An Isoform of Kalirin, a Brain-specific GDP/GTP Exchange Factor, Is Enriched in the Postsynaptic Density Fraction*

(Received for publication, May 17, 1999, and in revised form, December 6, 1999)

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Communication between membranes and the actin cytoskeleton is an important aspect of neuronal function. Regulators of actin cytoskeletal dynamics include the Rho-like small GTP-binding proteins and their exchange factors. Kalirin is a brain-specific protein, first identified through its interaction with peptidylglycine-α-amidating monooxygenase. In this study, we cloned rat Kalirin-7, a 7-kilobase mRNA form of Kalirin. Kalirin-7 contains nine spectrin-like repeats, a Dbl homology domain, and a pleckstrin homology domain. We found that the majority of Kalirin-7 protein is associated with synaptosomal membranes, but a fraction is cytosolic. We also detected higher molecular weight Kalirin proteins. In rat cerebral cortex, Kalirin-7 is highly enriched in the postsynaptic density fraction. In primary cultures of neurons, Kalirin-7 is detected in spine-like structures, while other forms of Kalirin are visualized in the cell soma and throughout the neurites. Kalirin-7 and its Dbl homology-pleckstrin homology domain induce formation of lamellipodia and membrane ruffling, when transiently expressed in fibroblasts, indicative of Rac1 activation. Using Rac1, the Dbl homology-pleckstrin homology domain catalyzed the in vitro exchange of bound GDP with GTP. Kalirin-7 is the first guanine-nucleotide exchange factor identified in the postsynaptic density, where it is positioned optimally to regulate signal transduction pathways connecting membrane proteins and the actin cytoskeleton.

Communication between membranes and the cytoskeleton involves small GTP-binding proteins of the Rho family and their regulators (1, 2). Many Dbl family members function as guanine-nucleotide exchange factors (3, 4), catalyzing the exchange of bound GDP with GTP on small GTP-binding proteins of the Rho family. Rho family proteins also regulate gene expression (5–7) and a growing number of other processes in cells (8).

Kalirin, a Dbl family member, was first identified as an interactor with the cytoplasmic COOH-terminal domain of membrane peptidylglycine-α-amidating monooxygenase, an important neuropeptide-processing enzyme (9, 10). Northern blot analysis identified several forms of Kalirin mRNA, with expression restricted to the central nervous system and highest levels in cerebral cortex and hippocampus. The Dbl homology (DH) domain of Kalirin, with its adjacent pleckstrin homology (PH) domain, is conserved among all Dbl family members (Fig. 1). Several Dbl family members, including Ost (11) and Tiam (12), are expressed in the brain, but only Kalirin is restricted to the central nervous system. AtT-20 corticotrope tumor cells overexpressing one form of Kalirin (Kalirin-8) and peptidylglycine-α-amidating monooxygenase developed longer and more branched neuritic processes (10). Both AtT-20 and Chinese hamster ovary cells overexpressing Kalirin-8 displayed rearrangements of the actin cytoskeleton (13).

Along with its DH and PH domains, Kalirin-8 has nine spectrin-like repeats, an Src homology 3 domain, and two proline-rich regions of the actin cytoskeleton (13).

Little is known about the normal forms of Dbl family proteins and their functions in neurons, since the majority of studies have utilized transfected cells, and endogenous proteins have been difficult to detect. We first searched for a form of rat Kalirin equivalent to human DUO. We demonstrate that a 7-kb transcript encodes a rat protein (Kalirin-7) equivalent to human DUO. Using a general Kalirin antiserum, we identify a family of endogenous Kalirin proteins in adult rat brain. Using an antisemir specific to the COOH terminus of Kalirin-7, we show that Kalirin-7 is highly enriched in the postsynaptic density fraction. We used cultured neurons to localize Kalirin-7 to spine-like structures and show that other forms of Kalirin are localized in the cell soma and neurites. We explore the ability of Kalirin-7 and its DH-PH domain to induce formation of lamellipodia and membrane ruffling by transient expression in NIH 3T3 fibroblasts and demonstrate that the DH-PH domain is an active guanine-nucleotide exchange factor for Rac1. Kalirin-7 may tether membrane proteins to the actin cytoskeleton, potentially providing a link between postsynaptic mem-

* This work was supported by National Institutes of Health Grants DA-00266 and DK-32948. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: DH, Dbl homology; PH, pleckstrin homology; PEST, Pro, Glu, Ser, Thr-rich protease-sensitive region (39); kb, kilobase pairs; bp, base pairs; nt, nucleotide(s); EST, expressed sequence tag; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; NPY, prepropeptide Y.

