

Actinfilin, a Brain-specific Actin-binding Protein in Postsynaptic Density*

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Ying Chen, Rachel Derin, Ronald S. Petralia‡, and Min Li§

From the Department of Physiology and §Department of Neuroscience, School of Medicine, Johns Hopkins University, Baltimore, Maryland 21205 and the ‡NIDCD, National Institutes of Health, Bethesda, Maryland 20892-8027

The dynamic assembly and disassembly of actin-based cytoskeleton is closely linked to the changes in the postsynaptic density in both number and shape, which is thought to be important in forming long-term memory. Thus, regulation of actin filaments may play a critical role in contributing to the formation of long-term memory. Here, we report the cloning of actinfilin, a brain-specific Kelch protein, which interacts with F-actin. Actinfilin contains an amino-terminal POZ/BTB domain and carboxyl positioned six tandem Kelch repeats that presumably form six blades of β -propeller structure of the Kelch domain. Co-immunoprecipitation analyses showed that the amino-terminal POZ domain mediated actinfilin-actinfilin interaction. The recombinant Kelch domain alone was sufficient to mediate binding to F-actin. Immunohistochemistry studies of rat brain sections suggested that actinfilin is broadly expressed in neurons of most regions of the brain. The subcellular localization of actinfilin was studied by biochemical fractionation and immunogold labeling. The results showed the postsynaptic density distribution of actinfilin. Together, these results indicate that actinfilin may be a key player in the actin-based neuronal function.

Cytoskeleton structures play a role in determining the static and dynamic morphology of subcellular structures in neurons. Activity-induced actin assembly and disassembly is thought to play a critical role in shaping the dendritic spine, a subcellular protrusion that is found in more than 90% of glutamatergic synapses. The changes in number, size, and morphology of spines are linked to long-term memory (1–6). Increasing evidence suggests that the dynamic reorganization of actin filament is probably mediated by several classes of molecules including small second messengers and regulatory protein factors that interact with G- or F-actin. Thus, molecular identification and functional understanding of regulatory components may provide important insights into both the dynamic morphology of synaptic structure and the activities that are coupled with

polymerization, stability, and subcellular anchoring of actin filaments.

CAP70¹ is a scaffolding protein that was purified from the kidney on the basis of its interaction with the carboxyl terminus of cystic fibrosis transmembrane conductance regulator (7). Like other PDZ domain-containing proteins, such as PSD95 or INAD (inactivation no afterpotential D), the CAP70 coding sequence is essentially composed of four tandemly positioned PDZ domains. In contrast to other known PDZ domain-containing proteins, CAP70 has the activity to dynamically alter the oligomerization state of the cystic fibrosis transmembrane conductance regulator channel, thereby inducing potentiation of cystic fibrosis transmembrane conductance regulator-induced chloride channel activity (7). The native CAP70 protein was found in actin-rich domains, such as the apical surfaces of epithelial cells in the small intestine and kidney as well as floppodia-like structures in cultured epithelial cells.² In addition, the CAP70 protein was also detected in neuronal tissue, where the membrane receptors that interact with CAP70 remain unknown. Interestingly, in contrast to what was found in the kidney and small intestine, the brain CAP70 protein was insoluble and co-sedimented with cytoskeletal components that are abundant in actin. However, purified recombinant CAP70 protein behaved as a monomer and displayed no detectable interaction with purified actin. Together, the evidence suggests that CAP70 may interact with actin filaments and/or other cytoskeletal components via an intermediate adaptor protein that specifically links CAP70 to cytoskeletal components, thereby connecting the CAP70 interacting cell surface receptors and ion channels to intracellular cytoskeleton structures.

To identify genes that encode CAP70 interacting proteins, we screened a yeast two-hybrid cDNA library using a GAL4-CAP70 fusion protein. The identified candidate cDNAs were tested using a combination of biochemical and molecular approaches. Here, we report on one of the identified cDNAs, which encodes a previously unknown actin-binding protein with restricted expression in the brain.

