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Autonomous functions for the Sec14p/spectrin-repeat region of Kalirin

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Abstract

Kalirin is a GDP/GTP exchange factor (GEF) for Rho proteins that modulates the actin cytoskeleton in neurons. Alternative splicing generates Δ -isoforms, which encode the RhoGEF domain, but lack the N-terminal Sec14p domain and first 4 spectrin-like repeats of the full-length isoforms. Splicing has functional consequences, with Kal7 but not Δ Kal7 causing formation of dendritic spines. Cells lacking endogenous Kalirin were used to explore differences between these splice variants. Expression of Δ Kal7 in this system induces extensive lamellipodial sheets, while expression of Kal7 induces formation of adherent compact, round cells with abundant cortical actin. Based on *in vitro* and cell-based assays, Kal7 and Δ Kal7 are equally active GEFs, suggesting that other domains are involved in controlling cell morphology. Catalytically inactive Kal7 and a Kalirin fragment which includes only Sec14p and spectrin-like domains retain the ability to produce compact, round cells and fractionate as high molecular weight complexes. Separating the Sec14p domain from the spectrin-like repeats eliminates the ability of Kal7 to cause this response. The isolated Sec14p domain binds PIP₂(3,5) and PIP₃, but does not alter cell morphology. We conclude that the Sec14p and N-terminal spectrin-like domains of Kalirin play critical roles in distinguishing the actions of full-length and Δ -Kalirin.

Keywords

alternative splicing; guanine nucleotide exchange factor; cytoskeleton; transferrin uptake; phosphatidylinositol; Kalirin 4

INTRODUCTION

Kalirin is a GDP/GTP exchange factor (GEF) for small GTP binding proteins of the Rho subfamily and plays an essential role in establishing and maintaining neuronal morphology. Reduced Kalirin levels block axon initiation and outgrowth from sympathetic neurons, and reduce dendritic complexity and linear spine density in mature hippocampal pyramidal neurons

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[1,2]. Over-expression of Kalirin increases axon initiation and outgrowth in sympathetic neurons and increases linear spine density in hippocampal and cortical neurons [1,3].

Studies aimed at elucidating the pathways leading to these dramatic morphological responses have focused on the two RhoGEF domains of Kalirin and the actions of their target Rho GTPases. However, the use of several promoters and alternate 3'-ends produce multiple Kalirin isoforms that include or eliminate domains other than the two RhoGEFs, suggesting that there are other functional attributes of Kalirin proteins that remain to be elucidated. In support of this hypothesis, the expression of many alternatively spliced Kalirin isoforms is tissue-specific and developmentally regulated [4–6].

The major isoform in the adult rat brain, Kal7, has a Sec14p domain, 9 spectrin-like repeats, a single RhoGEF domain and a class I PDZ binding motif (Fig. 1) [3]. Use of a different promoter and an alternate internal translational start site yields Δ Kal7, which lacks the Sec14p domain and the first 4 spectrin-like repeats. Transcripts encoding Δ Kal7 are more prevalent than transcripts encoding Kal7 in the adult rat cortex, but the longer Kalirin protein is more prevalent [4]. The functional consequences of many Kalirin splicing events remain to be explored. Since Kal7, 8, 9, and 12 each have Δ -isoforms, we decided to evaluate the functional significance of this common Kalirin alternative splicing event [4].

Here we show that exogenous Kal7 and Δ Kal7 produce different phenotypes and adopt different subcellular localizations when expressed in cortical neurons or in non-neuronal cells. In exploring the underlying mechanism, we were surprised to find that neither the GEF activity nor the GEF domain of Kalirin was essential for many of its morphological effects. Differences in the actions of Kal7 and Δ Kal7 arise from unique properties of the Sec14p and N-terminal spectrin-like repeat regions, the domains in Kal7 that are missing in Δ Kal7. The majority of this region is expressed as a unique endogenous transcript designated Kal4.

Both Kal4 and Kal7 oligomerize, are largely insoluble, associate with the cytoskeleton and inhibit endocytosis, whereas Δ Kal7 does not. These different properties are consistent with the observed differences in morphological effect. Like spectrin itself, the Sec14p and spectrin-like repeat region of Kalirin can alter cell shape without any contribution from a GEF domain. In addition to assigning functional significance to the expression of Δ -vs. full-length Kalirin isoforms, our findings provide insight into a family of proteins that contain only a Sec14p domain and spectrin-like repeats [7].

EXPERIMENTAL

Construction and sources of plasmids

Several plasmids were used for transfection experiments; plasmids are numbered using the α -isoform numbering for Kalirin (Genbank U88157). The cDNAs for rat Kalirins 4, Δ 7 and 7 were subcloned into the pEAK 10 vector (Edge Biosystems; Gaithersburg, MD) with an N-terminal His-Myc tag [8]. Vectors encoding GST-KalSpec(4–7) and GST-KalSpec(4–6) were described previously [9]. Vectors encoding GST- Δ Kal7 and GST-KalSpec5 were constructed from the corresponding expression vectors [10]. Kal7(ND/AA) was generated by moving a fragment containing the N1415A and D1416A mutations from pEAK10.Kal-GEF1 into pEAK10.HisMyc.Kal7 (numbers are according to Accession # AAF66019, Kal12a) [1]. pCIneo.HisMycKalSec14p terminates with -LDYNH¹⁶². pCIneo.HisMycKalSec14pSpec_{1–4} terminates with -RKLLLD623. pCIneo.HA₂KalSpec_{1–4} has a dual HA tag fused to spectrin repeat 1 (E¹⁶³EWIE-) and terminates with -RKLLLD⁶²³. pEAK10.HisMyc.KalSolo terminates with -ARKKEF¹²⁵⁷. pCIneo.HA₂ Δ Sec14pKal7 has the same NH₂-terminal as pCIneo.HA₂KalSpec_{1–4}, but extends to the STYV1636 at the end of Kal7. All plasmids generated by PCR were verified by DNA sequencing. Kal-GEF1 (residues S¹²³¹-K¹⁵⁷⁵) and

psCEP.Kal7 Δ CT (lacking the last 60 amino acids of Kal7) were previously reported [3,11, 12]. Other plasmids used were pEGFP-N2 (Clontech; Palo Alto,CA) and pEAK10 (Edge Biosystems).

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 μ g/ml streptomycin sulfate, 25 mM HEPES, and 10% fetal bovine serum. Transient transfection was performed by mixing 330 μ l Opti-MEM (Life Technologies) with plasmid (0.1–2.4 μ g) and 330 μ l Opti-MEM with Lipofectamine 2000 (2 μ l per μ g DNA; Invitrogen; Carlsbad, CA). After 20–30 min at room temperature, the plasmid and lipid mixtures were combined and applied to cells. After 4–6 hr the DNA/lipid mixture was removed, cells were incubated in DMEM:F12, 10% serum for 1 day, and then 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 hr.

Primary cortical cultures were prepared from P1 rat pups as described [1,2]. Vectors encoding HisMycKal7 or HisMyc Δ Kal7 were introduced into dissociated cells using the rat neuron nucleofection kit (Amaza GmbH, Germany) as described [13]. Nucleofected neurons were plated, allowed to recover in DMEM for 3 hours and kept in maintenance medium for up to 3 weeks.

Immunostaining

Cells were plated in 24 well plates prepared by treating with UV light for 30 min, coating with polylysine (0.1 mg/ml in water) for 5 min and rinsing with DMEM:F12 containing serum. For some experiments cells were plated onto glass coverslips treated in the same way. After transient transfection, cells were fixed in 4% formaldehyde/phosphate buffered saline (PBS), permeabilized with PBS/0.75% Triton X-100, and blocked with 2 mg/ml BSA/PBS (block buffer) [14]. Each well was incubated with antibodies (9E10 [15], pan-Kalirin JH2581 [8, 16]) as indicated and fluorescently-tagged phalloidin (Sigma, St. Louis, MO; TRITC, 1:4000; FITC, 1:1000) diluted in block buffer. Unbound antibody was removed by washing with PBS, and primary antibody was visualized by incubating with an anti-rabbit-fluorescein isothiocyanate conjugate (Jackson ImmunoResearch, West Grove, PA; 1:500) or an anti-rabbit-AlexaFluor 350 conjugate (Molecular Probes; Eugene, Oregon) as indicated. After washing in PBS, cells were visualized with a Nikon epifluorescence microscope using a 40X objective. Some digital images were recorded using a Spot CCD camera (Diagnostic Instruments; Sterling Heights, MI). Where indicated, confocal images were acquired using a Zeiss LSM510 confocal microscope (Zeiss, Thornwood, NY) as described [1,2,17]. Z-stacks were taken using a 63X objective (0.3 digital zoom factor); images of the entire cell were generated with Zmaris 3.2 software (Bitplane AG, Zürich).