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Experiment 1. Domain structures and alignment of Kalirin-8 and related proteins. Domains indicated are DH, PH, PEST, fibronectin III-like (FnIII), and immunoglobulin-like (Ig). The region of the spectra domains of Kalirin used to immunize rabbits is indicated at the top (Kalirin-spectrin). For clarity, the 1899-residue form of rat Kalirin described earlier (10) is referred as Kalirin-8. UNC-73 A and B are alternatively spliced transcripts of a C. elegans gene involved in axon guidance and neuronal cell migration (16). TRIO is an interactor of transmembrane protein-tyrosine phosphatase LAR (15). Ost is an oncprotein cloned from an osteosarcoma cDNA library, which acts as a guanine-nucleotide exchange factor for RhoA and Cdc42 and binds activated Rac1 (11). Dbl is a guanine-nucleotide exchange factor for diffuse B-cell lymphoma, with guanine-nucleotide exchange factor activity for RhoA and Cdc42 (40). Dbl gene product was isolated from pBS.Kalirin-7 digested with the same two enzymes to generate pBS.Kalirin-7. The mammalian expression vector pSCEP was subcloned into pBS.Kalirin-8 digested with the same two enzymes to construct the other mammalian expression vector, pEAK10.His-Myc-Kalirin-7, was cloned into pSCEP.Myc-Kalirin-8 cut with the same two enzymes. An- tisense primer 5'-GGGAAAGCCGCGCTTTGGCATTTGTTT-TGG, bearing the restriction sites for NotI (underlined), and NotI (double underlined), pEAK10.His-Myc-DH-PH was constructed by in- corporating the NotI–NotI fragment of the DH-PH PCR product into pEAK10.His-Myc-cut with BspL111 and NotI. All constructs were verified by sequencing.

PCR sense primers were as follows: at 1–27 of P-CIP10a, 5'-GGCCTTGCTCCTGAGCCGACATAGT-3'; at 327–325 of P-CIP10b, 5'-GGCCTTGCTCCTGAGCCGACATAGT-3'; at 98–123 of human DUO, 5'-GGAGTTGAGCGGCGCTTTGGGACCAT-3'. Antisense primer was KAL 7-stop, nucleotides 4951–4925 of P-CIP10a (5'-AGTCCAGGCTGCGCGCTAAAAGCTAAG-3'). Reverse transcription was performed with Superscript II (Life Technologies, Inc.), and the resultant cDNA was amplified using the High Fidelity PCR System (Roche Molecular Biochemicals). Samples were denatured for 15 s at 94°C, annealed for 30 s at 62°C, and extended for 4 min at 72°C for a total of 30 cycles. Extension times were increased 20 s per cycle for the last 20 cycles.

Northern Analysis

A DUO-specific probe for use in Northern blot analysis was generated by RT-PCR. Total or poly(A)+ cerebral cortical RNA was reverse transcribed using oligo(dT)15. Amplification was carried out with Kalirin-7-specific sense 5'-GCACAGGCCCTCCGCAGGCACCAT-3' (nt 4885–4884) and antisense (5'-GGAAACATGTTGCCCTCTGA-3' (nt 4987–4967)) primers (nt numbers are for DUO). This 122-bp fragment was used to probe a Northern blot of rat cerebral cortex poly(A)+ mRNA.

Antiserum

Rabbit polyclonal antiserum JH2580–2582 was generated against Kalirin-spectrin-like repeats 4–7 (rat Kalirin-8, 517–576) (4). Affinity purification of these antisera did not alter the pattern observed on Western blots. Rabbit polyclonal antiserum JH2958 and JH2959 were generated at Covance (Denver, PA) by immunizing rabbits with the synthetic peptide comprising the COOH-terminal 19 residues of rat Kalirin-7 (5:487NLYPRHWHLPDGFSTYGDG) cross-linked to keyhole limpet hemocyanin with glutaraldehyde (2 mg of peptide, 5 mg of hemocyanin). This peptide was synthesized by Dr. Henry Keutmann (Endocrine Unit, Massachusetts General Hospital). Following ammonium sulfate precipitation, JH2959 was affinity-purified using the rat Kalirin-7 COOH-terminal peptide linked to Affi-Gel 10. Affinity-purified antiserum was eluted with 0.2 M glycine HCl, 0.1 M NaCl, 0.1% Triton X-100, pH 2.3, immediately neutralized with Tris-HCl, dialyzed into 100 mM sodium phosphate buffer, pH 7.4, and used at a dilution of 1:100.