EXPERIMENTAL PROCEDURES

Materials—Molecular cloning reagents were purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI). Glutathione-Sepharose 4B was from Amersham Biosciences. Rabbit anti-PSD95 antibody (1:5,000 for immunoblot) and rabbit anti-CAP70 antibody (1:2,000 for immunoblot) were generated against the GST (glutathione S-transferase) fusion protein. Goat anti-rabbit lactate dehydrogenase (1:500 for immunoblot) was purchased from Chemicon International (Temecula, CA). Mouse anti-c-Myc (9E10, 1:100 for immunoblot) and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF505655.

The amino acid sequence of actinfilin can be accessed through NCBI Protein Database under NCBI accession number gi17434913.

§ To whom correspondence should be addressed: Dept. of Physiology, WBSB 216, Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205. Tel.: 410-614-3692; Fax: 410-614-1001; E-mail: minli@jhmi.edu.

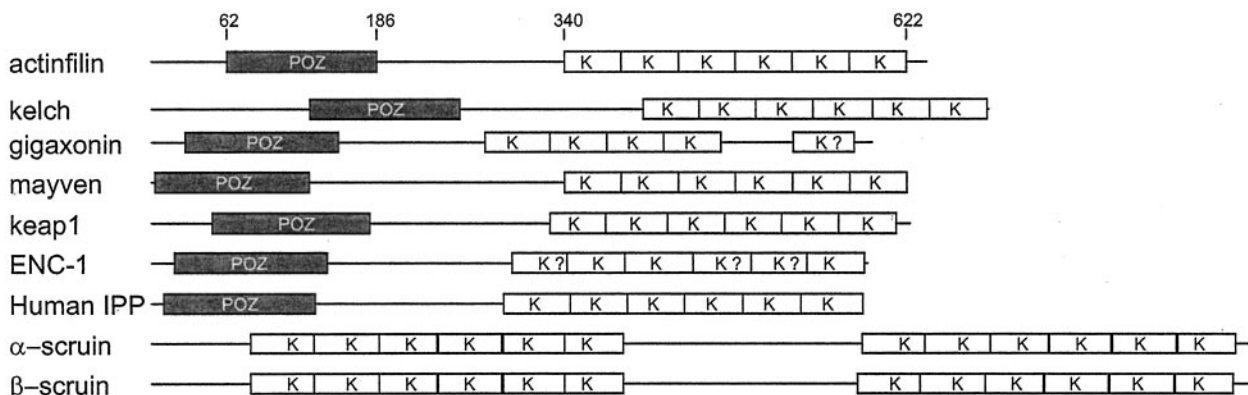
¹ The abbreviations used are: CAP70, cystic fibrosis transmembrane conductance regulator-associated protein of 70 kDa; GST, glutathione S-transferase; BTB, broad-complex, tramtrack and bric-a-brac; POZ, poxvirus and zinc finger; PSD95, postsynaptic density protein of 95 kDa; CHES, 2-(cyclohexylamino)ethanesulfonic acid.

² S. Wang and M. Li, unpublished results.

a.

MQPRGERPAGRTQSPHESSPGPGPEAPPPQPPAPEAERARPRQARPAAPMEGAMQLLSREGHSVA
 HNSKRHYHDAFVAMSRMRQGLLCDIVLHVAAKEIRAHKVVLASCSYPFHAMFTNEMSESRQTHVT
 LHDIDPQALDQLVQFAYTAIEIVVGEQVQTLPAASLLQLNGVRDACKFLLSQLDPSNCLGIRGF
 ADTHSCSDLLKAAHRYVLQHFVDVAKTEEFMLLPLKQVLELVSSDSLNVPSSEEDVYRAVLSWVKHD
 VDRRQHVPRMLMKCVRLPLLSRDFLLGHVDAESLVRHHPDCKDLLIEALKFHLLPEQRGVLTGTSRT
 RPRRCEGAGPVLFAVGGGSLFAIHGDCEAYDTRTRDRWHVVASMSTRRARVGVAAVGNRLYAVGGYD
 GTSDLATVESYDPVTNTWQPEVSMGTRRSCLGVAALHGLLYAAGGYDGASCLNSAERYDPLTGTWT
 SIAAMSTRRRYVRVATLDGNLYAVGGYDSSSHLATVEKYEPQVNSWTPVASMLSRSSAGVAVLEG
 ALYVAGGNDGTSCLNSVERYSTKAGAWESVAPMNIIRRSTHDLVAMDGWLYAVGGNDGSSSLNSIEK
 YNPRNTNKWVAASCMFTRRSSVGVAVLELLNFPSSPTLSVSSSTSL