When quantitative data were to be obtained, images of 10 randomly selected fields from all samples were acquired under the same non-saturating conditions. Micrographs were coded and scored blind. For deconvolution, images (0.5 μ m z-step; 15 to 20 μ m thick) recorded using a Hamamatsu digital camera with Openlab 5.0.2 were processed using Volocity 4.1.0 (Improvision; Lexington, MA); individual layers are shown.

Endocytosis of transferrin

pEAK Rapid cells transfected 24 h or 48 h earlier were rinsed with L15 and placed into an air incubator. Cells were then incubated in L15 containing transferrin (50 μ g/ml; Molecular Probes; AlexaFluor488 or AlexaFluor546) for 10 min. Following two quick rinses, cells were fixed as described above. Transfected cells were identified by GFP fluorescence or by staining the epitope-tagged protein as described above; nuclei were visualized with the Hoechst stain.

Western blot analysis and protein solubility

Cell extracts were fractionated on 4–15% gradient SDS-PAGE gels (BioRad; Hercules, CA) or 4–12% NuPAGE gels (Invitrogen) 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 described [14]. The rabbit antibody to the spectrin repeat region of Kalirin (JH2581) and monoclonal antibody to myc were described [8].

pEAK Rapid cells harvested 24 – 48 h after transfection were extracted into 20 mM NaTES, 10 mM mannitol, 1% TX-100, pH 7.4 (TMT). Soluble proteins (S) were separated from insoluble proteins by centrifugation at $14,000 \times g$ for 15 min or by centrifugation at $430,000 \times g$ for 15 min; the pellet (I) was solubilized using Laemmli sample buffer. Aliquots accounting for an equal percentage of each fraction were subjected to Western blot analysis using antibody to myc or HA.

Cells expressing Kal7 or Δ Kal7 were also analyzed using a modification of a protocol developed to extract spectrin from erythrocytes [18]. Cells scraped into spent medium were pelleted and sonicated in ice cold 20 mM NaTES, 10 mM mannitol (0.3 ml/well of a six-well plate). Nuclei and large debris were removed by centrifugation at 1000 rpm for 30 sec; the supernatant was centrifuged at $435,000 \times g$ for 15 min in a Beckman TL-100 centrifuge. The supernatant was removed and the pellet was resuspended and allowed to extract in 0.3 mM Na phosphate, 0.1 mM EDTA, pH 9.5 containing PMSF, X and Na orthovanadate for 30 min of ice. The sample was again centrifuged at $435,000 \times g$ for 15 min and the pellet was solubilized by incubation with SDS lysis buffer at 95°C for 5 min.

GEF assays

The GEF activity of Δ Kal7 and Kal7 was measured using cell-based assays and *in vitro* fluorescence assays. For cell based 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). Lysates prepared in MLB buffer (25 mM HEPES, 150 mM NaCl, 1% Nonidet P40, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, pH 7.5) with 0.3 mg/ml phenylmethyl sulfonyl fluoride (PMSF), 1.0 mM sodium vanadate, and protease inhibitors [19] were incubated with glutathione-agarose beads containing immobilized Pak-CRIB domain (residues 67–150; 10.0 μg) and unbound protein was removed with 3 washes of MLB buffer. Extract (50 μl) 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, chilled and brought to 100 mM $MgCl_2$ prior to incubation with GST-effector protein bound to glutathione-agarose beads. Transfected Kalirin proteins and bound Rac (Transduction Laboratories; R56220; 1:1000) were analyzed by Western blot.

For *in vitro* fluorescence based GEF assays, the activity of purified Kalirin proteins was assayed by following the release of the methylantraniloyl analog of GDP (GDP-MANT) from loaded GST-Rac1 with modifications of a previously described assay [20,21]. GST- Rac1 expressed in *E. coli* was purified using glutathione Sepharose [1], 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-Rho 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 NICKTM column (Pharmacia) equilibrated and eluted

in reaction buffer containing 10 mM MgCl₂. Fluorescence was measured by excitation at 355 nm and recording emission at 460 nm using a Wallac Victor² 1420 Multilabel 96-well plate reader. Reactions were initiated by adding GTP to 800 μM and starting the reaction with 20 μl of Kalirin in reaction buffer. Reaction rates were determined by subtraction of the intrinsic rate of loss of fluorescence (reaction lacking enzyme).

Subcellular Fractionation and Gel Filtration

Parietal cortex from adult rat brain was subjected to subcellular fractionation as described [1, 3]; the efficacy of the separation was verified using antisera to PSD95, synaptophysin, calnexin, NMDAR1 and glutamic acid decarboxylase. To obtain cytosol for gel filtration, adult rat cerebral cortex was homogenized in 320 mM sucrose, 10 mM Tris HCl, 3 mM MgCl₂, 1 mM EDTA, pH 7.0 and centrifuged at $435,000 \times g$ for 20 min; aliquots (1 mg protein) of the soluble fraction were applied to the gel filtration column. Extracts of transfected pEAK Rapid cells prepared using TMT as described above were also applied to the column.

Samples were analyzed on a 1.5×17 cm column of Sephacryl S-400 equilibrated and eluted with 20 mM Hepes, 100 mM NaCl, 0.05% TX-100, pH 7.0. Bovine serum albumin (1 mg) and phenol red (to mark the total volume, V_t) were added to each lysate as internal standards. The column was calibrated using blue dextran (V_0), thyroglobulin, catalase, bovine serum albumin, ovalbumin and cytochrome c; the void volume (V_0) was $0.32 \times V_t$ and BSA eluted at $0.54 + 0.01 (V_{BSA}/V_t)$. For unknowns, the elution positions of BSA and phenol red were determined by monitoring A₂₈₀ and A₅₆₀; elution positions are expressed as V_x/V_t .

Oligomerization of Kalirin

GST-ΔKal7, GST-KalSpec(4–7), GST-KalSpec(4–6) and GST-KalSpec5 bound to glutathione agarose equilibrated with phosphate buffered saline were cleaved with thrombin (1 mg GST-fusion protein; 10 units thrombin); released protein was recovered and pooled with a subsequent wash. After dialysis into 0.1 M NaHCO₃/0.5 M NaCl, recombinant protein was linked to 0.5 ml Affi-Gel 15 (BioRad); unreacted sites were blocked by incubation with 0.1 M ethanolamine HCl, pH 8.0. Linkage efficiency was verified by analyzing unbound protein. Binding assays were carried out in TMT; after incubation at 4°C for 2 h, beads were washed twice with TMT and once with the same buffer lacking TX-100. Bound protein was eluted using Laemmli sample buffer. HisMycΔKal7 expressed in insect cells using the Baculovirus expression system was purified using Talon resin as described (Clontech).

PIP Strips

Lipid strips from Echelon Bioscience (Salt Lake City, UT; P-6001) were blocked with 3% BSA (fatty acid free) in TTBS for 1 h at RT and then incubated overnight at 4 °C with GST or GST-Sec14p (10 μg/ml) in the same buffer. The strips were rinsed 6 times for 5 min in TTBS and incubated for 1 h at room temperature with monoclonal antibody to GST (Upstate). Bound antibody was visualized as described above; images were acquired using a GeneGnome digital imaging system.