Tissue Preparations

Parietal cortex and liver were dissected from adult female Harlan Sprague Dawley rats (Harlan), and tissues were homogenized in 10 volumes of 20 mM Tris-HCl (pH 7.5), containing phenylmethylsulfonyl fluoride (0.3 mg/ml), and a protease inhibitor mixture (lina trypsin inhibitor (50 μg/ml), leupeptin (2 μg/ml), benzamidine (16 μg/ml), and 1 mM sodium orthovanadate (5 μg/ml)) with five strokes of a glass Teflon homogenizer. Large debris and unbroken cells were removed by centrifugation at 5,000 × g for 5 min. The supernatant was centrifuged in a Beckman TL100 ultracentrifuge at 450,000 × gmax for 15 min to obtain crude soluble and crude particulate fractions.

Subcellular Fractions

Extracts of rat brain cortex and olfactory bulb were fractionated in isotonic buffer according to the method of Hutten et al. (18). An equal percentage of each fraction was analyzed by SDS-PAGE and Western blotting. Postsynaptic densities were purified from brain cortex and olfactory bulb from 10 female adult Holtzman rats (Harlan) using the protocol described by Carlin et al. (19) with the exception that the homogenization buffer A contained 4 mM HEPES, pH 7.5.

Transient Transfections

NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 containing 10% fetal bovine serum (HyClone) and 10% NuSerum (Collaborative Research). Briefly, cells grown on glass slides for 3 days to 40–60% confluence were transfected with 1 μg of plasmid DNA/4-cm² (pEAK10.His-Myc-DH-PH, pEAK10.His-Myc-Kalirin-7, or a plasmid carrying preproenkephalin Y (20)) and 4 μl of lipofectamine (Life
The Kalirin-spectrin repeat 1–3 probe detected mRNAs of 7.0 kb and larger. The Kalirin-spectrin repeat 1–3 probe detected mRNAs of 5.0 and 7.0 kb, while the Kalirin-spectrin repeat 1–3 probe detected mRNAs of 5.0 and 7.0 kb, while the Kalirin-spectrin repeat 1–3 probe detected mRNAs of 5.0 and 7.0 kb. The position of the peptide used to generate the Kalirin-7-specific antisera is indicated by the bar.

The DH-PH protein was enriched using the Talon affinity resin (Amersham Pharmacia Biotech). For expression of DH-PH, pEAK10-His-Myc.DH-PH using Lipofectamine (Life Technologies, Inc.). The DH-PH protein was enriched using the Talon affinity resin (Qiagen) following the manufacturer’s instructions. The GDP/GTP exchange assay was performed according to Debant et al. (15) and Steven et al. (16).

**RESULTS**

Cloning of Kalirin-7—Human DUO (14) and rat Kalirin-8 are >98% identical in their 1612-amino acid region of overlap, differing at only 22 positions (Fig. 2A). This degree of identity strongly suggests that DUO and Kalirin are encoded by equivalent human and rat genes, respectively. Human DUO and rat Kalirin-8 differ at their NH2- and COOH-terminal ends. Human DUO has a 22-residue N terminus preceding the homologous region, while two different rat Kalirin N termini (10a, 4 residues; 10b, 24 residues) were identified (10). The three NH2-terminal sequences share no sequence similarity. Recombinant Kalirin-8 included the 4-residue N terminus, because transcripts with this start site were more efficiently expressed following in vitro transcription/translation. At its COOH terminus, DUO contains 20 residues not present in Kalirin-8, which they were fed with growth medium. After 1 day, the medium was replaced with Dulbecco’s modified Eagle’s medium/F12 containing no serum for 16 h. Cells were fixed in 4% formaldehyde in phosphate-buffered saline (50 mM NaCl, pH 7.5, 150 mM NaCl) for 30 min at room temperature, permeabilized, and stained as described previously (17). Cell morphology was evaluated by analyzing approximately 30 transfected cells and counting the fraction of cells displaying a particular phenotype. Protein expression levels in transfected cells were compared by quantifying the intensities of immunofluorescence upon detection with Myc antibody, using the Scion image software.

**Primary Cultures of Rat Cortical and Olfactory Bulb Neurons**

Olfactory bulbs dissected from postnatal rat olfactory brains were dissociated by treatment with porcine pancreas trypsin (Sigma) (5 mg/ml) and Benzonase (EM Science) (4 mg/ml) for 20 min at 37 °C. Cells were plated onto polylysine-coated plastic slides and grown in Dulbecco’s modified Eagle’s medium/F12 containing 10% fetal bovine and 10% NuSerum. Plasma membrane preparations were isolated from cell cultures as described above. Rat cortical hemispheres dissected from embryonic day 17 or 18 rat embryos were dissociated by treatment with papain (Sigma) (200 units) for 40 min at 37 °C. Cells were plated onto polylysine- and laminin-coated glass coverslips and grown in Neurobasal medium (Life Technologies, Inc.) containing 2% B-27 supplement (Life Technologies, Inc.), 2.1 mM l-glutamine (Sigma), and 5 μg/ml gentamycin. After 7 days in culture, cells were fixed with 4% formaldehyde for 30 min and stained as described above.