b.



c.

| | |
|---------------------|---|
| actinfilin kelch R1 | VLFVAVGGGSLFAIHGDCEAYDTRTRDRWHVVASMSTRRARVGVAAVGN |
| actinfilin kelch R2 | RLYAVGGYDGTSDLATVESYDPVTNTWQPEVSMGTRRSCLGVAALHG |
| actinfilin kelch R3 | LLYAAGGYDGASCLNSAERYDPLTGTWTSIAAMSTRRRYVRVATLDG |
| actinfilin kelch R4 | NLYAVGGYDSSSHLATVEKYEPQVNSWTPVASMLSRSSAGVAVLEG |
| actinfilin kelch R5 | ALYVAGGNDGTSCLNSVERYSTKAGAWESVAPMNIIRRSTHDLVAMDG |
| actinfilin kelch R6 | WLYVAGGNDGSSSLNSIEKYNPRNTNKWVAASCMFTRRSSVGVAVLEL |

Fig. 1. *a*, deduced amino acid sequence of rat actinfilin (accession number AF505655) is shown and the regions corresponding to protein domains are *underlined*. A proline-rich region is located at the amino terminus, followed by a POZ/BTB domain. Six Kelch repeats are found at the carboxyl terminus. *b*, scale-proportioned schematic representation of the actinfilin and its homologous Kelch family proteins. The Kelch proteins are characterized by the tandemly positioned Kelch motifs, each with ~50 amino acids (represented by a *blank rectangle* with the letter *K*). The Kelch repeats in each protein may be variable in number. Many Kelch proteins also contain a POZ/BTB domain (represented by a *filled rectangle* and marked by *POZ*). In addition to actinfilin, the domain organization of the following proteins is also shown: *Drosophila melanogaster* Kelch protein (long form, gi1079096), *Homo sapiens* gigaxonin (gi11545731), *H. sapiens* Mayven (gi13431614), *Mus musculus* Keap1 (gi3894323), *M. musculus* ENC1 (gi6679645) (identical to *H. sapiens* NRP/B(gi3309573)), *H. sapiens* IPP (gi5174473), and *Limulus polyphemus* α - and β -scruin (gi2497944 and gi2497945). The *thicker lines* in α - and β -scruin between the two Kelch repeats are because of short amino acid gaps between the two domains. *c*, alignment of the six Kelch repeats of actinfilin. The Kelch motif is ~50 amino acids. The signature of the Kelch repeats is a double glycine (GG) and a tyrosine-tryptophan combination that is separated by six amino acids (as marked by an *underline*).

mouse anti-actin (C-2, 1:100 for immunoblot) were from Santa Cruz (Santa Cruz, CA). Mouse anti-FLAG (M5, 1:2,500 for immunoblot) and mouse anti-hemagglutinin (F-7, 1:2,500 for immunoblot) were from Sigma. All other reagents were purchased from Sigma, except as indicated.