RESULTS

Exogenous ΔKal7 and Kal7 produce different phenotypes when over-expressed in cortical neurons

Expression of ΔKal7 and Kal7 in rodent brain is first detectable around post-natal day 14 [1, 3]. To determine whether the different isoforms have different effects on neuronal morphology, dissociated neurons were transfected with vectors encoding ΔKal7 or Kal7 and examined sixteen days later. Low power images reveal ΔKal7 in the cell soma and extending into

dendrites (marked by staining for MAP2, a dendritic marker) (Fig. 1B, C). Exogenous Δ Kal7 is present in the sparse spine-like structures and filopodia observed along the dendrites of transfected cells, but most of the Δ Kal7 remains in the cell soma. In contrast, exogenous Kal7 is concentrated in dendritic processes, with little protein remaining in the cell soma (Fig. 1D, E). Kal7 is especially prevalent in spine-like structures, where it is concentrated at the spine-head. While expression of exogenous Kal7 causes early formation of dendritic spines [1,3], exogenous Δ Kal7 does not; linear spine density is approximately three-fold higher in neurons expressing Kal7 than in neurons expressing Δ Kal7.

Endogenous Δ Kal7 and Kal7 have different properties

We next wanted to investigate the properties of these splice variants that lead to these differing morphologies. Both Kal7 and Δ Kal7 are expressed in the adult rat brain. We first compared their subcellular localizations using standard procedures to prepare fractions enriched in post-synaptic densities, synaptic vesicles and cytosol (Fig. 2A). Based on content of PSD95, a marker for the post-synaptic density, Kal7 is enriched in this fraction (LP1); Δ Kal7 is not. Neither Kal7 nor Δ Kal7 is enriched in the synaptic vesicle fraction (LP2; synaptophysin positive). Δ Kal7 is most enriched in cytosolic fractions (S3, LS2) (Fig. 2A).

To further compare the properties of rat brain Kal7 and Δ Kal7, we fractionated cytosol on calibrated gel filtration columns (Fig. 2B). Only the small fraction of the total Kal7 ($M_r = 190$ kDa) that was recovered in the cytosol was analyzed. A peak of cytosolic Kal7 elutes between thyroglobulin (670 kDa) and apoferritin (474 kDa), suggesting the presence of oligomers, and higher molecular weight complexes that extend into the void volume. In contrast, Δ Kal7 ($M_r = 115$ kDa) fractionates as a single, homogeneous component; its elution position suggests that it forms smaller oligomers or has an asymmetric shape.

Δ Kal7 and Kal7 produce different phenotypes in non-neuronal cells

Spine formation is a complex process involving developmental changes in both the presynaptic and postsynaptic neurons. Therefore we sought a simple system in which to systematically compare the properties of Δ Kal7 and Kal7. pEAK Rapid cells were used previously to define the actions of the GEF1 domain of Kalirin [11]; these non-neuronal cells express very little endogenous Kalirin. Transfected cells were fixed and visualized with myc antibody to localize the transfected protein and with fluorescently tagged phalloidin to visualize filamentous actin. As observed in neurons, Δ Kal7 and Kal7 produce distinctly different phenotypes. Expression of Δ Kal7, much like expression of KalGEF1 or Rac1, produces cells with extensive, flattened lamellipodia filled with spokes of filamentous actin (Fig. 3A). In contrast, expression of Kal7 produces adherent compact, round cells with a prominent ring of cortical filamentous actin (Fig. 3A).

Both KalGEF1 and Δ Kal7 co-localize with the filamentous actin that forms the spoke-like structures radiating through the lamellipodia (Fig. 3A; arrows). Kal7 forms a continuous layer under a uniformly curved membrane, co-localizing with the filamentous actin that accumulates beneath the plasma membrane (Fig. 3B). Internal vacuoles outlined by Kal7 are often observed (Fig. 3B; red lines). Blebs emanating from the cell surface are seen occasionally; Kal7 extends into these blebs (Fig. 3B; red arrows). While Kal7 is concentrated at the plasma membrane, both Δ Kal7 and KalGEF1 are more diffusely distributed throughout the non-lamellipodial region of the cell (Fig. 3A).

Quantification of these data shows that the lamellipodial and compact, round phenotypes are highly penetrant (Fig. 3C). Cells expressing GFP extend short processes with small lamellipodia at their tips; the wild type morphology is referred to as flat. The effects of Δ Kal7 and KalGEF1 are indistinguishable, with most of the cells surrounded by extensive

lamellipodia. The effects of Kal7 are strikingly different; lamellipodia are never observed. Similar differences were noted when the morphological effects of Kal8 and Δ Kal8 were compared (data not shown).

Expression of Kal7, but not Δ Kal7, inhibits endocytosis

The accumulation of filamentous actin beneath the plasma membrane following expression of Kal7 suggested that endocytosis might be affected. To explore this possibility, clathrin-mediated endocytosis was assessed by monitoring uptake of transferrin. Transfected cells allowed to internalize FITC-transferrin for 10 min at 37 °C were rinsed and fixed. Micrographs taken under identical conditions (Fig. 4A) were coded and scored qualitatively for internalized transferrin (Fig. 4B). Expression of Δ Kal7 has no discernible effect on endocytosis. After a 10 min pulse, transferrin-positive puncta are broadly distributed throughout the cell; in some cells, puncta of internalized transferrin accumulate in the perinuclear region. In contrast, expression of Kal7 substantially alters transferrin uptake. In many Kal7 expressing cells, transferrin-positive puncta accumulate at the plasma membrane. The dramatic differences observed between the effects of the Δ and full-length Kalirin isoforms suggest that domains unique to the full-length isoform affect protein localization, protein/protein interactions and/or control the activity of the GEF domain.

The GEF activities of Δ Kal7 and Kal7 are indistinguishable

Since activated Rho GTPases are potent regulators of the actin cytoskeleton, we first explored the possibility that the GEF activities of Δ Kal7 and Kal7 differed. Myc-tagged Δ Kal7 and Kal7 were purified from transfected cells (Fig. 5A) and their GEF activities were assayed using saturating amounts of purified Rac1/GDP-Mant (Fig. 5B). Δ Kal7 and Kal7 catalyze nucleotide exchange at similar rates, $0.23 \pm 0.03 \text{ min}^{-1}$ and $0.21 \pm 0.01 \text{ min}^{-1}$, respectively, indicating that their intrinsic GEF activities for Rac1 do not differ. These rates are 4-fold lower than that of purified KalGEF1 ($0.82 \pm 0.36 \text{ min}^{-1}$) [11].

Despite having similar *in vitro* activities, other molecules may differentially modulate the GEF activity of Δ Kal7 and Kal7 in cells. The cell-based Pak-CRIB domain Rac activation assay was used to compare the ability of KalGEF1, Δ Kal7 and Kal7 to activate Rac in a cellular environment (Fig. 5C). Cells were transfected with different amounts of DNA in order to obtain similar levels of Kalirin expression. While Kal7 and Δ Kal7 produce similar amounts of Rac activation, neither isoform activates Rac to the level observed with KalGEF1 (Fig. 5C) [11]. As observed with the purified proteins, the catalytic activity of the first GEF domain of Kalirin is diminished when spectrin repeats 5 through 9 are present, but no further change in GEF activity is apparent when the Sec14p domain and first 4 spectrin repeats are appended.

GEF activity is not required for Kal7 to affect cell morphology

While the global GEF activities of cells expressing Δ Kal7 and Kal7 are similar, differential localization of the isoforms could allow GEF activity to play a key role in the morphological and functional differences observed. We therefore asked whether a catalytically active GEF domain were necessary for the unique morphological effects of Kal7. Replacement of Asn¹⁴¹⁵-Asp¹⁴¹⁶ near the C-terminus of the DH domain of KalGEF1 with Ala-Ala reduces its GEF activity ~50-fold [11]. The compact, round shape observed following expression of Kal7 is uniformly observed following expression of Kal7(ND/AA) (Fig. 5D), indicating that GEF activity does not play a major role in establishing this phenotype. However, the effects of Kal7 and Kal7(ND/AA) are not identical. Cells expressing Kal7(ND/AA) are irregularly shaped and frequently exhibit membrane blebs (Fig. 5D). Membrane blebs are visible because Kal7(ND/AA) remains associated with the plasma membrane (Fig. 5D, arrows); blebs are not observed as frequently in cells expressing Kal7.