GST-Rac1 was expressed in *Escherichia coli* and purified on glutathione affinity resin (Amersham Pharmacia Biotech). For expression of DH-PH, pEAK-Rapid cells (Edge Biosystems) were transiently transfected with pEAK10-His-DH-PH using Lipofectamine (Life Technologies, Inc.). The DH-PH protein was enriched using the Talon affinity resin (Qiagen) following the manufacturer’s instructions. The GDP/GTP exchange assay was performed according to Debant et al. (15) and Steven et al. (16).

To find out if rats express a form of Kalirin equivalent to human DUO, we performed RT-PCR on rat cerebral cortex...
poly(A)^+ RNA (Fig. 2B). A cDNA fragment of the predicted size was amplified with a sense primer common to human DUO and rat Kalirin-8 and an antisense primer specific to human DUO (based on human EST T74341). EST T74341 is identical to Kalirin and DUO up to their 3'-divergence point, after which it is identical to DUO. The polypeptide encoded by the PCR product was identical to Kalirin-8 until the 3'-divergence point, after which the 20 COOH-terminal residues were identical to the corresponding region of human DUO and were followed by a stop codon.

The 5'-ends of Kalirin-8 and DUO diverge at exactly the same point at which the two previously identified Kalirin 5'-ends (10a and 10b) diverged. To determine which of the three potential Kalirin 5'-ends encode Kalirin mRNAs with the DUO-like 3'-end, we amplified cDNA with sense primers specific for each of the three 5'-ends (10a, 10b, DUO) and a DUO-specific 3' antisense primer. Products of the predicted size (5 kb) were obtained with all three primer pairs, indicating that all three 5'-ends were present in mRNAs that include a DUO-type 3'-end. Southern analysis using a cDNA probe to Kalirin-spectrin domains 1–3 (nt 580–1531) was used to confirm that the amplified products correspond to Kalirin (Fig. 2C).

Northern blot analysis was performed to determine the sizes of the Kalirin mRNAs including various regions of Kalirin (Fig. 2D). A probe specific for the DUO-type 3'-end detected 5.0- and 7.0-kb Kalirin transcripts and no larger forms. Hybridization with a probe corresponding to the first three spectrin-like repeats of Kalirin (spectrin 1–3) revealed that the 7-kb transcripts, as well as several longer transcripts, contained this region, while the 5-kb transcript did not. A probe specific for the 10b 5'-end also identified 7-kb transcripts (Fig. 2D). Based on the PCR data shown in Fig. 2C, 7-kb Kalirin transcripts could also include the DUO- and 10a 5'-ends. Since the functional significance of the different 5'-ends is not yet apparent, the three Kalirin proteins encoded by the 7-kb mRNA will be referred to collectively as Kalirin-7 (Fig. 2E).

A full-length Kalirin-7 cDNA was constructed by joining the 5'-end of Kalirin-8, bearing the 10a 5'-end (10), with the DUO-type 3'-fragment. The open reading frame of Kalirin-7 encodes a protein of 1644 residues (190 kDa). The Kalirin-7 protein includes the 10a sequence, nine spectrin-like repeats, a DH domain, and a PH domain (Fig. 2E). It lacks the Src homology 3 domain present in Kalirin-8 and contains instead a unique 20-residue COOH terminus. The final COOH-terminal 3 residues correspond to the recognition motif for proteins with PDZ domains (S/T)XV, suggesting that Kalirin-7 and Kalirin-5, unlike larger Kalirin proteins, could interact with such proteins.

**Kalirin Antisera Detect Multiple Proteins in Rat Brain**—Two sets of antisera were used to detect endogenous Kalirin-related proteins. Kalirin-spectrin antisera JH2581 and JH2582 were raised against recombinant spectrin-like repeats 4 to 7 of Kalirin (13). We tested the cross-reactivity of the Kalirin-spectrin antibodies with the homologous fragments of TRIO and Ost.
The Kalirin-spectrin antisera had little (JH2581) or no (JH2582) cross-reactivity with the corresponding regions of TRIO and Ost (Fig. 3A). We used a 19-residue synthetic peptide corresponding to the unique COOH terminus of Kalirin-7 to generate Kalirin-7-specific antisera (JH2958 and JH2959).