Yeast Two-hybrid Screening—The full-length of CAP70 was subcloned into a yeast expression vector, pPC97, in a fusion with the GAL4 DNA-binding domain. This construct was transformed in yeast strain PJ69-4a (*MATa trp1-90; leu2-3,112 ura 3-52 his3-200 gal4 Δ gal80 Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*) (8) using the lithium acetate method (9). Transformants were selected on leucine-deficient (Leu⁻) medium and subsequently transformed with an oligo(dT)-primed rat hippocampal cDNA library subcloned into pPC86. Positive clones were selected on triple-deficient plates (Leu⁻, Trp⁻, and adenine⁻). Their plasmids were isolated using the RPM Yeast Plasmid isolation kit (Bio 101, Vista, CA) and transformed into *Escherichia coli* HB101. The *E. coli* clones containing the pPC86 construct were rescued and their plasmids were isolated. The positive clones were co-transformed into yeast PJ69-4a with either the bait vector or the original pPC97 vector to confirm the interaction.

Antibody Generation and Western Blot Analysis—The cDNA encoding the actinfilin NH₂-terminal domain (amino acids 1–344) was subcloned into the PMBP vector in a fusion with an NH₂-terminal MBP tag

and transformed in *E. coli* BL21. The fusion protein was purified by an amylose affinity column (New England Biolabs, Beverly, MA). The purified fusion protein was then used to immunize rabbits.

Various organs of Sprague-Dawley rats were ground in liquid nitrogen. Twenty milligrams of the powdered tissue was solubilized in 250 μ l of SDS solubilization buffer (containing 1% (w/v) CHES, 2% (w/v) SDS, 1% (w/v) dithiothreitol, 10% (v/v) glycerol). An equal amount of SDS sample buffer was then added. The samples were boiled for 5 min. A 5- μ l (200 μ g) quantity of each sample was loaded onto 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Schleicher & Schuell Inc.). A purified anti-actinfilin antibody (in 1:5,000 dilution) was used as the primary antibody for Western blot analysis. Goat anti-rabbit IgG was used as the secondary antibody and an ECL Western blot analysis system (Amersham Biosciences) was used to visualize the immunocomplex.

Immunohistochemistry Staining—Immunohistochemical staining of adult male Sprague-Dawley rat brain was performed on horizontal 40- μ m microtome sections using antibodies against actinfilin and PSD95. Biotin-conjugated anti-rabbit IgG secondary antibody and a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) were used to visualize the immunocomplex in the sections.

Protein Fractionation—Protein fractionation studies were performed using brain homogenates from adult Sprague-Dawley rats as described by