Δ Kal7 and Kal7 interact with the cytoskeleton in different ways

We next compared the ability of Kal7 and Δ Kal7 to associate with the particulate fraction following homogenization (Fig. 6). Cells were sonicated in a low ionic strength, neutral pH buffer containing 1% TX-100; soluble proteins and proteins solubilized by 1% TX-100 were recovered in the supernatant and the pellet, which includes cytoskeletal elements, was solubilized in SDS sample buffer (Fig. 6A). Like KalGEF1, most of the Δ Kal7 is recovered from the supernatant under these conditions; in contrast, only about one third of the Kal7 is soluble or solubilized by TX-100 (Fig. 6A).

Based on the association of Kal7 with the plasma membrane observed by immunostaining, the protocol developed to extract spectrin from erythrocyte ghosts was used to compare the behavior of Δ Kal7 and Kal7 [18]. Transfected cells were homogenized in low ionic strength, high pH buffer and soluble proteins (S) were separated from membranes, which were then solubilized using 1% TX-100 (M); the remaining pellet was solubilized using deoxycholic acid or SDS (P) (Fig. 6B). Most of the Δ Kal7 is soluble or can be solubilized with 1% TX-100. In contrast, almost no Kal7 is soluble; approximately one-third is membrane-associated and can be solubilized with TX-100. Two thirds of the Kal7 remains particulate even after incubation with 1% TX-100 (Fig. 6B). Both approaches suggest that differences in the manner in which Δ Kal7 and Kal7 interact with the cytoskeleton and cell membrane contribute to their different effects on cell morphology.

We next asked whether Kal7 and Δ Kal7 solubilized from pEAK Rapid cells using TX-100 fractionate in a manner similar to the Kal7 and Δ Kal7 present in rat brain cytosol (Fig. 6C). pEAK Rapid Δ Kal7 fractionates as a single, uniform component ($V/V_t = 0.49 \pm 0.01$), eluting at the same position as endogenous rat brain cytosolic Δ Kal7 ($V/V_t = 0.50 \pm 0.01$) (Fig. 2B). pEAK Rapid Kal7 is almost entirely recovered from the void volume of the column, with a small amount of material eluting between thyroglobulin and apoferritin, where much of the rat brain cytosol Kal7 elutes (Fig. 2B). Very little exogenous Kal7 is actually soluble (Fig. 6B) and the sample applied to the gel filtration column represents Kal7 solubilized by treatment with TX-100. The endogenous Kal7 recovered from rat brain cytosol (Fig. 2B) was not exposed to TX-100 and high molecular aggregates are not as prevalent. Taken together, our immunofluorescence, solubilization and gel filtration data indicate that Kal7 assembles into high molecular weight complexes beneath the plasma membrane.

The spectrin-repeat region of Kalirin forms oligomers

Differences in the behavior of Δ Kal7 and Kal7 during gel filtration led us to explore the possibility that Kalirin, like erythrocyte spectrin, forms oligomers [18]. A GST fusion protein containing spectrin repeats 4–7 [KalSpec(4–7)] was used for the initial studies because it proved to be readily soluble. Lysates of cells transiently expressing KalGEF1, Δ Kal7 or Kal7 were incubated with KalSpec(4–7) beads (Fig. 7A). While Δ Kal7 bound to KalSpec(4–7), KalGEF1 did not; Kal7 also bound, although not as well as Δ Kal7.

Since these interactions could be indirect, we next asked whether purified Δ Kal7 bound to KalSpec(4–7) beads (Fig. 7B). Binding saturated as the concentration of Δ Kal7 increased; Δ Kal7 binds to KalSpec(4–7) with nM affinity. The region of the spectrin-repeat region responsible for this interaction was explored by linking purified Δ Kal7, KalSpec(4–6) and KalSpec5 to beads (Fig. 7C). Similar amounts of purified Δ Kal7 bound to KalSpec5 and to each of the larger proteins, identifying the fifth spectrin repeat as the critical region. Coupled with its behavior during gel filtration, our data suggest that Δ Kal7 forms dimers.

We next evaluated the ability of Kal7 to interact with the various spectrin repeat regions. Transiently expressed Kal7 was less able than Δ Kal7 to interact with Δ Kal7, KalSpec5,

KalSpec(4–6) or KalSpec(4–7) (Fig. 7D). This difference may reflect the fact that almost all of the soluble Kal7 is already assembled into stable high molecular weight complexes.

The GEF domain is not required for Kal7 to affect cell morphology

To evaluate the role of the GEF domain of Kal7 in generating the compact round phenotype, we expressed a Kal7 variant truncated so that it lacks the entire GEF domain.

KalSec14pSpec_{1–9} terminates after the ninth spectrin repeat and lacks a GEF domain (Fig. 8A); since zebrafish Solo, a homologue of Kalirin, is composed of a Sec14p domain and spectrin-like repeats with no GEF domain [7], we refer to this molecule as KalSolo. Most of the cells expressing KalSolo have a compact, round phenotype resembling that observed in cells expressing Kal7 (Fig. 8A). KalSolo is associated with the plasma membrane, but is not uniformly distributed beneath the cell surface. Where the cells contact the substrate, patches of KalSolo are apparent (red lines); sections through the middle or top of the cells reveal discrete puncta of Kal7 at the plasma membrane (red arrows). Some of the cells expressing KalSolo exhibit a less dramatic change in shape, remaining rather flat, with KalSolo still associated with the plasma membrane. Lamellipodia are never observed. The morphological effects of Kal7, Kal7/ND/AA, Kal7 lacking its PDZ-binding motif and KalSolo are summarized in Fig. 8B. Neither GEF activity, the GEF domain nor the PDZ-binding motif is necessary for cells to adopt the compact round morphology.

The solubility properties of these Kalirin mutants were next investigated (Fig. 8C). Like Kal7, Kal7(ND/AA), Kal7 Δ CT and KalSolo are recovered primarily from the TX-100 insoluble cytoskeletal fraction (Fig. 8C). KalSolo is even less soluble than Kal7, with over 95% of the protein recovered from the TX-100 insoluble cytoskeletal fraction. The effects of KalSolo indicate that the non-enzymatic domains of Kal7 act autonomously, contributing to the unique effects of Kal7 on cytoskeletal organization and to its lack of solubility. Although transcripts encoding a protein homologous to KalSolo were not identified in mammals (see Supplementary Data), transcripts encoding a non-catalytic endogenous isoform, Kal4, are expressed. Kal4 includes the Sec14p domain and first four spectrin repeats of Kalirin; in addition, Kal4 includes part of the fifth spectrin repeat, which is present in both Kal7 and Δ Kal7, terminating after its unique fifth spectrin-repeat (Fig. S1).

Kal4 causes the formation of compact, round cells

Expression of Kal4 produces a phenotype somewhat similar to the Kal7 phenotype, with compact, round cells lacking filopodia or lamellipodia (Fig. 9A). Filamentous actin is quite uniformly distributed along the plasma membrane. Images through the middle and top of the cells reveal multiple plasma membrane blebs, resulting in a jagged appearance (Fig. 9A, arrows). Approximately a third of the Kal4-expressing cells are flat; Kal4 is membrane-associated, with foci of staining along the plasma membrane (Fig. 9A, C). Spectrin repeat 5, which plays a key role in the dimerization of Δ Kal7, occurs in modified form at the C-terminus of Kal4. To evaluate the importance of this spectrin repeat region, we truncated Kalirin after the 4th spectrin repeat, generating KalSecSpec_{1–4}. The truncated protein is expressed at low levels in only a small percentage of cells. A significant fraction of the KalSecSpec_{1–4} expressing cells are flat, somewhat resembling GFP-expressing control cells (Fig. 9B, C). Instead of forming a continuous layer under the plasma membrane, discrete puncta and clumps of KalSecSpec_{1–4} are associated with the cell surface (red lines); KalSecSpec_{1–4} often extends into membrane blebs (red arrows). Lamellipodia are not seen in cells expressing Kal4 or KalSecSpec_{1–4}.