The two Kalirin-spectrin antisera and a Kalirin-7 antiserum were used to detect Kalirin proteins in crude soluble and particulate fractions of rat brain cortex homogenate (Fig. 3B). Both Kalirin-spectrin antisera detected a family of proteins in the soluble and particulate fractions. The bands recognized by both Kalirin-spectrin antisera and not present in the liver, a tissue that does not express detectable levels of Kalirin mRNA by Northern blotting (10), were assumed to represent forms of Kalirin: 115, 190, 370, 420, and 470 kDa (Fig. 3B). The 150-kDa protein is considered nonspecific because it is not detected by both Kalirin-spectrin antisera and corresponds to a cross-reactive protein in liver. The 190-kDa Kalirin protein was enriched in the particulate fraction.

The Kalirin-7 antibodies detected a major 190-kDa protein and a minor 115-kDa protein (Fig. 3B). Proteins of the same size were detected by both Kalirin-spectrin antisera. The 190-kDa protein detected by the Kalirin-7 antiserum is the mass predicted for Kalirin-7, and the 115-kDa protein is small enough to be encoded by the Kalirin-5 transcripts. Staining by the Kalirin-7 antiserum was eliminated by inclusion of excess antigen (Fig. 3C). Since Kalirin-7 is enriched in the particulate fraction (Fig. 3B), the abundance of Kalirin-7 in extracts varies with the extraction conditions used. In particulate fractions of adult rat cerebral cortex, Kalirin-7 was one of the most abundant Kalirin proteins. To our surprise, we found it difficult to detect a protein with the mass of Kalirin-8 (217 kDa), suggesting that Kalirin-8 is not an abundant isofrom.

To confirm the identity of this 190-kDa protein as Kalirin-7, we performed an immunodepletion experiment (Fig. 3D). Since we used a crude soluble fraction of the rat brain to reduce background problems during immunoprecipitation, Kalirin-7 was not enriched in the starting material (IN-Cortex-S). Kalirin-7 antibody was incubated with the sample, and antibody-bound proteins were collected with protein A-agarose beads. Incubation with the Kalirin-7 antibody specifically depleted the 190-kDa Kalirin protein from the brain homogenate, while the other Kalirin-related proteins remained in the flow-through fraction (F.T.).

Preincubation of the Kalirin-7 antiserum with antigenic peptide or use of preimmune serum prevented immunoprecipitation of the 190-kDa protein, demonstrating that binding to the antibody was specific. Further work is in progress to uncover the identity of the other Kalirin forms; the 115-kDa protein specifically bound to the Kalirin-7 antibody could represent the protein encoded by the Kalirin-5 transcript.

Subcellular Distribution of Kalirin Proteins in Rat Brain—
Based on Western blot analysis of cerebral cortex homogenates, Kalirin-7 is a major form of Kalirin in the rat brain and is recovered primarily from particulate fractions. To further investigate its localization, we used differential centrifugation to prepare fractions enriched in different organelles (Fig. 4A) (18); antibodies to specific marker proteins were used to verify the identity of each subcellular compartment. We used a method optimized for the isolation of synaptosomes, pinched off presynaptic boutons with their enclosed synaptic vesicles and cytosol (18); the apposed postsynaptic membrane remains strongly attached (18, 19). An equal proportion of each subcellular fraction was analyzed.

As expected, using the Kalirin-spectrin antibody, multiple Kalirin proteins were identified in the soluble, cytosolic fraction (fraction S3). Fraction P3, enriched in internal membranous organelles such as the endoplasmic reticulum and Golgi apparatus (as indicated by BiP and TGN38 markers, respectively), contained more Kalirin-7 than did the cytosolic fraction (S3); the other forms of Kalirin were equally abundant in P3 and S3. The crude synaptosomal pellet (P2) was subjected to hypotonic lysis and then centrifuged to pellet a fraction enriched in synaptosomal plasma membranes and attached postsynaptic membranes (LP1). The resultant supernatant was separated into fractions enriched in synaptosomal cytosol (LS2) and synaptic vesicles (LP2). A cytosolic marker (GAD-65), was recovered from S3 and LS2; very little Kalirin was recovered in LS2. In contrast, the synaptosomal plasma membrane fraction (LP1) was substantially enriched in Kalirin-7. The synaptic vesicle fraction (as shown by the synaptophysin/p38 marker) did not contain detectable levels of any Kalirin proteins. A significant proportion of the total Kalirin-7 was recovered in the synaptosomal membrane (LP1) fraction, while the higher
molecular weight Kalirin proteins were evenly distributed among the cytosolic (S3), P3, and LP1 fractions.

