the coordinated control of multiple RhoGTPases [1,7].

The Kalirin GEF domains bind to RhoGTPases and the isolated GEF1 domain catalyzes nucleotide exchange on Rac1 and RhoG [8–10]. Kal-GEF1 is dominant in superior cervical ganglion cultures, as exogenous Kal-GEF1 produces initiation of new axons and Kal-GEF2 is without effect on axon initiation [10]. Likewise, Kal7, an isoform containing GEF1, but lacking GEF2, plays a role in Ephrin-induced spine formation in cortical neurons that requires its GEF activity [11]. In contrast, expression of exogenous Kal-GEF2 in cortical neurons causes axons to lengthen in a RhoA-dependent manner while expression of Kal-GEF1 causes the Rac1-dependent retraction of processes [12]. In this example, Kal-GEF2 dominates the morphological response, suggesting that the opposing

morphological activities of the two GEF domains are controlled. Thus, the response of cells to Kalirin will be dependent upon the RhoGTPase-effector systems and Kalirin binding proteins present in each type of cell. In order to compare the activities of the two Kalirin GEFs, we have chosen a hEK-293 cell variant because it allows consistent scoring of morphological responses and because its high transfection efficiency allows evaluation of the mechanism underlying the morphological responses.

Our experiments indicate that Kal-GEF1 is an active GEF, but has GEF-independent activities that may be just as important as its ability to activate RhoGTPases. While the catalytic activity of most GEFs is clearly essential for their effects on cell morphology and transformation (e.g., [13]), GEF-independent activities of RhoGEFs are not unprecedented. For example, an αPIX mutant devoid of exchange factor activity still activates Pak, and the RhoGEF Vav plays a role in the maturation of myeloid cells that does not require its GEF activity [14,15]. GEFs may act through non-catalytic interactions with RhoGTPases. RhoGEFs such as Lfc, Ost/Dbs, and Kal-GEF2 bind to selected GTPases without catalyzing nucleotide exchange [12,16].

Members of the Kalirin/Trio family are also known to have GEF-activity-independent effects. Trio-GEF1 binds Filamin A, a protein involved in cross-linking actin filaments [17], and Trio-GEF1-mediated membrane ruffling in fibroblasts requires Filamin A, but not the catalytic activity of Trio [18]. Tara, another actin binding protein, also interacts with Trio-GEF1, although the relationship of this interaction to exchange activity is unknown [19]. Trio-GEF1 mutants lacking GEF activity activate JNK kinase by an unknown mechanism [20]. Worms expressing an Unc-73 mutant devoid of GEF activity toward Rac show a pathfinding error that lacks complete penetrance [21]. While this is attributed to parallel pathways, another explanation is GEF-independent effects of Unc-73 [21]. Finally, dTrio-GEF2 does not activate any of the six *Drosophila* RhoGTPases [7], suggesting that it functions in a manner independent of its GEF activity, or may require accessory factors for activity. In this study, we found that Kalirin GEF1 can induce lamellipodia independent of its GEF activity.

Experimental procedures

Cell culture and transient transfection

pEAK Rapid cells (Edge Biosystems, Gaithersburg, MD) were maintained in DMEM:F12 medium containing 200 U/ml penicillin G, 20 μg/ml streptomycin sulfate, 25 mM HEPES, and 10% fetal bovine serum and were passaged weekly. Transient transfections were performed by mixing 300 μl Opti-MEM (Life Technologies) with plasmid (0.02–0.1 μg) and 300 μl Opti-MEM with Lipofectamine 2000 (Invitrogen; Carlsbad, CA; 2 μl) for each well of a twelve-well plate. After 30 min at room temperature, the plasmid and lipid mixtures were combined and applied to cells. After 4–6 h, cells were rinsed twice with Opti-MEM and incubated in DMEM:F12, 10% serum. After 1 day, cells were incubated in serum-free DMEM:F12 containing insulin–transferrin–selenium (Invitrogen), HEPES buffer, penicillin–streptomycin, plus 1 mg/ml bovine serum albumin (BSA; fatty acid free) for 24 h prior to extraction or fixation.

Construction and sources of plasmids

Several plasmids were used for transfection experiments. Kal-GEF1 (residues S^{1271}–K^{1575}) and Kal-GEF2 (residues A^{1850}–V^{2222}), were generated by PCR and introduced with a His-Myc tag into the pEAK10 vector (Edge Biosystems; Gaithersburg, MD; numbers are according to accession number AAF66019, Kal12a) [10]. ΔKal7 was subcloned into pEAK10 containing a His-Myc-tag [4]. A mutant Kal-GEF1 plasmid was constructed by PCR mutagenesis to introduce N1427A and D1428A mutations based on studies of Dbl [GEF1(ND/AA)] [16]. A fragment containing these mutations was subcloned into ΔKal7 [Peak10.ΔKal7(ND/AA)]. pEGFP.GEF1 was generated by subcloning Kal-GEF1 into pEGFP.N2 (Clontech; Palo Alto, CA). Trio-GEF1 (residues G^{1211}–K^{1550}; accession number NM_007118) was generated by PCR and subcloned into PEAK10 with a His-Myc tag. The PakAID expression construct was generated by PCR from human Pak (amino acids 83–149) and subcloned in frame with GFP into the pEGFP.N2 vector. All plasmids generated by PCR were verified by DNA sequencing. Other plasmids used in transfection were pEGFP.N2, pEAK10, PEAK10.βGal (Edge Biosystems), a constitutively active mutant of human Rac1 [pCEV.Rac1(Q61L); ca-Rac, a gift from Dr. Richard Cerione (Cornell University, Ithaca, NY)], and a dominant negative Rac1 mutant [pCEV.Rac(T17N); dnRac1, a gift from Dr. Silvio Gutkind (National Institute of Dental Research, National Institutes of Health, Bethesda, MD)]. Wild-type (pEBB.HA-Pak1) and mutant (pEBB.HA-Pak1-kd) human Pak1 with a C-terminal HA-tag were gifts from Dr. Bruce Mayer (University of Connecticut Health Center, Farmington, CT). pEBB.HA-Pak1-kd contains a K299R mutation that inactivates Pak kinase [22,23]. An expression construct for dominant negative N-WASP with the verprolin-homology, Cofilin-homology, acidic (VCA) domain deleted and pGEX.Pak-PBD (residues 67–150) domain were also gifts from Dr. Bruce Mayer [24]. The vector encoding GFP-actin was from Clontech.

Immunostaining

Cells were plated in 12-well plates which were prepared by exposure to UV light for 30 min, coating with polylysine (0.1 mg/ml) for 5 min and rinsing with DMEM:F12. After transient transfection, cells were fixed in 4% formaldehyde/

phosphate buffered saline (PBS), permeabilized with PBS/0.075% Triton X-100, and blocked with 2 mg/ml BSA/PBS [25]. Each well was incubated with one of the following antibodies diluted in block buffer as indicated: myc monoclonal antibody (9E10) [26], Rac (BD Biosciences), Phospho-Pak(P-Thr^{423}) (Rockland Immunochemicals; Gilbertsville, PA), Wave2 (Upstate USA; Charlottesville, VA), N-WASP [24], or Cortactin (Abcam; Cambridge, MA). Samples were washed with PBS and incubated with a goat-anti-rabbit- or goat-anti-mouse fluorescein isothiocyanate conjugate (CalTag Laboratories; Burlingame, CA; 1:500), goat-anti-rabbit-Cy3 (Santa Cruz), goat-anti-rabbit ALEXA-350 (Molecular Probes; Eugene, OR), and/or TRITC-phalloidin (Sigma, St. Louis, MO; 25 ng/ml). After washing in PBS, cells were visualized with a Nikon epifluorescence microscope using a 20× or 40× objective, and digital images were recorded using a Spot CCD camera (Diagnostic Instruments; Sterling Heights, MI) or a Hamamatsu digital camera and Improvision Openlabs 3.1.7 software. For deconvolution, images were processed using Volocity 3.0 (Improvision; Lexington, MA). The morphology of transfected cells was scored in a double-blinded manner. Images were recorded and at least 50 cells from six images and two or more experiments were scored. The fraction of cells having each of 4 morphologies was calculated for each field of cells. Samples were compared by a rank-order Kruskal–Wallis test to determine significant differences (http://faculty.vassar.edu).

Time-lapse video microscopy

pEAK Rapid cells were transfected with GFP actin or co-transfected with Kal-GEF1 or KAL-GEF1 (ND/AA) and after 24 h placed in CFSM with 25 mM HEPES buffer without phenol red. Time-lapse images were recorded every 15 s for 20 min using a heated stage (37°C).