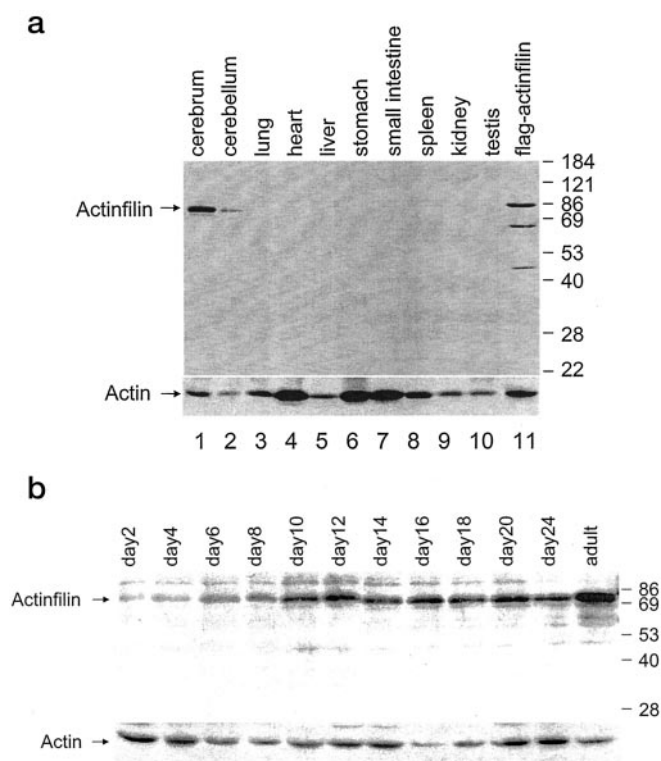


FIG. 2. *a*, tissue distribution of rat actinfilin protein. Total tissue lysates (200 μ g) from 10 major rat tissues were subject to 11% SDS-PAGE. A polyclonal antibody against MBP-actinfilin (amino acid 1–344) fusion protein was used for immunoblot detection. The actinfilin protein is marked on the *left* and the molecular weight standards are marked on the *right*. Anti-actin antibody was used to calibrate the amount of protein loading and efficient protein transfer. *b*, expression of actinfilin during postnatal development of the rat brain. Tissue lysates were prepared from rat brains that were harvested every other day from days 2 to 20, plus day 24, and adult rat. A total of 200 μ g of tissue lysates from each sample were analyzed by Western blotting. The actinfilin protein is indicated on the *left* and the protein molecular weight standards are marked on the *right*. Anti-actin antibody was used to calibrate the amount of protein loading and efficient protein transfer.

Carlin *et al.* (10). Briefly, ~5 g of fresh cerebellum was homogenized in 50 ml of 320 mM sucrose buffer (320 mM sucrose, 1 mM NaCO_3). The sample was saved as a brain lysate and centrifuged for 10 min at $1,400 \times g$. The supernatant was saved and the pellet was then homogenized again in 50 ml of the same buffer and spun for 10 min at $710 \times g$. The supernatant was pooled with the supernatant from the previous spin and spun for a further 10 min at $755 \times g$. The supernatant was then saved as s2 and the pellet was resuspended in 50 ml of the same buffer and saved as s2, then the pellet was discarded. The supernatant was spun again for 10 min at $13,800 \times g$ and saved as s5. The resultant pellet was resuspended and homogenized in 12 ml of a low-salt buffer (320 mM sucrose, 1 mM NaCO_3) (the sample was saved as p5) and loaded onto a sucrose gradient (0.85 M sucrose, 1.0 M sucrose, and 1.2 M sucrose in 1 mM NaCO_3). After a 2-h spin at $82,500 \times g$, the sample above 0.85 M sucrose, the sample between 0.85 M and 1.0 M sucrose, the sample between 1.0 and 1.2 M sucrose, and the sample of the pellet were resuspended in 30 ml of a low-salt buffer yielding 6g1, 6g2, 6g3, and 6g4 fractions, respectively. The 6g3 was referred to as the synaptosomal fraction in previous literature (11). The 6g3, the synaptosomal fraction, was solubilized by adding 30 ml of 1% ice-cold Triton X-100 in a low-salt buffer for 15 min and centrifuged at $32,000 \times g$ for 20 min. The pellet was resuspended in 1.25 ml of the low-salt buffer as the Triton 1 PSD fraction. Samples of the supernatant and the pellet were saved as s7 and Triton 1. The Triton 1 fraction was loaded onto a sucrose gradient (1.0 M sucrose, 1.5 M sucrose, and 2.0 M sucrose in 1 mM NaCO_3). The PSD fraction was recovered after a 2-h spin at $201,800 \times g$. The PSD fraction was resuspended in 6 ml of the low-salt buffer (the pellet was saved as the PSD fraction). The PSD fraction was then further solubilized by adding 6 ml of 1% Triton X-100 (1% Triton X-100 in 150 mM KCl). The PSD fraction was centrifuged at $210,800 \times g$ for 20 min. The pellet was resuspended in 200 μ l of 40 mM Tris-HCl (pH 8.0) and saved as

the Triton 2 PSD fraction. An aliquot of 5 μ l of the saved samples was separated on a 10% SDS-polyacrylamide gel, and immunoblotted with anti-PSD95 antibody, anti-actinfilin antibody, anti-actin, or anti-lactate dehydrogenase antibody.