The solubility properties of these truncated proteins were assessed (Fig. 9D). Kal4, an 80 kDa protein (predicted mass, 82 kDa), is as insoluble as Kal7. KalSecSpec_{1–4} (67 kDa; predicted mass 74 kDa) is almost entirely insoluble. Gel filtration of the soluble Kal4 yields a major peak

in the void volume that trails to the elution position expected of a monomer (Fig. 9E). Consistent with a key role for spectrin repeat 5 in the oligomerization of Kalirin, transiently expressed Kal4 binds to recombinant KalSpec(4–7) (data not shown).

The Sec14p domain is necessary for formation of compact, round cells

To determine whether the Sec14p domain of Kal7 plays a role in its ability to cause the formation of compact, round cells, we expressed Δ Sec14Kal7, which lacks this domain (Fig. 10A). Cells expressing Δ Sec14Kal7 exhibit a variety of phenotypes, but only $27 \pm 9\%$ would be classified as compact and round (vs. $90 \pm 3\%$ for Kal7 assayed simultaneously). Extensive lamellipodial sheets often extend across the surface of the dish and ruffles commonly extend from the dorsal surface of the cells. Cells expressing Δ Sec14Kal7 have filopodia resembling those on wildtype cells. The Δ Sec14Kal7 protein is found throughout the cell, often decorating internal vacuolar structures (Fig. 10A). Δ Sec14Kal7 does not localize to the plasma membrane.

Yeast Sec14p is a lipid transfer protein [22]. Since its Sec14p domain is essential to the actions of Kal7, we next asked whether the Kalirin Sec14p domain binds lipids (Fig. 10B). Purified GST-Sec14p was incubated with a membrane spotted with different lipids (PIP strip). Bound GST-Sec14p was detected using a GST antibody. Kal-Sec14p preferentially binds to PI(3,5)P2 and to PI3P, phosphoinositides known to play a role in endocytosis [23]. This is consistent with the fact that over-expression of Kal7 inhibits endocytosis while over-expression of Δ Kal7 does not.

Expression of the isolated Sec14p domain of Kalirin had little effect on cell morphology; small lamellipodial extensions were commonly observed and 93% of the cells had the Flat morphology common to wildtype cells (Fig. 10C). Exogenous Sec14p was not concentrated at the plasma membrane. Upon subcellular fractionation, most of the Sec14p protein is recovered from the TX-100 soluble fraction (Figs. 10D); the rest is associated with the cytoskeletal fraction. Fractionation of recombinant Sec14p on the gel filtration column yields a major peak at the elution position expected for monomeric Kalirin Sec14p (Fig. 10E). The spectrin repeat region of Kalirin, not its Sec14p domain, causes the formation of high molecular weight complexes.

DISCUSSION

We wanted to determine if there were functional differences between the Δ - and full length isoforms of Kalirin. By comparing Kal7 to Δ Kal7, we discovered potent effects of the Sec14p domain and N-terminal spectrin-like repeats that are clearly distinct from those of the GEF domain. These non-enzymatic domains affect endocytosis, solubility, oligomerization, binding to the cytoskeleton and subcellular localization. Additional studies will be needed to understand how the actions of these domains are integrated to control the actions of Kalirin.

The Δ -isoform of Kal7 does not stimulate spine formation

Exogenous Kal7 stimulates the formation of dendritic spines by hippocampal and cortical neurons [2,3,13,24]. Antisense or shRNA mediated specific knockdown of Kal7, which also decreases Δ Kal7, reduces linear spine density along the dendrites of pyramidal neurons and reduces the number of excitatory synapses on the dendrites of GABAergic interneurons [13]. The GEF activity of Kal7 and its PDZ-binding motif, which interacts with PSD95, both play essential roles in the effects of Kal7 on spine formation and function [3,24]. Although Δ Kal7 has the same GEF domain and PDZ binding motif, we show here that exogenous Δ Kal7 does not increase spine formation like Kal7.

In the simple test system used here, both KalSolo and Kal4, which lack a GEF domain and a PDZ binding motif, produce phenotypes similar to that of Kal7. Amongst the 69 human RhoGEF family members, only Kalirin, Trio, Dbl and Dbs (Ost) have spectrin-like repeats [25]. With nine spectrin repeats, Kal7 is half the size of α - and β -spectrin. Our data suggest that Kal7, like α - and β -spectrin, forms high molecular weight complexes that participate in the formation of a membrane skeleton. Lacking the Sec14p and first four spectrin repeats, Δ Kal7 forms dimers, but does not form high molecular weight complexes; its exogenous expression produces effects indistinguishable from those of the isolated Kal-GEF1 domain.

The Sec14p/spectrin repeat region of Kalirin affects cell morphology

Expression of KalSolo (Sec14p domain and 9 spectrin repeats) or Kal4 (Sec14p domain and 5 spectrin repeats) causes pEAK Rapid cells to adopt a compact round morphology. KalSolo and filamentous actin both accumulate beneath the plasma membrane. While Kal4 and SecSpec₁₋₄ accumulate under the plasma membrane, filamentous actin is not especially prominent when these truncation mutants of Kalirin are expressed. These observations suggest that the spectrin repeat region of Kalirin contributes to the structural integrity of the compact, round cells. Loss of the KalGEF1-mediated interaction with filamin A (ABP280) in KalSolo and Kal4 may contribute to the less regular cell shape observed in cells expressing these constructs.

Proteins in the spectrin family have well established roles linking the plasma membrane to the underlying actin cytoskeleton [26]. Erythrocyte spectrins associate with the plasma membrane both directly, through their PH domains and interactions with specific integral membrane proteins, and indirectly, through ankyrin. Erythrocyte spectrins interact with actin both directly and indirectly; these interactions are essential to membrane elasticity and stability [26–28].

Alternative splicing yields non-catalytic isoforms of Kalirin

While less prevalent than longer Kalirin transcripts, Kal4 is expressed in the rat CNS, with levels increasing during development. In many spectrin family members, the rigid structure of the spectrin repeat regions ensures proper spacing between N- and C-terminal functional groups [28,29]. The nine spectrin repeats of Kal7 separate the Sec14p domain, which can bind phosphatidylinositol (3,5)P₂, from both the GEF domain and the PDZ binding motif. Natural products such as Kal4 and zebrafish Solo contain only one functional domain and would be expected to alter the function of the Kalirin meshwork by uncoupling membrane attachment sites from the GEF domain and from the PDZ binding motif. Consistent with this role, the phenotypes of cells expressing Kal7 and KalSolo or Kal4 do differ.

Δ Kal7 forms soluble dimers while Kal7 forms insoluble oligomers

As observed before, a substantial amount of the endogenous rat brain Δ Kal7 is soluble, while very little rat brain Kal7 is soluble. Gel filtration identified endogenous Kal7 in high molecular weight complexes. Since Δ Kal7 does not exhibit this behavior, neither the Kal-GEF1/filamin A interaction [11], the Kal-PH1/TrkA interaction [26–28] nor interactions with PDZ binding domain proteins such as neurabin, ZO-1, ZO-2 and afadin [24] plays an essential role in it. Although Kal7 and Δ Kal7 may differ in the ability of their spectrin repeat regions to bind to integral membrane proteins like peptidylglycine Δ -amidating monooxygenase [12] and membrane associated proteins like Arf6-GDP [10], the dramatic differences observed in transfected cells indicate inherent differences in the properties of these two proteins.

Each of the spectrin repeat containing fragments of Kalirin examined was recovered almost entirely from the cytoskeletal pellet. In addition to a Sec14p-mediated association with membranes, the spectrin repeat region of Kalirin clearly contributes to its lack of solubility. Using purified Δ Kal7, we identified a role for spectrin repeat 5 in the formation of Kalirin

oligomers. While Δ Kal7 forms what appear to be dimers, the additional spectrin repeats in Kal7 allow it to form larger oligomers. As for Kal7, gel filtration reveals high molecular weight complexes containing KalSolo. Kal4, with its five spectrin-like repeats, also forms high molecular weight complexes. The Sec14p domain of Kalirin fractionates as a monomer, identifying the spectrin repeats as responsible for formation of high molecular weight complexes. The spectrin repeat regions of erythroid spectrin are responsible for protein/protein interactions yielding dimers, tetramers and larger oligomers, allowing spectrin to perform a structural function.