Western blot analysis

Cell extracts were fractionated on 4–15% gradient SDS-PAGE gels (BioRad; Hercules, CA) and transferred to PVDF membranes (New England Nuclear; Boston, MA). Blocking, incubating with antibodies, washing, and visualization with ECL reagent (Amersham; Piscataway, NJ) were as previously described [25]. Pak1 polyclonal antiserum was from Santa Cruz (La Jolla, CA). Polyclonal antibody (JH2581) to the Kalirin spectrin domains was previously described [9]. Monoclonal Rac antibody was from BD Biosciences (San Jose, CA). Densitometry was performed using NIH Scion Image software.

In vitro assays of GEF activity

The activity of purified Kalirin proteins was assayed by following the release of the methylanthraniloyl analog of GDP (GDP-MANT) from loaded GST-Rac1 with modifications of a previously described assay [27,28]. GST-fusion proteins (Rac1) expressed in *E. coli* were purified as described [10], dialyzed against 50 mM HEPES, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.6, to remove any bound nucleotide, and then dialyzed against 50 mM HEPES, 100 mM NaCl, 1 mM DTT. His-tagged Kalirin proteins were purified from transfected pEAK-Rapid cells using His-bind resin as described by the manufacturer (Novagen, Madison, WI), with elution in buffer containing 300 mM imidazole.

Before each assay, the dialyzed GST-Rac1 protein (5–67 μM) was loaded with GDP-MANT (50–100 μM) in a volume of 80 μl. Reactions were supplemented to 10 mM $MgCl_2$ and unbound GDP-MANT was removed using a G50 NICK™ column (Pharmacia) equilibrated and eluted in reaction buffer containing 10 mM $MgCl_2$. Fluorescence was measured using excitation at 355 nm and recording emission at 460 nm using the Wallac Victor^2 1420 Multilabel 96-well plate reader. Reactions were initiated by adding GTP to 800 μM followed by 20 μl of purified GEF in reaction buffer; the order of addition could be reversed without effect. Initial velocities were determined by subtracting the buffer blank. Kinetic constants were derived by fitting data to an Eadie–Hofstee plot [29]. Significant differences were determined using a *t* test.

Effector-binding GTPase activation assays

Activation of Rac in pEAK Rapid cells transfected with Kalirin plasmids was analyzed using GST-effector protein binding assay kits (Upstate Biosystems, Lake Placid, NY). For Rac activation assays, cell supernatants prepared in MLB buffer (25 mM HEPES, 150 mM sodium chloride, 1% Nonidet P40, 10 mM magnesium chloride, 1 mM EDTA,10% glycerol, 0.3 mg/ml phenylmethyl sulfonyl fluoride [PMSF], 1.0 mM sodium vanadate, and protease inhibitors [pH 7.5] [30]) were incubated with glutathione-agarose beads containing immobilized GST-Pak-PBD (residues 67–150; 10.0 μg); unbound protein was removed with 3 washes of MLB buffer. Extract (50 μl) was incubated with 10 mM EDTA and 0.1 mM GTPγS (positive control) or 1 mM GDP (negative control) for 20 min at 30°C, cooled and brought to 10 mM magnesium chloride prior to incubation with GST-effector protein bound to glutathione-agarose beads. Bound Rac was analyzed following denaturation and Western blot analysis and quantitation by densitometry. Transfection of myc-Kal-GEF1 was confirmed by Western blot analysis.

Pak kinase assays

Pak kinase assays were performed as described [22]. Cell extracts (400 μl) were prepared from transfected pEAK Rapid cells (35 mm dish) using TMT buffer (20 mM sodium TES, 10 mM mannitol, 1% Triton X-100), 0.3 mg/ml PMSF, and 1.0 mM sodium vanadate. Endogenous Pak was

immunoprecipitated by incubation of extract (200 μl diluted with 900 μl MLB buffer) with 50 μl Protein A Sepharose beads (Sigma) containing 10 μl of Pak1 antibody (Santa Cruz) for 2 h. Beads were washed twice with MLB buffer and three times with KLB buffer (25 mM Tris, pH 7.4, 150 mM sodium chloride, 5 mM EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and 10% glycerol). Immunoprecipitated Pak–antibody complexes were incubated with 100 μg of Histone H4 in 50 μl of kinase assay buffer (50 mM HEPES, pH 7.4, 10 mM magnesium chloride), 100 μM ATP, and 10 μCi [^{32}P]γ-ATP for 15 min at 30°C. Reactions were quenched by boiling in SDS-PAGE sample buffer. Samples were fractionated by SDS-PAGE, discarding the portion below ~5 kDa, transferred to PVDF membranes, and exposed to X-ray film. Band intensities were densitized, quantified using Scion Image (NIH), and plotted. Statistical analysis of Pak kinase assays was performed using nonparametric one-way ANOVA with a Newman–Keuls algorithm to determine significant differences.

Co-immunoprecipitation of Kalirin-GEF1 with Pak and Filamin A

For Pak co-immunoprecipitation experiments, pEAK Rapid cells were transiently transfected with pEBB.HA-Pak (1.0 μg)[22] and pEAK vectors encoding various Kalirin proteins (0.1 μg for Kal-GEFs, 0.5 μg for ΔKal7). Cell extracts prepared in MLB buffer were immunoprecipitated with an HA antibody (12CA5) [31] immobilized on Protein G-agarose (Sigma, St. Louis, MO), washed three times with MLB buffer and then eluted by boiling into SDS-PAGE sample buffer and analyzed by Western blotting with myc antibody (9E10) and Pak antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

For Filamin A co-immunoprecipitation experiments, transiently transfected pEAK Rapid cells were extracted in 20 mM PIPES, 2 mM Na_2EDTA, 50 mM NaF, 10 mM $Na_4P_2O_7$,1 mM Na_3VO_4, 1% TX-100, pH 7.5 containing protease inhibitors. Cleared cell lysates were incubated with myc antibody at 4°C for 2 h, debris was removed by centrifugation and supernatants were applied to Protein G agarose. After a 1-h incubation at 4°C, resin was washed twice with extraction buffer containing 0.25 M NaCl, and once with 50 mM NaPi, pH 7.5. Proteins were eluted by boiling into SDS-PAGE sample buffer and analyzed by Western blotting with a myc antibody to verify immunoprecipitation of myc-tagged proteins (4 to 15% gradient gel) and a Filamin A antibody (Chemicon, MAB 1678) (4% gel) to detect co-immunoprecipitation of endogenous Filamin A.

Binding of Kal-GEF1 to the Pak-PDB domain

Cell extracts prepared from pEAK Rapid cells transfected with myc-tagged Kal-GEF1 were incubated in 500 μl MLB buffer with GST or GST-Pak-PBD (10 μg) for 1 h at 4°C with agitation. Debris was removed by centrifugation for 15 min at 12,000 × g and supernatants were incubated with glutathione-agarose for 1 h. The resin was washed 3 times with MLB buffer, and bound proteins were boiled into Laemmli SDS-PAGE sample buffer. Bound proteins were detected by Western blot analysis with a myc antibody.

Results

Kal-GEF1, but not Kal-GEF2, induces formation of lamellipodia

Since the effects of the two RhoGEFs of Kalirin on neurons vary with cell-type and/or developmental stage [10,12], we compared the effects of exogenously expressed Kalirin GEFs on the organization of filamentous actin in a simple system, pEAK Rapid cells (a hEK-293 cell variant). Serum-starved cells were fixed and endogenous filamentous actin was visualized with TRITC-phalloidin, while epitope-tagged Kal-GEFs were localized using a myc antibody. Control non-transfected cells generally had several short processes filled with filamentous actin, with filopodial projections at their tips (Fig. 1B, and higher power image in C; solid arrows); stress fibers were not prominent in these cells and filamentous actin was localized in the perinuclear region and under the plasma membrane. No myc-staining was visible. The morphology of cells expressing green fluorescent protein (GFP) was unaltered (not shown).