Co-immunoprecipitation—COS7 cells were transfected with different combinations of constructs for co-immunoprecipitation in heterologous cells. At 48 h post-transfection, the cells were washed twice with phosphate-buffered saline and lysed in 200 μ l of lysis buffer (10 mM Tris-HCl (pH 8.0), 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 10 mM NaN_3 , and a protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 1 μ g/ml pepstatin)) at room temperature for 30 min. The supernatant was collected after centrifugation of the lysate at $27,000 \times g$ for 1 h at 4 $^\circ\text{C}$. An aliquot of 50 μ l of the supernatant was incubated with the corresponding antibody and 20 μ l of protein A-Sepharose (1:1 slurry) overnight at 4 $^\circ\text{C}$. The mixture was washed three times with phosphate-buffered saline (containing 0.5 M NaCl). The proteins were eluted with 30 μ l of SDS sample buffer. An aliquot of 10 μ l from each sample was loaded onto 10% SDS-polyacrylamide gels. Western blot analysis was performed with the corresponding antibody.

Co-sedimentation—Globular actin (G-actin) was prepared from rabbit skeletal muscle (12) and gel purified on Sephacryl S-300 (a gift of Hua Chen and Eaton E. Lattman).

The ability of GST fusion proteins to associate with F-actin was examined for bacterially expressed GST fusion proteins using modified co-sedimentation assays (13). GST fusion protein (1 μ M, final concentration) and G-actin (10 μ M, final concentration) were mixed in 300 μ l of F-actin polymerization buffer (5 mM imidazole (pH 7.0), 50 mM KCl, 1 mM MgCl_2 , 0.2 mM ATP, 1 mM EGTA, and 0.5 mM dithiothreitol) at 4 $^\circ\text{C}$ overnight. The samples were centrifuged at $100,000 \times g$ for 20 min at 4 $^\circ\text{C}$. The pellets were resuspended in 20 μ l of SDS sample buffer. An aliquot of 10 μ l from each sample was analyzed by SDS-PAGE, and visualized with Coomassie Blue.

Immunogold and Electron Microscopy—The hippocampus and cerebellum of an adult rat were processed for post-embedding immunogold labeling as described previously (14, 15). In addition, similar regions were examined in a mouse (not shown). Animals anesthetized and perfused were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85-23; NIDCD protocol number 874-98). Fixed brain sections were frozen in liquid propane in a Leica CPC (Vienna, Austria) and were freeze-substituted in a Leica AFS. For immunolabeling, 10-nm immunogold (Amersham Biosciences) was used.

RESULTS

Molecular Cloning of Actinfilin—Using CAP70 as a bait for the yeast two-hybrid screen, we have identified several cDNA fragments whose open reading frames encode candidate interacting proteins for CAP70. In addition to known genes, such as synaptic RAS-GTPase activating protein (synGAP) (11, 16) and SAP90/PSD-95-associated proteins (17), both of which are present in the nervous system and are found in a macromolecule known as the postsynaptic density, we also found several cDNA fragments encoding a novel Kelch domain protein. Because of its interaction with actin as shown in the later part of this paper, we called it “actinfilin” (accession number AF505655).

The amino acid sequence of the cloned rat actinfilin was then used as a probe, which identified a human EST (AL555656) available in the NCBI data base that encodes a stretch of amino acid sequences nearly identical to the reported amino acid sequence shown (Fig. 1a). However, the upstream sequence from the putative methionine displays significant sequence divergence from that of the human sequence. The human chromosomal localization of the *actinfilin* gene was assigned to chromosome 1 (NT_025635.7, NCBI gi17434913), suggesting that *actinfilin* is a single copy gene.

Actinfilin sequence characteristics begin with a proline-rich domain located at the NH_2 terminus, which is followed by an amino-terminal BTB or POZ domain (see review in Ref. 18). A cluster of six tandemly positioned Kelch domains is located on the carboxyl terminus (see review in Ref. 19) (Fig. 1, a and c). The BTB/POZ domain is a conserved protein-protein interaction domain that has been found in diverse proteins ranging