The Sec14p domain of Kalirin binds phosphoinositides and is necessary for localization of Kal7 to the plasma membrane

Kal7, but not Δ Kal7, can interact with membrane phospholipids through its Sec14p domain. Sec14 is a member of the lipid transfer family of proteins, which includes proteins that bind phosphatidylinositol, phosphatidylcholine, phosphatidylserine, α -tocopherol, and retinal [30–32]. Of the lipid transfer domains with known function, the N-terminal Sec14 domain of Kalirin is most homologous to yeast Sec14, a phosphatidylinositol-phosphatidylcholine transfer protein involved in protein trafficking from the Golgi that is also required for endocytosis [30,33].

A small subset of the human RhoGEFs (Kalirin, Trio, Dbs, and Dbl) contain Sec14p domains [25]. As for Kalirin, alternative splicing determines whether a Sec14p domain is included in Dbs and Dbl; protein localization and signaling ability differ for Dbs and Dbl isoforms having or lacking this Sec14p domain [34,35]. The Sec14p domain of Dbs binds to its PH domain, inhibiting transforming activity; removal of the Sec14p domain greatly increases transforming activity [35]. While the Sec14p domains of Kalirin, Dbs, and Dbl interact with the $G_{\beta\gamma}$ subunits of heterotrimeric G proteins [36], the functional significance of this interaction is not clear.

Like the Sec14p domain of Kalirin, the Sec14p domains of Dbs and Dbl bind to phosphoinositides [34,35]. The Dbl Sec14p domain binds best to phosphatidylinositol monophosphates (PI3P, PI4P and PI5P) and the Dbs Sec14p domain prefers phosphatidylinositol bisphosphates [PI(4,5)P₂, PI(3,5)P₂, PI(3,4)P₂]. The binding of KalSec14p to PI3P and PI(3,5)P₂ suggests roles in early and late phases of endocytosis, respectively [23]. This is consistent with the fact that transferrin uptake is inhibited by expression of full-length Kal7 but not by expression of Δ Kal7.

Kal7 and Δ Kal7 have similar guanine nucleotide exchange activity

Since many mammalian RhoGEFs were identified as oncogenes activated by mutation or deletion of domains adjoining the GEF domain [37,38], the first hypothesis we tested was that the GEF activities of Δ Kal7 and Kal7 differed. *In vitro* and cell-based GEF assays indicate that the GEF activities and substrate specificity of Kal7 and Δ Kal7 are indistinguishable [11].

The fact that an inactive mutant of Kal7 exerts cytoskeletal effects similar to those of Kal7 was the first indication that non-catalytic regions play a key role in the formation of compact round cells. Plasma membranes form blebs when adhesion to the underlying cytoskeleton is lost or when gaps form in the cytoskeleton [39]. The fact that blebs are observed more frequently in Kal7(ND/AA)-expressing cells than in Kal7-expressing cells suggests that Rho proteins activated by KalGEF1 prevent this from occurring. A role for Rac in maintaining the actin/spectrin scaffold in erythrocytes is supported by deficits observed in erythrocytes lacking both Rac1 and Rac2 [40]. The controlled budding and vesiculation of membranes allows the partitioning of membrane components that remain attached to the underlying cytoskeleton from those that do not [41] and Kalirin is well designed to participate in a process of this type.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Reference List

1. May V, Schiller MR, Eipper BA, Mains RE. Kalirin Dbl-homology guanine nucleotide exchange factor 1 domain initiates new axon outgrowths via RhoG-mediated mechanisms. *J Neurosci* 2002;22:6980–6990. [PubMed: 12177196]
2. Ma XM, Huang JP, Wang Y, Eipper BA, Mains RE. Kalirin, a multifunctional Rho GEF, is necessary for maintenance of hippocampal pyramidal neuron dendrites and dendritic spines. *J Neurosci* 2003;23:10593–10603. [PubMed: 14627644]
3. Penzes P, Johnson RC, Sattler R, Zhang X, Haganir RL, Kambampati V, Mains RE, Eipper BA. The neuronal Rho-GEF Kalirin-7 interacts with PDZ domain-containing proteins and regulates dendritic morphogenesis. *Neuron* 2001;29:229–242. [PubMed: 11182094]
4. Johnson RC, Penzes P, Eipper BA, Mains RE. Isoforms of kalirin, a neuronal Dbl family member, generated through use of different 5'- and 3'-ends along with an internal translational initiation site. *J Biol Chem* 2000;275:19324–19333. [PubMed: 10777487]
5. McPherson CE, Eipper BA, Mains RE. Genomic organization and differential expression of Kalirin isoforms. *Gene* 2002;284:41–51. [PubMed: 11891045]
6. McPherson CE, Eipper BA, Mains RE. Kalirin expression is regulated by multiple promoters. *J Mol Neurosci* 2004;22:51–62. [PubMed: 14742910]
7. Sato T, Mishina M. Representational difference analysis, high-resolution physical mapping, and transcript identification of the zebrafish genomic region for a motor behavior. *Genomics* 2003;82:218–229. [PubMed: 12837271]
8. Penzes P, Johnson RC, Alam MR, Kambampati V, Mains RE, Eipper BA. An isoform of kalirin, a brain-specific GDP/GTP exchange factor, is enriched in the postsynaptic density fraction. *J Biol Chem* 2000;275:6395–6403. [PubMed: 10692441]
9. Mains RE, Alam MR, Johnson RC, Darlington DN, Back N, Hand TA, Eipper BA. Kalarin, a multifunctional PAM COOH-terminal domain interactor protein, affects cytoskeletal organization and ACTH secretion from AtT-20 cells. *J Biol Chem* 1999;274:2929–2937. [PubMed: 9915831]
10. Koo TH, Eipper BA, Donaldson JG. Arf6 recruits the Rac GEF Kalirin to the plasma membrane facilitating Rac activation. *BMC Cell Biol* 2007;8. [PubMed: 17326840]
11. Schiller MR, Blangy A, Huang JP, Mains RE, Eipper BA. Induction of lamellipodia by Kalirin does not require its guanine nucleotide exchange factor activity. *Exp Cell Res* 2005;307:402–417. [PubMed: 15950621]
12. Alam MR, Johnson RC, Darlington DN, Hand TA, Mains RE, Eipper BA. Kalirin, a cytosolic protein with spectrin-like and GDP/GTP exchange factor-like domains that interacts with peptidylglycine alpha-amidating monooxygenase, an integral membrane peptide-processing enzyme. *J Biol Chem* 1997;272:12667–12675. [PubMed: 9139723]
13. Ma XM, Wang Y, Ferraro F, Mains RE, Eipper BA. Kalirin-7 Is an Essential Component of both Shaft and Spine Excitatory Synapses in Hippocampal Interneurons. *J Neurosci* 2008;28:711–724. [PubMed: 18199770]
14. Schiller MR, Mains RE, Eipper BA. A neuroendocrine-specific protein localized to the endoplasmic reticulum by distal degradation. *J Biol Chem* 1995;270:26129–26138. [PubMed: 7592816]
15. Kari B, Lussenhop N, Goertz R, Wabuke-Bunoti M, Radeke R, Gehrz R. Characterization of monoclonal antibodies reactive to several biochemically distinct human cytomegalovirus glycoprotein complexes. *J Virol* 1986;60:345–352. [PubMed: 3021969]