**Kalirin-7 Is Highly Enriched in the Postsynaptic Density Fraction**—Since a significant amount of the Kalirin-7 fractionated with the synaptosomal membrane fraction (LP1), we explored the possibility that Kalirin-7 was concentrated at postsynaptic densities. The postsynaptic density fraction prepared by the method of Carlin et al. (19) is enriched in postsynaptic densities (electron-dense structures located on the postsynaptic sides of neuronal synapses) along with the apical presynaptic membranes. These structures are enriched in receptors and ion channels along with cytoskeletal proteins and scaffolding proteins thought to anchor receptors and signaling molecules (22).

We generated a synaptosomal fraction (P2) by sucrose gradient centrifugation (19, 23). The proteins of the postsynaptic density are resistant to detergent extraction (19, 23). We compared the behavior of Kalirin-7 to that of two PSD markers (PSD95 (23) and Chapsyn-110 (24)) and a presynaptic membrane-associated protein, Munc18-1 (25). Equal amounts of protein from each fraction were examined. Kalirin-7 was highly enriched in the fraction that remained insoluble after one or two rounds of Triton extraction (1-Triton, P and 2-Triton, P (Fig. 5)). The higher molecular weight Kalirin proteins were progressively depleted from the Triton-insoluble pellets by Triton extraction. Kalirin-7 was enriched in the postsynaptic density fraction (“2-Triton”-P), PSD-95 and Chapsyn-110 co-fractionated with Kalirin-7 throughout the Triton extractions. Munc18-1, a peripheral membrane protein (25), was progressively depleted from the Triton-insoluble pellets.

Extraction of the 1-Triton pellet with Sarcosyl (N-lauroylsarcosinate), a much stronger detergent, leaves behind a “postsynaptic density core,” which contains a more restricted set of proteins (22). After extraction with Sarcosyl, a smaller but still significant fraction of the Kalirin-7 remains in the “postsynaptic density core” (Sarcosyl-P) (19, 23). Chapsyn-110 and PSD95 are more prevalent in the “postsynaptic density core” than Kalirin-7.

**Kalirin Immunostaining in Cultured Neurons**—To date, few endogenous Dbl-family members have been localized at a subcellular level in their native tissues. To examine the cellular and subcellular localization of Kalirin proteins, we prepared primary cultures of cortical and olfactory bulb neurons. To determine whether Kalirin were expressed in neurons and/or glia, we stained cultures simultaneously with a polyclonal Kalirin-spectrin antibody and a monoclonal antibody for neuron-specific tubulin (Fig. 6). Staining of the same cells with both the Kalirin-spectrin and neuron-specific tubulin antibodies showed that Kalirin proteins were expressed in neurons (Fig. 6) in both types of culture. All neuron-specific tubulin-positive cells also expressed Kalirin, and all cells that stained with Kalirin also expressed neuron-specific tubulin.

Western blot analysis indicated that only the higher molecular weight forms of Kalirin were expressed in the olfactory bulb at this developmental stage; Kalirin-7 was undetectable (Fig. 7A). In contrast, a significant amount of 190-kDa Kalirin-7 was expressed in the cerebral cortex at this developmental stage. Thus, by using the Kalirin-spectrin antibody to visualize Kalirin proteins in olfactory bulb neurons, we localized the higher molecular weight forms of Kalirin, not Kalirin-7 (Fig. 7B). The higher molecular weight Kalirin proteins were most prevalent in the cell soma (s), often concentrated in the perinuclear region, but they were also detectable in processes (p) (Fig. 7B). To detect the higher molecular weight forms of Kalirin in cortical neurons, we stained 2-day-old cultures, which express very low levels of Kalirin-7 (Fig. 7, C and D). The staining patterns observed with the Kalirin-spectrin antibody in cultured cortical neurons resembled the patterns observed in cultured olfactory bulb neurons.

Because the DH domain of Kalirin binds specifically to nucleotide-depleted Rac1 (9) and is highly similar to the first DH domain of TRIO and UNC-73, both nucleotide exchange factors for Rac1, we stained neurons simultaneously with Kalirin-spectrin and Rac1 antibodies (Fig. 7, B–D). Rac1 was especially enriched in neuronal processes and the cortical (subplasmalemmal) region. The high molecular weight Kalirin proteins in olfactory bulb neurons were detected in these same regions but were more prevalent in the cell soma (Fig. 7B). In cortical neurons, Kalirin staining coincides with Rac1 in the subplasmalemmal regions of the soma (s) and in some of the processes (p) (Fig. 7, C and D). In long processes (p), Rac1 was significantly enriched in the distal portion, where Kalirin was also enriched (Fig. 7D).