Expression of Kal-GEF1 consistently produced a dramatic change in cell shape, with collapse of the filopodial processes and induction of massive lamellipodia in 89% of the transfected cells (Fig. 1, flat and pinwheel bars). Our morphological scoring assay segregates lamellipodia into two groups: those that are flattened sheets with filamentous actin concentrated at the periphery (flat) and those that have discrete spokes of filamentous actin arranged in a pinwheel- or rib-like pattern. Pinwheel lamellipodia (Fig. 1B inset; hatched bars in graph) were significantly induced over control, but were far less prevalent than flat lamellipodia (open arrows; black bars in graph). Consistent with the previously established role of Rac1 in lamellipodial formation (e.g., [32]), the morphology of Kal-GEF1 cells was similar to that of cells transfected with constitutively active (ca) Rac1 (Fig. 1B). Kal-GEF1 was found throughout the cytosol, but could be seen aligned with the filamentous actin in the lamellipodia (open arrows). In contrast, expression of Kal-GEF2 did not alter actin organization when compared to control; stress fibers were not induced. Kal-GEF2 was found throughout the cell and often concentrated in the filopodia, with no noticeable effect on cell morphology. This system and morphological assay allowed us to investigate the mechanism by which Kal-GEF1 affects cell morphology.

Kalirin GEF1 activates Rac and Pak, proteins involved in forming lamellipodia

Results from Fig. 1 and previous studies indicate that Kal-GEF1 activates Rac1, a GTPase known to induce lamellipodia, but its catalytic efficiency toward Rac1 was not determined. We quantified the ability of Kal-GEFs purified from transfected pEAK Rapid cells to activate GST-Rac1 purified from *E. coli* (Fig. 2A). Consistent with its morphological effects on pEAK Rapid cells, Kal-GEF1 activates Rac1 with a K_m of 0.42 μM and k_{cat} of 1.9 ± 0.03 min^{-1}, whereas Kal-GEF2 produced no detectable activation of Rac1 (Fig. 2B). Similar results were obtained in live cell GEF assays and in yeast two hybrid based GEF assays. Kal-GEF1 also activates RhoG, another GTPase known to induce formation of lamellipodia [10,33] (data now shown).

The *Drosophila* ortholog of Kalirin has a strong genetic interaction with *Drosophila* Pak. Pak is a known downstream target of Rac and induces lamellipodia [7,34–37]. The kinase activity of Pak is activated upon the binding of the GTP-bound form of Rac1 or Cdc42 to a sequence near its NH_2-terminus (the PBD) [38,39]. Since Kal-GEF1 activates Rac, we next tested whether the isolated Kal-GEF domains activated endogenous Pak in pEAK Rapid cells. Cells were again transfected with a constitutively active Rac (caRac), Kal-GEF1, or Kal-GEF2. Endogenous Pak was immunoprecipitated and its kinase activity was assayed by incubation with Histone H4 in the presence of [^{32}P]-γ-ATP (Fig. 2C). caRac caused a 2-fold elevation of Histone H4 phosphorylation when compared to control cells; this is consistent with the levels of activation found by other investigators [22,40]. Kal-GEF1 caused an 8-fold activation of Pak kinase activity, while Kal-GEF2 produced no significant activation of Pak kinase.

Several controls were performed to ensure that Pak kinase activity was assessed accurately. Pak antibody bound to Protein A-Sepharose did not phosphorylate Histone H4. The specificity of the Pak antibody was evaluated by Western blot analysis; pEAK Rapid cells showed a single cross-reactive protein of the expected size (65 kDa) (not shown). Immunoprecipitation of Pak from extracts was confirmed by Western blot analysis (not shown). To ensure that kinases in cell extracts were not binding non-specifically to Protein A-Sepharose, extracts from cells transfected with Kal-GEF1

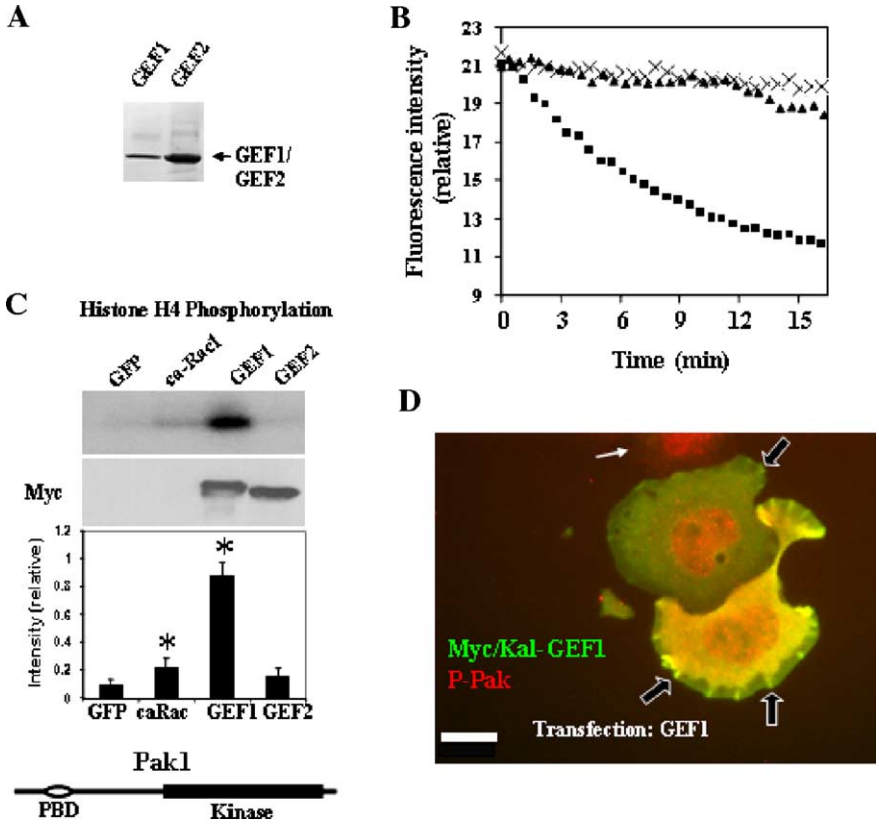

Fig. 2. Kal-GEF1 activates Rac and Pak. (A) Kal-GEF1 (2.0 μg) and Kal-GEF2 (3.2 μg) purified from transiently transfected pEAK Rapid cells using His-Bind Resin were fractionated by SDS-PAGE and stained with Coomassie Blue. (B) Fluorescence-based GEF assays were performed by following loss of fluorescence of GTPase bound GDP-MANT. Reactions contained 50 μM GST-Rac1/50 μM GDP-MANT and buffer (X), 0.670 μM Kal-GEF2 (▲), or 0.670 μM Kal-GEF1(■). (C) Constitutively active Rac1 (caRac1), Kal-GEF1, and Kal-GEF2 were expressed in pEAK-Rapid cells. Endogenous Pak was immunoprecipitated and Pak kinase activity was assayed by measuring phosphorylation of Histone H4; asterisks indicate significant differences from control ($P < 0.001$). The p21-Rac/Cdc42 binding domain (PBD) and kinase domains of Pak1 are drawn to scale. (D) pEAK Rapid cells transfected with Kal-GEF1 were immunostained with myc antibody and phospho-Thr^{423} Pak antibody (P-Pak). Arrow indicates a non-transfected cell and open arrow indicates co-localization of Kal-GEF1 and P-Pak in the spokes of the lamellipodia. Scale bar = 20 μm.

were applied to Protein A-Sepharose in the absence of Pak antibody; no Histone H4 phosphorylation was detected.

We next examined whether activated Pak co-localized with Kal-GEF1 in lamellipodia. Cells transfected with myc-Kal-GEF1 were immunostained with myc antibody and with a Pak antibody specific for P-Thr^{423}, a residue in the activation loop of the kinase domain that is phosphorylated when Pak is activated [35]. Phospho-Pak co-localized with Kal-GEF1 in the spokes of the lamellipodia (Fig. 2D). Together, these experiments suggest that Kal-GEF1 induces lamellipodial formation through activation of Rac1 and hence its downstream effector, Pak.

Kalirin GEF1 induces lamellipodial formation through a GEF-activity-independent mechanism

To determine whether activation of Rac1 or RhoG by Kal-GEF1 was necessary for lamellipodial formation, Kal-GEF1 and a dominant negative (dn) Rac1 (T17N) or dominant negative (dn) RhoG (RhoG-F37A) were co-transfected into PEAK Rapid cells and lamellipodial formation was scored as in Fig. 1. Kal-GEF1 induced flat and pinwheel lamellipodia in 89% of the transfected cells (shown again as part of Fig. 3 for comparison). Co-expression of Kal-GEF1 with dominant negative Rac or dominant negative RhoG did not inhibit the formation of flattened or pinwheel lamellipodia (Fig. 3; $P < 0.001$). Transfection of dominant negative Rac1 or RhoG alone did not significantly affect cell morphology. These experiments provide evidence that induction of lamellipodia by Kal-GEF1 does not require Rac or RhoG.