16. Field J, Nikawa J, Broek D, MacDonald B, Rodgers L, Wilson IA, Lerner RA, Wigler M. Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. *Mol Cell Biol* 1988;8:2159–2165. [PubMed: 2455217]
17. Sobota JA, Ferraro F, Back N, Eipper BA, Mains RE. Not all secretory granules are created equal: Partitioning of soluble content proteins. *Mol Biol Cell* 2006 Dec;17:5038–5052. [PubMed: 17005911]
18. Morrow JS, Marchesi VT. Self-assembly of spectrin oligomers in vitro: a basis for a dynamic cytoskeleton. *J Cell Biol* 1981;88:463–468. [PubMed: 7204503]
19. Husten EJ, Eipper BA. Purification and characterization of PAM-1, an integral membrane protein involved in peptide processing. *Arch Biochem Biophys* 1994;312:487–492. [PubMed: 8037462]
20. Snyder JT, Worthylake DK, Rossman KL, Betts L, Pruitt WM, Siderovski DP, Der CJ, Sondek J. Structural basis for the selective activation of Rho GTPases by Dbl exchange factors. *Nat Struct Biol* 2002;9:468–475. [PubMed: 12006984]
21. Zhang B, Zhang Y, Wang Z, Zheng Y. The role of Mg²⁺ cofactor in the guanine nucleotide exchange and GTP hydrolysis reactions of Rho family GTP-binding proteins. *J Biol Chem* 2000;275:25299–25307. [PubMed: 10843989]
22. Sha BD, Phillips SE, Bankaitis VA, Luo M. Crystal structure of the *Saccharomyces cerevisiae* phosphatidylinositol-transfer protein. *Nature* 1998;391:506–510. [PubMed: 9461221]
23. Di Paolo G, De Camilli P. Phosphoinositides in cell regulation and membrane dynamics. *Nature* 2006;443:651–657. [PubMed: 17035995]
24. Penzes P, Beeser A, Chernoff J, Schiller MR, Eipper BA, Mains RE, Huganir RL. Rapid Induction of Dendritic Spine Morphogenesis by trans-Synaptic EphrinB-EphB Receptor Activation of the Rho-GEF Kalirin. *Neuron* 2003;37:263–274. [PubMed: 12546821]
25. Rossman KL, Der CJ, Sondek J. GEF means go: Turning on Rho GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol* 2005;6:167–180. [PubMed: 15688002]
26. De Matteis MA, Morrow JS. Spectrin tethers and mesh in the biosynthetic pathway. *J Cell Sci* 2000;113(Pt 13):2331–2343. [PubMed: 10852813]
27. Broderick MJF, Winder SJ. Towards a complete atomic structure of spectrin family proteins. *J Struct Biol* 2002;137:184–193. [PubMed: 12064945]
28. Liu SC, Derick LH, Palek J. Dependence of the permanent deformation of red blood cell membranes on spectrin dimer-tetramer equilibrium: implication for permanent membrane deformation of irreversibly sickled cells. *Blood* 1993;81:522–528. [PubMed: 8422468]
29. Lee A, Morrow JS, Fowler VM. Caspase remodeling of the spectrin membrane skeleton during lens development and aging. *J Biol Chem* 2001;276:20735–20742. [PubMed: 11278555]
30. Bankaitis VA, Malehorn DE, Emr SD, Greene R. The *Saccharomyces cerevisiae* SEC14 gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi complex. *J Cell Biol* 1989;108:1271–1281. [PubMed: 2466847]
31. Zhao R, Fu X, Li Q, Krantz SB, Zhao ZJ. Specific interaction of protein tyrosine phosphatase-MEG2 with phosphatidylserine. *J Biol Chem* 2003;278:22609–22614. [PubMed: 12702726]
32. Zimmer S, Stocker A, Sarbolouki MN, Spycher SE, Sassoon J, Azzi A. A novel human tocopherol-associated protein: cloning, in vitro expression, and characterization. *J Biol Chem* 2000;275:25672–25680. [PubMed: 10829015]
33. Kean LS, Fuller RS, Nichols JW. Retrograde lipid traffic in yeast: identification of two distinct pathways for internalization of fluorescent-labeled phosphatidylcholine from the plasma membrane. *J Cell Biol* 1993;123:1403–1419. [PubMed: 8253840]
34. Ueda S, Kataoka T, Satoh T. Role of the Sec14-like domain of Dbl family exchange factors in the regulation of Rho family GTPases in different subcellular sites. *Cell Signal* 2004;16:899–906. [PubMed: 15157669]
35. Kostenko EV, Mahon GM, Cheng L, Whitehead IP. The Sec14 homology domain regulates the cellular distribution and transforming activity of the Rho-specific guanine nucleotide exchange factor Dbs. *J Biol Chem* 2005;280:2807–2817. [PubMed: 15531584]
36. Nishida K, Kaziro Y, Satoh T. Association of the proto-oncogene product dbl with G protein betagamma subunits. *FEBS Lett* 1999;459:186–190. [PubMed: 10518015]

37. Whitehead IP, Campbell S, Rossman KL, Der CJ. Dbl family proteins. *Biochim Biophys Acta* 1997;1332:F1–23. [PubMed: 9061011]
38. Zheng Y. DBL family guanine nucleotide exchange factors. *Trends Biochem Sci* 2002;25:724–732.
39. Sheetz MP, Sable JE, Dobereiner HG. Continuous membrane-cytoskeleton adhesion requires continuous accommodation to lipid and cytoskeleton dynamics. *Ann Rev Biophys Biomol Struct* 2006;35:417–434. [PubMed: 16689643]
40. Kalfa TA, Pushkaran S, Mohandas N, Hartwig JH, Fowler VM, Johnson JF, Joiner CH, Williams DA, Zheng Y. Rac GTPases regulate the morphology and deformability of the erythrocyte cytoskeleton. *Blood* 2006;108:3637–3645. [PubMed: 16882712]
41. Knowles DW, Tilley L, Mohandas N, Chasis JA. Erythrocyte membrane vesiculation: Model for the molecular mechanism of protein sorting. *Proc Nat Acad Sci USA* 1997;94:12969–12974. [PubMed: 9371784]
42. Tabaska JE, Zhang MQ. Detection of polyadenylation signals in human DNA sequences. *Gene* 1999;231:77–86. [PubMed: 10231571]

Figure 1. Kal7, but not Δ Kal7, stimulates spine formation

(A) Kalirin-7 and Δ Kal7 are diagrammed. Dissociated cortical neurons prepared from P1 rat pups were transfected with vectors encoding Δ Kal7 (B,C) or Kal7 (D,E), plated onto polylysine-coated coverslips and maintained in culture for 16 days before fixation. Exogenous protein was visualized using antisera to myc (Cy3-tagged second antibody) (B,D) and microtubule associated protein 2 (MAP2; Alexa633-tagged antibody); C and E, merged images. Thick red arrows, cell soma; thin red lines, dendritic branch points; white arrows, dendritic spines or filopodia. A few spines appear on the dendrites of non-transfected neurons after about 14 days *in vitro*. Scale bar applies to all images; Δ Kal7 image shows saturated cell soma in order to make processes visible.

Figure 2. Endogenous Kal7 and Δ Kal7 have different properties

A. Adult rat parietal cortex was fractionated as described in Methods. Equal amounts of protein (30 μ g) from each fraction were analyzed. Kalirin was visualized using a Kal-spectrin antibody (left) or a Kal7-specific antibody (right, upper panel). The identity of the subcellular fractions was verified using antibodies: S3 (cytosol; glutamic acid decarboxylase; not shown); P3 (Golgi and endoplasmic reticulum; calnexin; not shown); LP1 (synaptosomal membranes; PSD95); LS2 (synaptosomal cytosol); LP2 [synaptic vesicles, synaptophysin (Synapto)]. **B.** Aliquots of adult rat brain cytosol (equivalent to S3) were separated by gel filtration (Methods); aliquots of fractions were subjected to Western blot analysis using a Kal7-specific antibody. Recombinant Kal7 and Δ Kal7 were analyzed in the left-hand lane (not shown); an aliquot of column input was analyzed in the right-hand lane (not shown). The void volume (V_0) and elution volumes and masses of thyroglobulin, apoferritin and bovine serum albumin (BSA) are indicated.