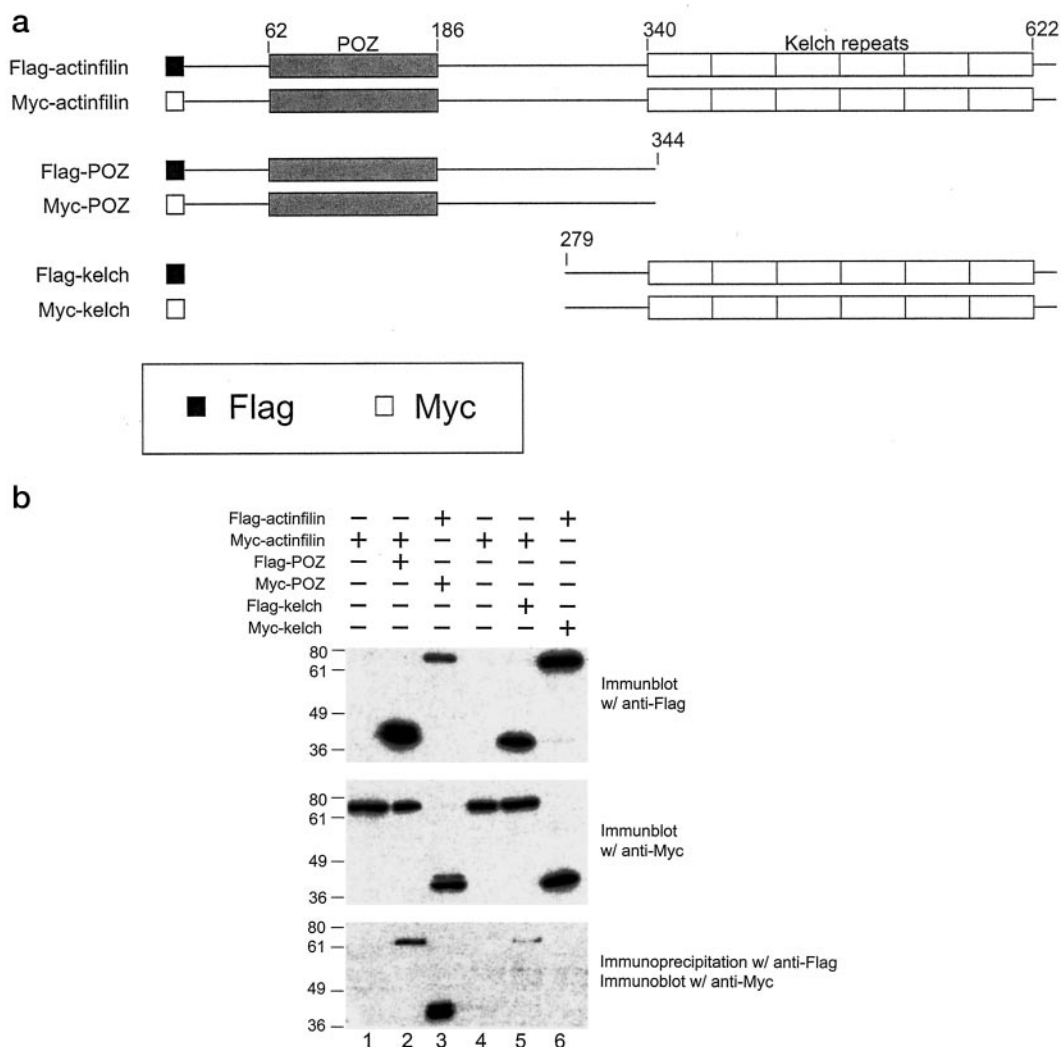


FIG. 3. *a*, schematic diagrams of FLAG and Myc fusions of the full-length or the truncated forms of actinfilin. The amino acid positions corresponding to the actinfilin amino acid sequences are indicated. The *small rectangular box* at the amino terminus represents the fusion tag, the *filled box* represents the FLAG tag, and the *empty box* represents the Myc tag. The actinfilin with COOH-terminal (amino acids, 344–640) deletion is designated as POZ and the actinfilin with the NH₂-terminal (1–279) deletion is designated as Kelch. *b*, actinfilin-actinfilin association in transfected COS7 cells. COS7 cells were transfected using different combinations of plasmids as indicated at the top. The top two panels are immunoblots probed with antibodies as indicated on the right. The cell lysates were immunoprecipitated with the anti-FLAG antibody and the immunoprecipitants were subjected to 11% SDS-PAGE gel and detected by the anti-Myc antibody (bottom panel). The protein molecular weight standards are marked on the left.