To determine whether Kal-GEF1 was acting through activation of other GTPases, we tested whether its GEF activity was required for induction of lamellipodia. We first generated a Kal-GEF1 mutant lacking catalytic activity. Replacement of an Asn-Asp sequence near the C-terminus of the DH domain in several RhoGEFs reduces GEF activity

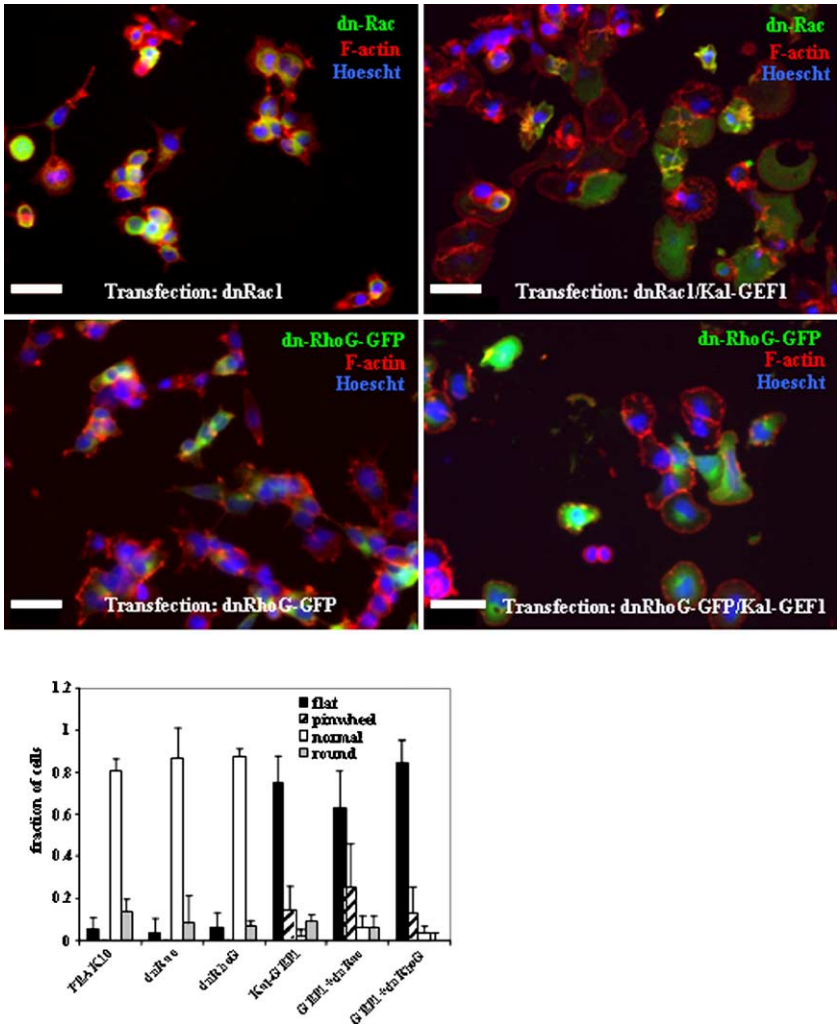

Fig. 3. Lamellipodia induced by Kal-GEF1 are not blocked by dominant negative Rac1 or RhoG. pEAK Rapid cells were transfected with dn-Rac or dn-RhoG-GFP, or co-transfected with Kal-GEF1 as indicated. Cells were immunostained for transfected dn-Rac or visualized directly (dn-RhoG-GFP) (green) and immunostained for filamentous actin (red) and nuclei (blue). Cells co-transfected with dn-Rac (stained green) and Kal-GEF1 are assumed to express Kal-GEF1. Scale bar = 20 μm. Bar graph shows cells scored as in Fig. 1.

~100-fold [1,16,41]. The analogous residues in Kal-GEF1 (N^{1427} and D^{1428}) were each replaced with Ala [GEF1(ND/AA)]. GEF1(ND/AA) showed no detectable activity toward Rac1 in a fluorescence based biochemical GEF assay (Fig. 4A). Since we observed a 50-fold increase over basal activity using equimolar Kal-GEF1, our data indicate that GEF1(ND/AA) has less than 2% of the activity of wild-type Kal-GEF1. This corresponds to a rate of less than one molecule/h/molecule of GEF1(ND/AA). A similar lack of activity for Kal-GEF1(ND/AA) was observed using a Pak-PBD pulldown assay to measure activation of Rac in pEAK Rapid cells (Fig. 4B) [38]. GEF1(ND/AA) did not activate Rac above levels observed in control cells expressing GFP (Fig. 4B), nor did it significantly activate Rac1 or RhoG in yeast two-hybrid-based GEF assays (data not shown) [42].

Knowing that Kal-GEF1(ND/AA) had little if any GEF activity, we next transfected cells with GEF1(ND/AA) and scored them for morphological changes. To minimize a possible contribution from any residual catalytic activity of the mutant GEF domain, transfections were carried out with limiting amounts of plasmid (100 ng plasmid/well). Despite its inability to activate RhoGTPases, expression of Kal-GEF1(ND/AA) in pEAK Rapid cells still induced lamellipodia (flat and pinwheel) in 69% of the transfected cells (Figs. 4C, D). The lamellipodia formed following expression of Kal-GEF1(ND/AA) had a unique phenotype. While cells expressing Kal-GEF1 generally had flattened,