With time, cortical cultures express Kalirin-7 along with the high molecular weight Kalirin isofoms (Fig. 7A). Thus, by staining older cortical cultures with the Kalirin-7 antibody, we asked whether Kalirin-7 exhibited a distinct pattern of localization (Fig. 7E). This allowed us to compare the staining with the Kalirin-spectrin and Kalirin-7 antibodies. Kalirin-7 was visualized as beads along the sides of neurites and the cell soma (Fig. 7E). This staining pattern is typical of synaptic proteins and is dramatically different from that of the higher molecular weight forms of Kalirin (Fig. 7, C and D). Little Kalirin-7 was detected in the cell body or the cytoplasm of neurites. Kalirin-7 was undetectable in 1-day-old cortical cultures. Rac1 was detectable in the processes and at the plasma membrane, in areas immediately adjacent to Kalirin-7. Signals from both Kalirin antisera in both types of culture were blocked by preincubation with the appropriate antigen (not shown), demonstrating that the signal was specific.

**Transient Expression of Kalirin-7 and DH-PH Domain of Kalirin in NIH 3T3 Fibroblasts**—To determine whether Kalirin affects the cytoskeletal organization of NIH 3T3 fibroblasts in a manner typical of factors that activate Rho-like small GTP-binding proteins, we transiently transfected these cells with plasmids encoding full-length Kalirin-7 or its isolated DH/PH domain (DH-PH) (Fig. 8). This system has allowed sensitive and specific detection of the activation of particular...
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Rho-like proteins, through analysis of rearrangements in the actin cytoskeleton and effects on cellular morphology that are specific for each Rho subfamily member (8).

As a control, we transfected fibroblasts with a plasmid encoding preproenpeptide Y (NPY), a soluble, secreted protein that should not affect cellular morphology and the actin cytoskeleton (Fig. 8A). NPY was localized to the Golgi area and punctate structures in processes. NPY-expressing cells were polygonal with concave edges and were indistinguishable from nontransfected cells. The actin cytoskeleton was not different from nontransfected cells. Thus, neither this method of transfection nor expression of a soluble, secreted protein affected the cytoskeleton and cell morphology.

Fibroblasts expressing full-length Kalirin-7 exhibited a markedly different cellular morphology from that of nontransfected cells (Fig. 8B). Kalirin-7 expressing cells were mostly flat, with massive generation of lamellipodia (95% of transfected cells). Kalirin-7-expressing cells also had fewer stress fibers, and actin staining was generally punctate. Kalirin-7 was distributed throughout the entire cytoplasm, with more intense staining in the perinuclear region. In some cases, we observed Kalirin-7 enrichment at the edges of protruding lamellipodia. Filamentous actin was also enriched at the edges of the lamellipodia.

To determine whether induction of these changes in cell morphology and cytoskeletal organization resembled those caused by activation of Rac1, we expressed constitutively active Rac1 (Rac1-Q61L) (5) in NIH3T3 cells (Fig. 8D). These cells also exhibited massive lamellipodia formation, a phenotype similar to that induced by Kalirin-7 or its DH-PH domain. The punctate localization of filamentous actin observed in cells expressing Kalirin-7 or DH-PH was not observed in cells expressing Rac1-Q61L.

In Vitro GDP/GTP Exchange Activity of the DH-PH Domain of Kalirin—The DH-PH domain of Kalirin is most closely homologous to the DH1-PH1 domains of TRIO (15) and UNC-73 (16); these proteins are both exchange factors for Rac1. Moreover, Kalirin-8 was previously shown to bind nucleotide-depleted Rac1, but not nucleotide-depleted RhoA or Cdc42 (10). Hence, we tested whether the DH-PH domain of Kalirin could activate in vitro the release of bound GDP from Rac1 (Fig. 9). The His.Myc-tagged DH-PH domain of Kalirin-7 was expressed transiently and enriched by binding to a metal chelate resin. While in the presence of buffer only, the release of [3H]GDP from Rac1 was slow; in the presence of the DH-PH domain of Kalirin-7, the release of [3H]GDP was significantly accelerated.

DISCUSSION

Based on analysis of both RNA and protein, the Kalirin gene encodes a variety of proteins in the rat central nervous system.
The Kalirin-spectrin antibodies detected proteins ranging in size from 115 to 470 kDa in homogenates of adult rat brain. Kalirin mRNAs ranging in size from 5 to over 11 kb were detected on Northern blots (Fig. 2). The probability that these proteins are splice variants is supported by the existence of at least three different Kalirin NH2-terminal sequences (10a, 10b, and DUO) and at least two different COOH-terminal sequences (Kalirin-7 and -8); the higher molecular weight forms of Kalirin may include COOH-terminal domains similar to those of TRIO and UNC-73A (Fig. 1).