Figure 3. Δ Kal7 and Kal7 produce distinctly different morphological responses in non-neuronal cells

A. pEAK Rapid cells were transfected with expression vectors encoding GFP or HisMyc-tagged KalGEF1, Δ Kal7 and Kal7 (HisMyc tag, green triangles). Cells were serum-starved for 24 h and transfected proteins were visualized with myc antibody (green) while filamentous actin was visualized using TRITC-phalloidin (red). Empty arrows indicate spokes of filamentous actin in lamellipodial sheets. Images are representative at least 100 cells examined for each construct. **B.** Deconvolved images showing localization of Kal7 (myc) and filamentous actin (phalloidin). Red arrows, places where plasma membrane separates from filamentous actin; red lines, internal myc-positive structures. **C.** Coded micrographs from three or more

separate transfections were scored for cell shape and then decoded; standard deviation is shown. The control, KalGEF1 and Kal7 expressing cells in panel **A** illustrate Flat, Lamellipodial and Compact/Round cells, respectively.

Figure 4. Expression of Kal7 disrupts endocytic trafficking

A. pEAK Rapid cells expressing GFP, Δ Kal7 or Kal7 were incubated with AlexaFluor546-transferrin (50 μ g/ml) for 10 min. Snapshot from a deconvolved image is shown. Coded micrographs of internalized transferrin and exogenous protein were evaluated for level and localization of transferrin uptake (**B**). Dashed white arrows, cells scored as WT; white arrows, cells scored as EDGE.

Figure 5. The GEF activity of Kal7 is not necessary for formation of compact round cells
A. Coomassie stained gel of purified Δ Kal7 and Kal7 used in GDP-Mant assays **B.** Time course of release of GDP-Mant from Rac1/GDP-Mant (20 μ M) following addition of buffer, Δ Kal7 or Kal7. Similar results were obtained in 3 additional experiments. **C.** Activation of endogenous Rac in pEAK Rapid cells transfected with vectors encoding myc-tagged KalGEF1 (5, 10, 20 ng), Δ Kal7 (200, 400 ng) or Kal7 (100, 200 ng). Kalirin expression was quantified using myc antibody (x-axis). Total Rac2 and Rac2-GTP (bound to Pak-CRIB resin) were quantified using a Rac antibody [11]. Similar results were observed in 3 additional experiments. **D.** pEAK Rapid cells expressing Kal7/ND/AA were fixed and stained for myc (red) and filamentous actin

(phalloidin, green). Arrows mark blebs, where the cortical filamentous actin separates from the myc-positive plasma membrane; #, non-transfected cells.

Figure 6. The properties of ΔKal7 and Kal7 differ

A. pEAK Rapid cells were extracted into 20 mM NaTES, 10 mM mannitol, 1% TX-100, pH 7.4. Soluble proteins (S) were separated from insoluble proteins by centrifugation at $14,000 \times g$ for 15 min or by centrifugation at $430,000 \times g$ for 15 min; the pellet (I) was solubilized using Laemmli sample buffer. Aliquots accounting for an equal percentage of each fraction were subjected to Western blot analysis using antibody to myc. Data from 4 to 10 similar experiments were quantified; error bars show standard deviation. **B.** A protocol used to solubilize spectrin from erythrocyte ghosts was adapted for analysis of transfected ΔKal7 and Kal7; data were quantified and recovery in the soluble (S), membrane-associated (M) and pellet (P) fractions are plotted. Error bars, range from two independent experiments. **C.** TMT extracts of pEAK

Rapid cells expressing Kal7 and Δ Kal7 were centrifuged before application to the calibrated S-400 Sephacryl column; BSA was included in each sample as an internal standard. The void volume (V_0) and predicted elution positions of marker proteins are indicated. Vertical line marks position at which molecular weight standards were analyzed by SDS-PAGE; this lane was eliminated from the figure.

A. Binding to KalSpec(4-7)

B. Purified ΔKal7

C. Specificity

D. Kal7 vs. ΔKal7

Figure 7. Oligomerization of Kalirin

A. TMT extracts of pEAK Rapid cells transiently expressing KalGEF1, ΔKal7 and Kal7 were tumbled with KalSpec(4-7) or control (linked to an irrelevant peptide) beads; inputs (5%) and eluates (75%) were fractionated by SDS-PAGE and visualized with Myc antibody. Percent bound is indicated below the gel. **B.** Purified ΔKal7 diluted to the indicated concentration was tumbled with KalSpec(4-7) or control beads; binding was quantified by comparison to inputs analyzed at the same time. **C.** Purified ΔKal7 was tumbled with equivalent amounts of ΔKal7, spectrin repeat 5 (SR5), KalSpec(4-6) (Sp4-6), KalSpec(4-7) (Sp4-7) or albumin beads; bound ΔKal7 was visualized using Myc antibody. **D.** The ability of ΔKal7 and Kal7 to bind to

these same fragments of Kalirin was assessed using lysates of transiently transfected pEAK Rapid cells; percent bound is indicated below each blot.

Figure 8. Kalirin mutants lacking the GEF domain affect cytoskeletal organization

A. pEAK Rapid cells transiently expressing KalSolo were fixed and visualized using antibody to myc (red), FITC-phalloidin (green) and TO-PRO-3 (DNA; blue). Stacks of confocal images were acquired and sections through the bottom, middle and top of the cells are shown; the y-z image for the position indicated is shown to the right. Red lines, patches of Kalirin along the bottom surface of the cell; red arrows, myc-positive clusters, some of which extend from the surface; green arrows, subplasma membrane filamentous actin. **B.** The effects of KalSolo, Kal7/ND/AA and Kal7ΔCT on cell morphology were evaluated as in Fig. 3C; GFP and Kal7 data are from Fig. 3C. **C.** The solubility of the Kalirin proteins indicated was assessed as in Fig. 6A. KalSolo has an apparent mass of 153 kDa and a predicted mass of 147 kDa.

Figure 9. Kal4 produces effects similar to those of Kal7

pEAK Rapid cells transiently expressing Kal4 (**A**) or SecSpec₁₋₄ (**B**) were fixed and stained for exogenous protein (myc; red), filamentous actin (FITC-phalloidin; green) and TO-PRO-3 (DNA; blue). For Kal4, single images from z stacks are shown; for SecSpec₁₋₄, a single image through the middle of the cell is shown. Red lines, punctate myc staining at the plasma membrane; red arrows, blebs filled with myc staining; green arrows, subplasma membrane filamentous actin. **C.** Coded micrographs were scored for morphology. **D.** Protein expression and solubility were assessed by Western blot analysis of TX-100 soluble (S) and insoluble (P) fractions. Data for GFP and Kal7 are from Fig. 3C. **E.** The TMT soluble fraction from cells

expressing Kal4 was loaded onto the S-400 column with BSA and phenol red as internal standards; the molecular weight standards were loaded between fractions 34 and 35.

Figure 10. The Sec14p and spectrin regions of Kal4 are both essential

A. pEAK Rapid cells expressing Δ Sec14Kal7 were fixed and stained for myc (red) and filamentous actin (FITC-phalloidin; green); myc staining alone (left) and the merged image (right) are shown. A single image through the bottom of the cells is shown. Red arrow, vacuole; green arrow, lamellipodia; green line, filopodia. **B.** pGEX vectors encoding GST-Sec14p or GST alone were expressed in BL21 and the fusion protein or GST were purified using glutathione agarose. The purified proteins (10 μ g/ml) were incubated with PIP strips (**Methods**) and bound protein was visualized with antibody to GST. **C.** pEAK Rapid cells expressing Sec14p were visualized as in **A.** Lamellipodia and filopodia are present in cells expressing Sec14p. **D.** pEAK Rapid cells expressing Sec14p were extracted in TMT; equal

aliquots of the soluble (S) and particulate (P) fractions were analyzed by SDS-PAGE and Sec14p was visualized using antibody to myc; most of the protein is soluble. **E.** Recombinant HisMycSec14p purified from bacteria was applied to the S-400 column with BSA and phenol red as internal standard. The calculated V_0 and elution positions of BSA and cytochrome c (determined in a separate analysis) are indicated.