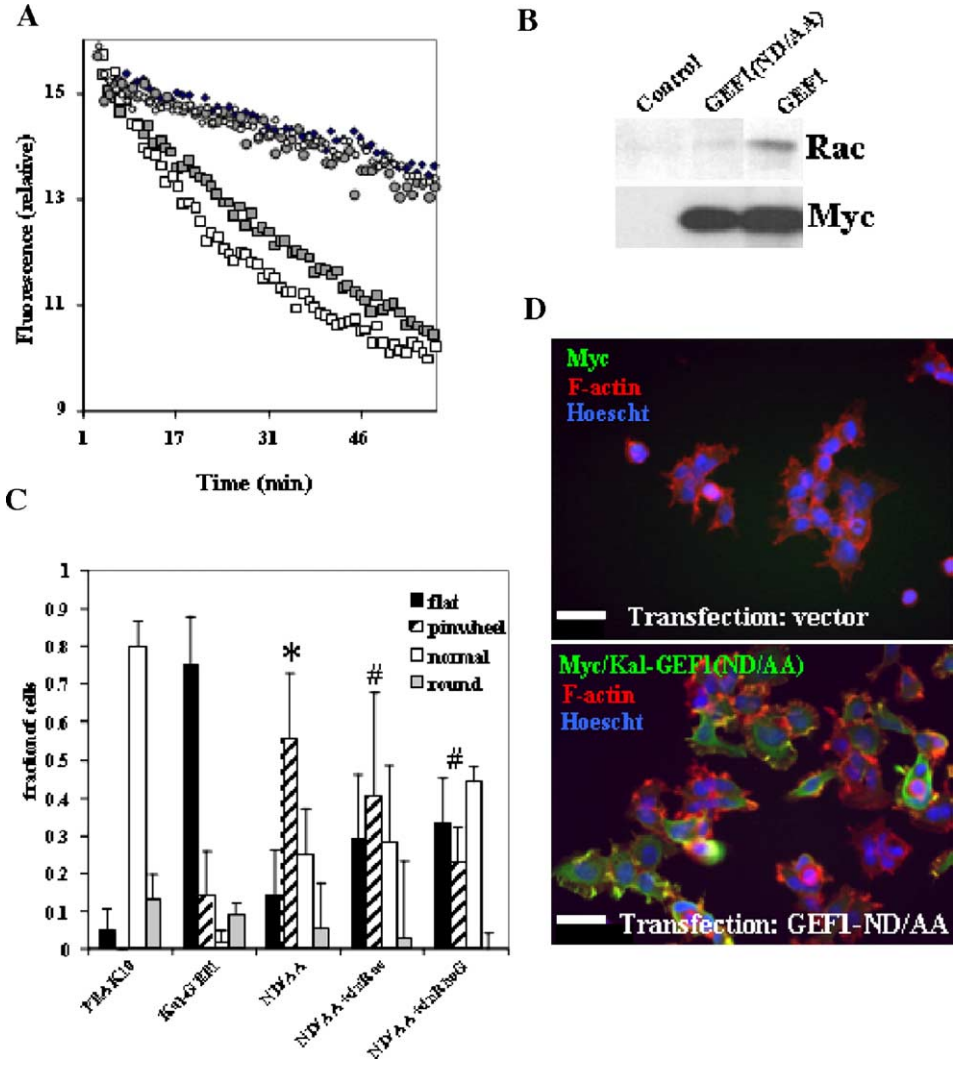

Fig. 4. GEF activity of Kal-GEF1 is not required for induction of lamellipodia. The catalytic activity of Kal-GEF1(ND/AA) was assessed using in vitro GEF assays (A) and effector binding assays (B). (A) For in vitro GEF assays, 30 μM Rac1/GDP-MANT was incubated with buffer (♦), Kal-GEF1 [170 nM (■) or 670 nM (□)], or GEF1(ND/AA) [170 nM (●) or 670 nM (>)]. (B) Cell-based GEF assay measuring levels of activated endogenous Rac following transfection of Kal-GEF1 or Kal-GEF1(ND/AA) (0.1 μg DNA). Western blots show equal levels of expression of Kal-GEF1 and GEF1(ND/AA) (myc antibody). GFP was used as the control. (C, D) Cells were transfected with the constructs indicated (0.5 μg dnRac or dnRhoG; 0.1 μg Kal-GEF) and cell morphology was scored as in Fig. 1; $*P < 0.001$ for induction of pinwheel cells by GEF1(ND/AA) when compared to control cells; $^{\#}P < 0.05$ for pinwheel cells in doubly vs. GEF1(ND/AA) transfected cells. (D) Representative images of control cells and cells transfected with GEF1(ND/AA) stained as in Fig. 1 are shown. Scale bar = 20 μm.

rather than pinwheel lamellipodia (5:1 ratio), cells expressing Kal-GEF1(ND/AA) generally had pinwheel lamellipodia (1:4 ratio; $P < 0.001$). These results demonstrate that the GEF-dependent and GEF-independent mechanisms of lamellipodial formation produce different structures.

Like wild-type Kal-GEF1, Kal-GEF1(ND/AA) localized with filamentous actin in the lamellipodial structures, concentrated in actin-rich spokes (Fig. 4D). As expected, formation of lamellipodia in response to GEF1(ND/AA) was not inhibited by co-expression of dnRac1 or dnRhoG, with 69 and 57% of cells having lamellipodia, respectively (Fig. 4C). Co-expression of dnRac1 or dnRhoG did reduce the fraction of cells with pinwheel lamellipodia in comparison to cells expressing Kal-GEF1(ND/AA) alone ($P < 0.05$). Collectively, these experiments indicate that GEF activity is not required for Kal-GEF1 to induce lamellipodia.

Induction of pinwheel lamellipodia by inactive Kal-GEF1 requires Pak kinase activity

Another Rho GEF, αPIX (Pak interacting exchange factor), activates Pak by both GEF-activity-dependent and GEF-activity-independent processes [15,34,36]. To determine whether Pak plays a role in the GEF-activity-independent lamellipodial formation induced by Kal-GEF1(ND/AA), we asked whether expression of dominant negative Pak could block this activity. A Pak expression construct containing a K299R mutation known to abolish kinase activity (Pak-kd) was used [23]. Cells expressing Pak-kd alone were similar in morphology to cells transfected with empty vector (Fig. 5A). As noted above, cells transfected with Kal-GEF1(ND/AA) produced primarily pinwheel lamellipodia. Co-transfection of Kal-GEF1(ND/AA) and Pak-kd virtually eliminated pinwheel lamellipodia

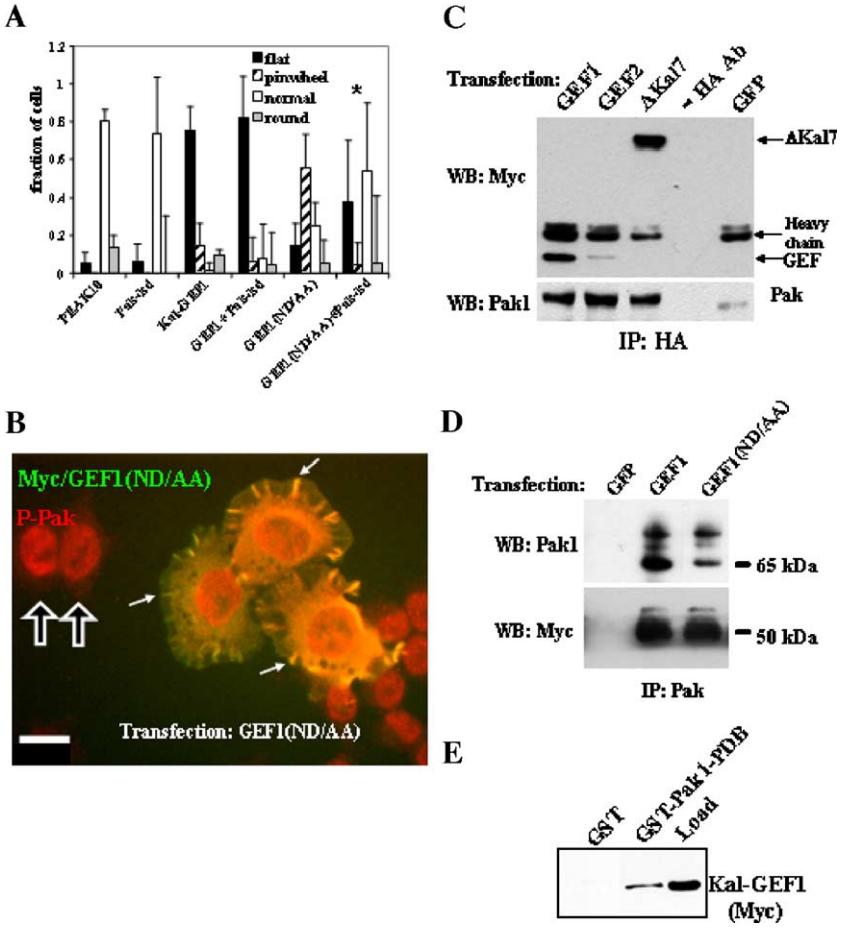

Fig. 5. Pak kinase activity is necessary for lamellipodia induced by GEF1(ND/AA). (A) Cells transfected with the indicated vectors were scored for altered cell morphology as in Fig. 1; *$P < 0.001$ for pinwheel cells in Kal-GEF1(ND/AA) cells with and without Pak-kd. (B) pEAK Rapid cells transfected with a vector encoding myc-Kal-GEF1(ND/AA) were immunostained with myc antibody (green) and P-Pak antibody (P-Pak; red). Arrows indicate actin spokes in the lamellipodia and unfilled arrows indicated non-transfected cells. Scale bar = 20 μm. (C) Kal-GEF1 and ΔKal7 co-immunoprecipitate with Pak. Extracts from cells co-transfected with HA-Pak and myc-Kalirin were immunoprecipitated with an HA antibody and analyzed by Western blotting with a Pak antibody to confirm immunoprecipitation of Pak and with a myc antibody to determine whether Kalirin was co-immunoprecipitated. (D) Kal-GEF1 and GEF1(ND/AA) co-immunoprecipitate with Pak1. Experiment was the same as in panel C except that a Pak antibody was used for the immunoprecipitation. (E) GST-Pak1-PBD assay. Extracts prepared from cells expressing Kal-GEF1 were incubated with GST or GST-Pak1-PBD as indicated; Western blot with myc antibody shows bound epitope-tagged Kal-GEF1.

(Fig. 5A, asterisk). These results indicate that Pak kinase activity is required for induction of pinwheel lamellipodia by Kal-GEF1(ND/AA). Pak-kd, like dnRac, or dnRhoG did not block the appearance of flat lamellipodia induced by active Kal-GEF1 (Fig. 5A).

Kal-GEF1 activation of Pak does not require GEF activity

The inhibitory effect of kinase-dead Pak on pinwheel lamellipodia induced by Kal-GEF1(ND/AA) suggested that GEF1(ND/AA) expression induced activation of Pak. To test this possibility, cells transfected with Kal-GEF1(ND/AA) were immunostained with the phospho-Pak(Thr^{324}) antibody. Staining for phospho-Pak increased in intensity following expression of Kal-GEF1(ND/AA) and phospho-Pak co-localized with GEF1(ND/AA) in the spokes of the lamellipodia (Fig. 5B, arrows). Non-transfected cells showed weak P-Pak immunostaining in the cytosol (unfilled arrows), with stronger staining in the nucleus. These results suggest that Kal-GEF1 can activate Pak by a pathway that does not require its GEF activity. Active Pak is known to phosphorylate and activate myosin light chain kinase and LIMK, a cofilin kinase. These proteins may play an important role in the mechanism by which lamellipodia are formed independent of the GEF activity of Kalirin.