In this study, we focused on characterizing one of the most abundant Kalirin transcripts, Kalirin-7. Kalirin-7 is the rat equivalent of human DUO and differs from the previously characterized Kalirin transcript, Kalirin-8, at the 3'-end. While the COOH-terminal region of Kalirin-8 includes an Src homology 3 domain and several PEST sequences, the COOH-terminal region of Kalirin-7 lacks these domains and has instead a 20-amino acid segment whose sequence could allow interactions with PDZ domain-containing proteins. Although heterogeneity is observed at the 5'-end of Kalirin-7, with three different 5'-sequences expressed with the Kalirin-7 3'-end (Fig. 2, C and D), the functional significance of these differences is not clear.

Using antisera to the spectrin-like repeat region of Kalirin, we were able to detect the naturally occurring forms of Kalirin in rat brain. Few Dbl family members have been characterized in their native tissues. Although more work is required to determine the identity of each of the forms of Kalirin, concomitant use of the Kalirin-spectrin and Kalirin-7 antisera clearly indicates that the 190-kDa Kalirin-7 protein and the higher molecular weight Kalirin isoforms occupy different subcellular locations. This conclusion is supported both by subcellular fractionation of rat brain and immunofluorescence localization of Kalirin in cultured neurons. The distinctive subcellular distribution of Kalirin-7 suggests that its functions are distinct from those of the higher molecular weight forms of Kalirin.

Biochemically, Kalirin-7 is enriched in the postsynaptic density fraction, while the higher molecular weight Kalirin proteins are equally distributed between the cytosol and membranous organelles (Fig. 5). By immunostaining, Kalirin-7 is enriched in neurites in structures with the properties of spines, while the Kalirin-spectrin antiserum visualizes a substantial amount of Kalirin in the cell soma. Depletion of Munc18-1, a protein associated with the presynaptic membrane by strong protein-protein interactions, from the postsynaptic density fraction suggests that Kalirin-7 is enriched on the postsynaptic density.
side. The COOH terminus of Kalirin-7 (STYV) matches the recognition sequence for proteins with PDZ domains (Thr/Ser/Val) (26, 27). Many of the proteins identified in the postsynaptic density fraction (e.g. PSD-95 and Chapsyn-110; markers shown in Fig. 5) have PDZ domains. Kalirin-7 localization to the PSD fraction may require interactions of its unique COOH terminus with PSD-localized PDZ domain proteins.

We show that expression of Kalirin-7 or its DH-Ph domain in 3T3 cells alters cell morphology and the actin cytoskeleton in a manner similar to that of activated Rac1, with disruption of stress fibers and formation of lamellipodia and ruffles (1, 2). The first DH domain of TRIO and the DH domain of UNC-73B are highly similar to Kalirin, and both activate Rac1 in vitro and in transfected cells. In vitro, the DH-PH domain of Kalirin is indeed a GDP/GTP exchange factor for Rac1. Since Kalirin-7 can induce Rac1-like cytoskeletal rearrangements in 3T3 cells, Kalirin-7 may fulfill a similar function at the sites of its localization in vivo in neurons.

Kalirin-7 is the first guanine nucleotide exchange factor localized to the postsynaptic density. Rho-like proteins have not yet been implicated in synaptic function, but actin filaments are abundant in postsynaptic densities and dendritic spines (29). Cytoskeletal rearrangements are believed to be important in receptor clustering (30), dendritic remodeling (31, 32), generation and dynamics of the postsynaptic density. Thus, Kalirin may participate in the generation and dynamics of the postsynaptic density.

Based on immunostaining and subcellular fractionation, the higher molecular weight forms of Kalirin are found in both the cell soma and in neurites. Kalirin may subserve different functions in these different locations. Overexpression of Kalirin-8 in AtT-20 cells affected the biosynthetic and endocytic trafficking of a membrane protein localized to the trans-Golgi network and immature secretory granules. Kalirin proteins localized to the cell soma may play a role in the trafficking of both newly synthesized and endocytosed membrane proteins. In cultured cortical neurons, the higher molecular weight forms of Kalirin were abundant in neuritic processes. A role for Kalirin in neurite growth is suggested by the fact that, when stably expressed in AtT-20 cells, Kalirin-8 induced formation of longer and more branched neurites (9). Small GTP-binding proteins of the Rho subfamily have been implicated in dendritic growth and remodeling in cortical neurons (35) and axonal development in cerebellar Purkinje cells (33). In general, Rac1 appears to mediate neurite extension (36–38). In C. elegans, UNC-73 is involved in axon growth (16). Kalirin forms are homologous to UNC-73 and may fulfill a similar function in mammalian neurons.