The immunostaining experiments suggest that Kalirin induces activation of Pak in a GEF-activity independent manner, so we used co-immunoprecipitation to ask whether Kal-GEFs bind to Pak [15]. pEAK Rapid cells were co-transfected with vectors encoding hemagglutinin (HA)-tagged Pak1 [22] and myc-tagged Kal-GEF1 or Kal-GEF2 or GFP. Detergent solubilized cell extracts were immunoprecipitated with an HA monoclonal antibody, fractionated by SDS-PAGE and analyzed by Western blotting (Fig. 5C). Probing with a polyclonal antibody to Pak1 confirmed immunoprecipitation of the Pak protein (Fig. 5C, bottom panel). When the same blot was probed with a myc antibody to detect epitope-tagged Kalirin, Kal-GEF1 was found to co-immunoprecipitate with Pak. Although Kal-GEF2 weakly co-precipitated with Pak, the interaction of Pak with Kal-GEF1 was much more robust. Similar co-immunoprecipitation experiments using a Pak antibody showed that both Kal-GEF1 and GEF1(ND/AA) co-immunoprecipitate with Pak (Fig. 5D); the immunoreactive bands above 75 kDa are phosphorylated Pak and are recognized by a phospho-Pak antibody. ΔKal7, a natural Kalirin isoform that contains GEF1, also immunoprecipitated with Pak, indicating that full-length Kalirin proteins can also interact with Pak. Ras, a soluble cytosolic protein used as a negative control, was not detected in the immunoprecipitates, demonstrating that the interaction of Kalirin with Pak was specific (not shown).

We next asked whether Kalirin might also play a role in the activation of Pak. Pak is activated by binding of Rac1-GTP or Cdc42-GTP to its autoinhibitory PBD (Fig. 2C). Other proteins such as αPIX, DCSAM, and Filamin A bind at or near the PBD domain and activate Pak [15,37,43]. Since Kal-GEF1 co-immunoprecipitated with Pak, induced phosphorylation of the autoactivation loop of Pak in cells, and co-localized with phospho-Pak, we asked whether Kalirin bound to the Pak-PBD domain. We used extracts of pEAK RAPID cells expressing Kal-GEF1 and a myc antibody to evaluate binding of Kal-GEF1 to GST-Pak-PBD resin (Fig. 5E). Kal-GEF1 bound to GST-Pak-PBD but not to GST (Fig. 5E). Collectively, these experiments demonstrate that Kal-GEF1 binds directly or indirectly to Pak-PBD, relieving autoinhibition and activating Pak kinase.

Kal-GEF1 interacts with Pak indirectly through Filamin A

We next attempted to determine whether Kal-GEF1 interacts directly with Pak-PBD by using purified Kal-GEF1 and GST-Pak-PBD. No binding was observed when purified proteins were used, indicating that the interaction is indirect or requires a specific modification (data not shown). Several reports suggested that the interaction of Kal-GEF1 with Pak-PBD might be mediated through the actin cross-linking protein Filamin A (also known as ABP-280), which contains 24 tandem Ig domain repeats. Both Pak and Trio-GEF1 bind to Filamin A; the PH1 domain of Trio binds to a fragment of Filamin A that includes part of tandem repeat 23 and all of tandem repeat 24 while the PBD domain of Pak1 binds to tandem repeat 23 [18,37]. The interaction of Filamin A with Pak induces the kinase activity of Pak [37].

Since the Filamin A binding domain of Trio-PH1 shares 87% amino acid identity with Kal-PH1 and Kal-GEF1(ND/AA) induces activation of Pak in cells, we assessed the ability

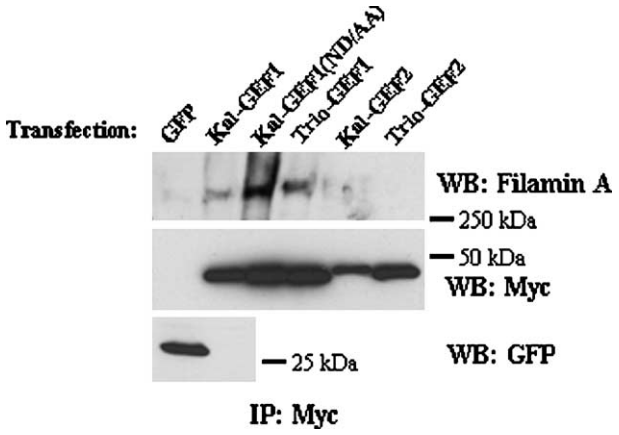

Fig. 6. Kal-GEF1 interacts with Filamin A. Filamin A co-immunoprecipitates with Trio-GEF1, Kal-GEF1, and Kal-GEF1(ND/AA). Extracts of pEAK Rapid cells expressing GFP (control) or the indicated myc-tagged GEF domain were immunoprecipitated with monoclonal antibody to myc. Filamin A was detected in immunoprecipitates by Western analysis with a Filamin A antibody. Western blots showing immunoprecipitation of myc-tagged GEF domains and expression of GFP are shown.

of Kal-GEF1 and Kal-GEF1(ND/AA) to interact with Filamin A. Endogenous Filamin A was readily detected in extracts of pEAK RAPID cells (data not shown). Cells transiently expressing GFP, one of the four GEF domains of Kalirin or Trio, or Kal-GEF1(ND/AA) were immunoprecipitated with a myc antibody and co-immunoprecipitation of Filamin A was assessed by Western blot analysis. Filamin A co-immunoprecipitated with Trio-GEF1 as expected and also with both Kal-GEF1 and Kal-GEF1(ND/AA); no Filamin A was detected in immunoprecipitates prepared from cells expressing GFP, Trio-GEF2, or Kal-GEF2 (Fig. 6) [18]. Immunoprecipitation of Myc-tagged proteins and expression of GFP was confirmed by Western analysis. Together, these results indicate that Kal-GEF1 and Kal-GEF1(ND/AA) exist

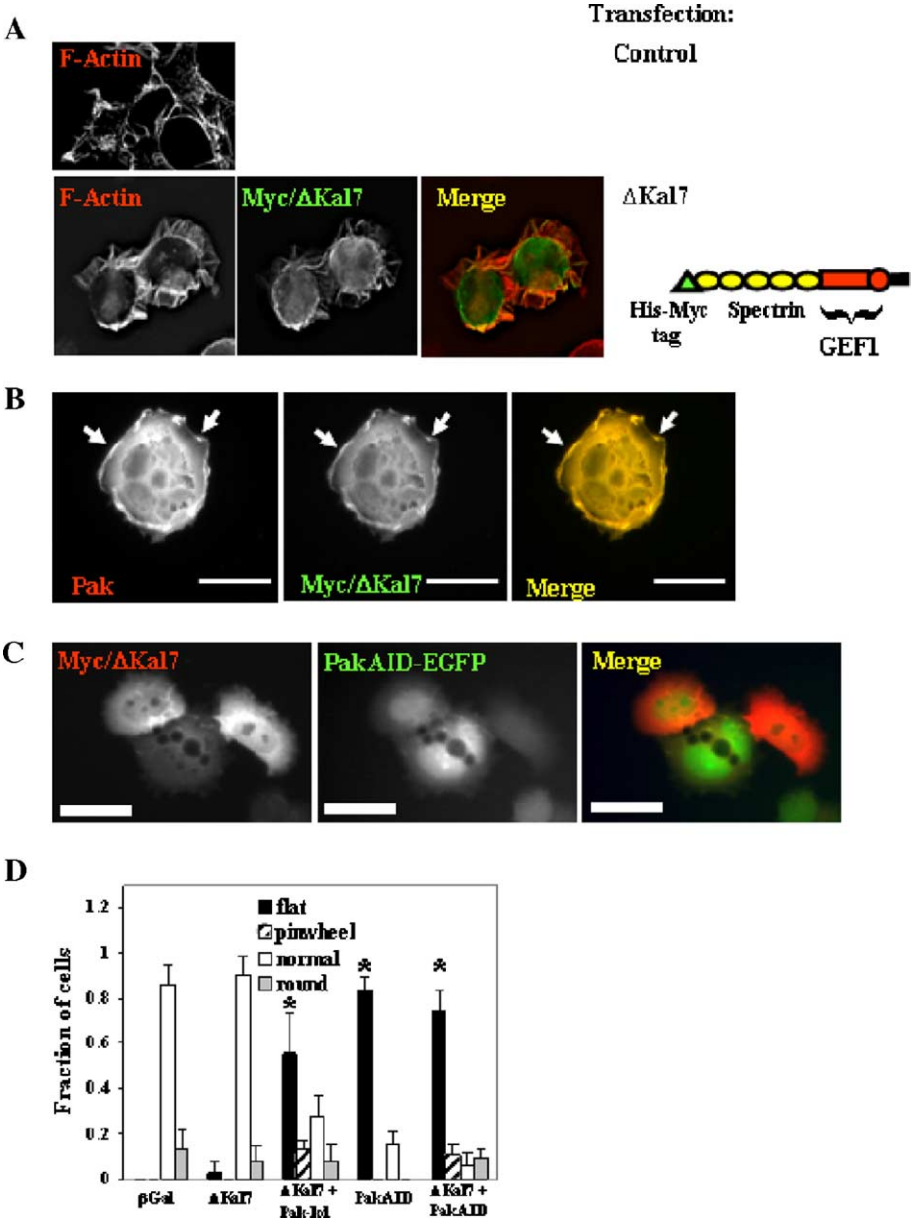

Fig. 7. A natural isoform of Kalirin induces lamellipodia and co-localizes with Pak. (A) PEAK Rapid cells transfected with expression vectors for pEAK10.βGal (control) or myc-ΔKal7 were stained for epitope-tagged Kalirin (green) and filamentous actin (red) as in Fig. 1. Images are snapshots from deconvolved Z-stacks. (B) ΔKal7 co-localizes with Pak in ruffles. pEAK Rapid cells immunostained for transfected myc-tagged ΔKal7 with myc (green) and endogenous Pak1 with a Pak antibody (Santa Cruz; red). Arrow indicated membrane ruffles. Scale bar = 10 μm. (C) Flattened lamellipodia induced by expression of ΔKal7 (red) are not inhibited by co-expression of a Pak-AID/GFP chimera (AID = autoinhibitory domain; green). Scale bar = 10 μm. (D) ΔKal7 induces flattened and pinwheel lamellipodia similar to Kal-GEF1. pEAK Rapid cells were transfected with the expression constructs indicated and scored for cell morphology as in Fig. 1. At least 25 cells were counted for each category and error bars are standard deviations for triplicate observations. Asterisks indicate that flattened lamellipodia were significantly different from control cells transfected with a βGal expression vector, and that co-expression of Pak-kd or PakAID did not significantly inhibit formation of flattened lamellipodia in response to ΔKal7 ($P < 0.05$).

in a complex with Filamin A and Pak, inducing activation of Pak.

A natural Kalirin isoform induces formation of lamellipodia and co-localizes with Pak

We next wanted to know if our experiments with isolated GEF domains reflected the behavior of naturally occurring full-length Kalirin. Kalirin is extensively spliced, producing at least 11 distinct isoforms. ΔKal7, the shortest natural isoform, includes Kal-GEF1 along with 5 spectrin-like repeats and a short C-terminal extension terminating with a functional PDZ binding motif [44]. Expression of ΔKal7 induces the formation of pinwheel lamellipodia (Figs. 7A, D) and flattened lamellipodia (Figs. 7B, C, D). Like Kal-GEF1, ΔKal7 also co-localized with Pak in lamellipodia and in membrane ruffles (Fig. 7B, arrows), and ΔKal7 also co-immunoprecipitates with Pak (Fig. 5C).

We next wanted to assess the role of Pak in the response of pEAK rapid cells to ΔKal7. In addition to the kinase dead Pak vector used to analyze the effects of Kal-GEF1, we used the autoinhibitory domain of Pak fused to GFP (PakAID). PEAK rapid cells expressing ΔKal7 alone or ΔKal7 along with Pak-kd or PakAID were scored for their morphological response (Figs. 7C and D). Cells expressing both ΔKal7 and PakAID continued to exhibit flattened lamellipodia; although the relative amounts of ΔKal7 and PakAID vary in the three cells shown in Fig. 7C; all have flattened lamellipodia. As observed for Kal-GEF1 (Fig. 5A), quantification of these images demonstrates that neither Pak-kd nor PakAID inhibits the formation of flattened lamellipodia in response to ΔKal7. These results support our conclusion

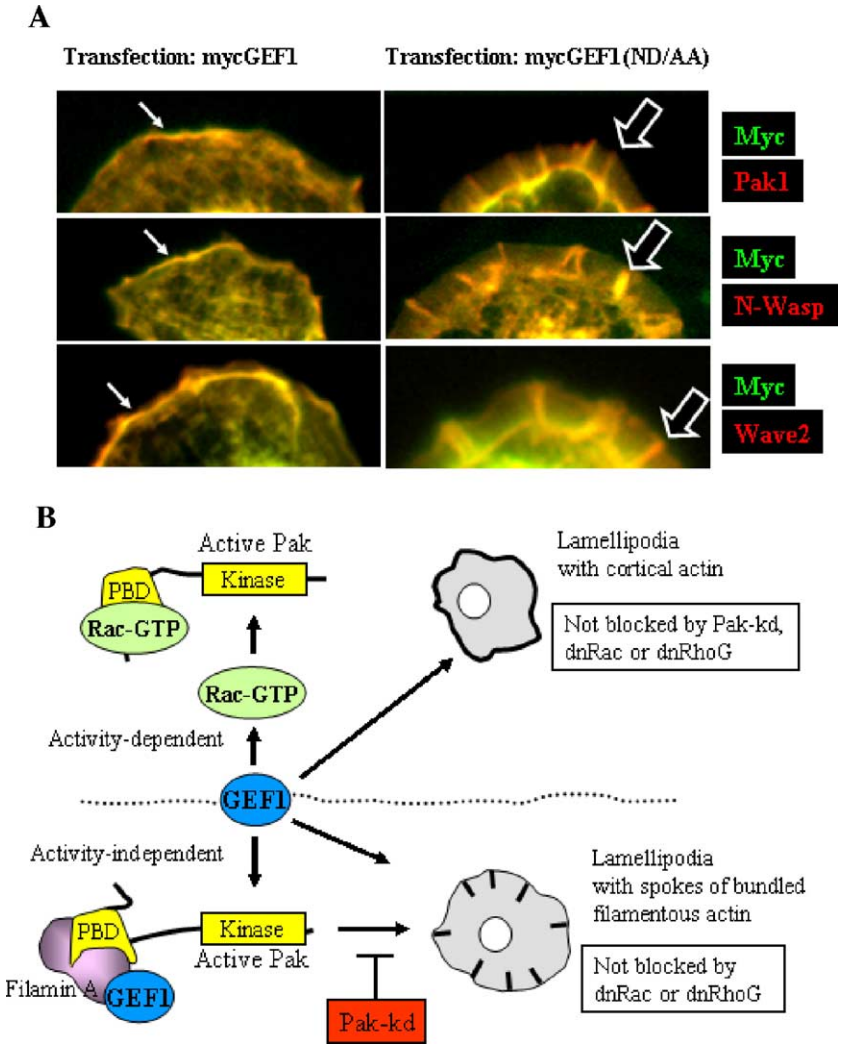

Fig. 8. Kal-GEF1 and GEF1(ND/AA) co-localize with Pak, N-Wasp, and Wave2 in lamellipodia. (A) pEAK Rapid cells were transiently transfected with a plasmid encoding myc-Kal-GEF1 or myc-GEF1(ND/AA) and stained with monoclonal antibody to the myc epitope (green) and polyclonal antibodies to endogenous proteins important for lamellipodial formation (red). Arrows indicate membrane ruffles and unfilled arrows indicate spokes of the pinwheel lamellipodia. (B) Model illustrating Kal-GEF1(ND/AA) activity-independent route to lamellipodial formation.

that Pak does not play an essential role in this morphological response and Kal-GEF1 functions much like a natural isoform of Kalirin.

Lamellipodia induced by Kal-GEF1(ND/AA) share properties with lamellipodia induced by other mechanisms

Since our experiments indicated that Kal-GEF1 induces lamellipodia despite inactivation of its GEF activity, we next explored potential downstream targets. Several pathways are known to affect formation of lamellipodia [35,45–47]. Rac-GTP activates Pak, which can phosphorylate and activate myosin light chain kinase and affect myosin motors. Pak also phosphorylates LIMK, which phosphorylates cofilin, inducing depolymerization of actin. Rac-GTP also binds to IRSp53, linking it to Wave2, a member of the Wiscott–Aldrich Syndrome protein (WASP) family. Wave2, like N-WASP, nucleates actin polymerization through the Arp2/3 complex [45].

To ask whether these proteins are similarly co-localized in lamellipodia induced by Kal-GEF1 and Kal-GEF1(ND/AA), we examined the localization of endogenous Pak, N-Wasp, and Wave2 in transfected cells. Kal-GEF1 co-localized with Pak, N-Wasp, and Wave2 in the lamellipodia, with staining especially concentrated at the leading edge of the lamellipodium (Fig. 8A, arrows). These experiments suggest that the lamellipodia induced by Kal-GEF1 are similar to those induced by Rac1-3, RhoG, and Rab5, in other systems [33,48]. Pak, N-Wasp, and Wave2 also co-localized with Kal-GEF1(ND/AA), most notably in the spokes of the pinwheel lamellipodia (unfilled arrows), with much less staining at the cell periphery. While the same molecules are localized to filamentous actin within flat and pinwheel lamellipodia, the structural organization is strikingly different.

Another way to assess differences between the activities of Kal-GEF1 and Kal-GEF1(ND/AA) is time-lapse video microscopy. Control cells transfected with GFP-actin and exhibiting normal morphology were compared to cells co-transfected with GFP-actin and either Kal-GEF1 or Kal-GEF1(ND/AA) that had pinwheel lamellipodia; cells were examined over a 20-min period (Supplemental data in Appendix A). Control cells formed blebs and exhibited filopodial movement, but little motility. In contrast, cells expressing either Kal-GEF1 or GEF1(ND/AA) and GFP-actin exhibited a rapid centripetal flow of GFP-actin, a known property of lamellipodia [47]. In GEF1(ND/AA) cells, we frequently observed splitting of the actin spokes in the pinwheel lamellipodia, suggesting a difference in actin bundling.

Discussion

Kalirin and its homologues are important in the development and maintenance of actin-rich structures in neurons including growth cones, dendrites, and spines [10,11,44,49]. Since different alternatively spliced Kalirin isoforms having one or two GEF domains are localized to different neuronal structures, we needed a system to systematically investigate the mechanism by which Kalirin affects the actin cytoskeleton. We decided to use a hEK-293 cell variant, pEAK Rapid cells, because they are easily transfectable at high efficiency (>70% in most experiments) and produce consistent morphological responses that are amenable to quantitative scoring. Using this approach, we showed that Kal-GEF1 induced two types of lamellipodia, one requiring its GEF activity and one independent of its GEF activity (Fig. 8B).

Kal-GEF1 activates Rac and Pak, and induces lamellipodia

Kal-GEF1, like a constitutively active mutant of Rac1, strongly induced the formation of lamellipodia, whereas Kal-GEF2 did not significantly affect cell morphology. Rac1 and Pak are known targets of Kal-GEF1 and biochemical assays demonstrate that Kal-GEF1 has a turnover rate of more than 100 molecules/h with Rac1-GDP as its substrate. Furthermore, expression of Kal-GEF1 strongly activated Pak kinase activity, consistent with the fact that Kal-GEF1 activates Rac1, which binds to and activates Pak. Since phosphorylation of Pak in its autoactivation loop at Thr^{423} is important for Pak activation, we consider immunostaining with this antibody to be a measure of activated Pak. Neuronal growth cones, with their prominent lamellipodia, share many features with the lamellipodia formed in non-neuronal cells. Motile dendritic spines contain actin-rich lamellipodia at the tips of spine heads [50]. These results are consistent with the ability of a cell permeant peptide inhibitor of Pak activity to block Kalirin-induced spine formation [11].

Kal-GEF1 induces lamellipodia by two pathways

While investigating the mechanism by which Kal-GEF1 induces lamellipodial formation, we were surprised to find that neither dominant negative Rac1 nor dominant negative RhoG blocked this response. Since these dominant negative Rho GTPases were highly expressed, these results suggested that another RhoGTPase was the target of Kal-GEF1, or that GEF activity was not required for the response. Based on studies of other RhoGEFs [1,16], an ND/AA mutation was introduced into Kal-GEF1, eliminating its exchange activity [1,16,41]. Expression of Kal-GEF1(ND/AA) still induced formation of lamellipodia, demonstrating the existence of a GEF-activity-independent pathway.

Kal-GEF1 induces different types of lamellipodia

There was a distinct difference between the flat lamellipodia with cortical actin prevalent in cells expressing

caRac or Kal-GEF1 and the lamellipodia with prominent actin spokes or ribs prevalent in cells expressing GEF1(ND/AA). One possible explanation is that the pinwheel lamellipodia formed in response to Kal-GEF1(ND/AA) may serve as precursors to the flattened lamellipodia observed in cells transfected with ca-Rac1 or Kal-GEF1. Kal-GEF1 co-localized with filamentous actin, N-Wasp, Wave2, Pak1, and phospho-Pak in ruffles at the edges of the lamellipodia. The same proteins co-localized with Kal-GEF1(ND/AA) in the actin-rich spokes of the pinwheel lamellipodia. The simplest explanation is that Kal-GEF1(ND/AA) cells lack a factor that converts actin-rich spokes or ribs into cortical actin localized to the edge of the lamellipodium (Fig. 8B). The missing factor would presumably be dependent on activation of a RhoGTPase because fewer cells with flattened lamellipodia are induced by Kal-GEF1(ND/AA). However, these two types of lamellipodium may arise through completely different mechanisms and additional studies will be needed to determine how the two types of lamellipodia relate to each other.

The role of PAK in lamellipodia induced by Kal-GEF1

Several pieces of evidence identify Pak as an important player in both pathways by which Kalirin induces lamellipodia. Kal-GEF1 strongly activated the kinase activity of Pak based on in vitro kinase assays and expression of either Kal-GEF1 or Kal-GEF1(ND/AA) resulted in co-localization of phospho-Pak with filamentous actin and the myc-tagged GEF in lamellipodia. However, expression of Pak-kd failed to block lamellipodial formation in response to Kal-GEF1, indicating that Pak kinase was not required. Thus, while Kal-GEF1 clearly activated both Rac and Pak, neither of these proteins alone was necessary for lamellipodial formation. Kal-GEF1 may activate another RhoGTPase that induces lamellipodia (e.g., TCL, Rac2, Rac3, or Cdc42). If a cell expresses redundant Rho GTPases, there may be no single RhoGTPase that is essential for lamellipodial formation [33]. This may also explain why Pak is also not required for induction of flattened lamellipodia.

Unlike Kal-GEF1, Pak kinase activity is essential for Kal-GEF1(ND/AA)-induced pinwheel lamellipodia. Pak complexes with Kal-GEF1, as evidenced by co-immunoprecipitation and GST-Pak-PBD binding assays using cell extracts containing Kal-GEF1. In addition, both Pak and phospho-Pak are co-localized with Kal-GEF1(ND/AA) in the actin-rich spokes of lamellipodia. However, the purified proteins did not interact in in vitro binding experiments (data not shown), suggesting an indirect interaction. The interaction of Kal-GEF1 with Pak may be mediated through the actin cross-linking protein Filamin A. Filamin A was previously shown to bind the GEF domain of Trio and the CRIB domain of PAK, and our experiments show that Kal-GEF1 and the GEF1(ND/AA) mutant co-immunoprecipitate with Filamin A [18,37]. Since Filamin A can activate Pak in a manner that does not require GTPases, a ternary complex between GEF(ND/AA), Filamin A, and Pak provides a likely mechanism for GEF(ND/AA)-mediated activation of Pak kinase. Consistent with the effects of Filamin A on Pak activity, recruitment of Pak to the membrane is an event known to activate Pak in a GEF-independent manner [51]. The mechanism by which Kal-GEF1 activates Pak is distinct from that of αPIX, another RhoGEF. The SH3 domain of αPIX binds to the Pro-rich region of Pak, activating Pak through both GEF-dependent and GEF-independent mechanisms [15].

Pak activates multiple pathways influencing the cytoskeleton

Expression of constitutively active Pak induces lamellipodia [52,53]. Pak phosphorylation activates the regulatory myosin light chain subunit and inhibits myosin light chain kinase. Pak phosphorylates and activates LIM kinase, which can phosphorylate cofilin, reducing its affinity for F-actin. Pak phosphorylates the microtubule catastrophe factor stathmin, the focal adhesion complex scaffold paxillin, and the intermediate filament protein vimentin; each of these could play a role in the adhesive and cytoskeletal dynamics involved in lamellipodial formation [54]. Kal-GEF1(ND/AA)-induced activation of Pak could affect lamellipodial formation through several of these downstream pathways [35]. The interactions of Kalirin and Pak with Filamin A could modulate GEF-activity-dependent and -independent effects on all three major cytoskeletal elements. The GEF-dependent and GEF-independent induction of lamellipodia by Kalirin are both likely to play important roles in the formation, organization, and plasticity of neuronal structures. Furthermore, GEF-activity-independent interactions with downstream effectors may be a general property of RhoGEFs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2005.03.024.

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