from cell surface membrane proteins, such as a Shaker-type potassium channel (20), to nuclear transcription factors, such as a promyelocytic leukemia zinc finger (21). The six Kelch repeats position with little or no intervening sequences and share significant homology at the amino acid level. The signature “GG . . . Y-X6-W” motif is perfectly conserved in all six Kelch repeats, which further supports the idea that they form six blades of a typical β -turn propeller structure as seen in galactose oxidase from *Hypomyces rosellus* (19). Interestingly, the overall domain organization of actinfilin bears a significant similarity to the other previously known Kelch domain-containing proteins as shown in Fig. 1*b*. The proline-rich region and the restricted tissue distribution are unique to actinfilin in the Kelch family proteins. Indeed, the *Drosophila* Kelch protein and actinfilin share the same overall domain organization. The amino-terminal domain of the *Drosophila* Kelch is known for its role in subcellular localization (22). Thus, it is possible that the proline-rich domain in actinfilin serves as an interaction site with another signaling protein containing proline-binding motifs, such as Src homology 3 or Homer domains.

An antibody has been generated using a recombinant fu-

sion protein corresponding to amino acids 1–344 of rat actinfilin cDNA. The resultant antiserum identified a fusion polypeptide only from cells transfected with the epitope-tagged actinfilin (Fig. 2*a*, lane 11). In the native tissue, the antibody identified a polypeptide of approximately 73,000 daltons, consistent with the predicted molecular weight derived from the amino acid sequence. To determine the tissue distribution of actinfilin, we performed an immunoblot to test for actinfilin expression in a panel of selected tissue extracts. The actinfilin protein was mainly found in the nervous system, especially in the cerebrum and the cerebellum (Fig. 2*a*). In contrast, crude lysates from other tissues although giving rise to an actin signal as detected by immunoblot, displayed no detectable amount of actinfilin. Using reverse transcriptase-PCR, the actinfilin mRNA message was abundant in the cortex, the cerebellum, and the hippocampus. The signal was also detectable in the testis, and to a lesser extent in the thymus.³ During development, the expression of act-

³ Y. Chen and M. Li, unpublished results.

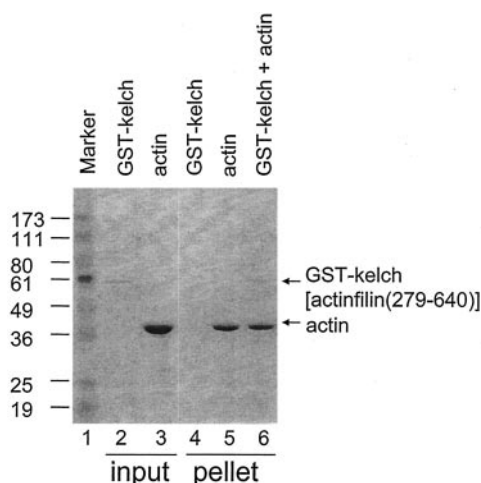


FIG. 4. Co-sedimentation assay of the GST-Kelch domain and F-actin. The ability of actinfilin-Kelch to associate with F-actin was tested as described under "Experimental Procedures." The protein molecular weight standards are shown in lane 1 and marked on the left side of the Coomassie Blue-stained SDS-PAGE gel. The expected proteins are indicated on the right side. Lane 2, the input of the GST-Kelch fusion protein (10 μ g); lane 3, the input of actin protein (50 μ g); lane 4, the sediments of GST-Kelch without actin; lane 5, the F-actin pellet without GST-Kelch protein. Lane 6, the F-actin and GST-Kelch mixture pellet.

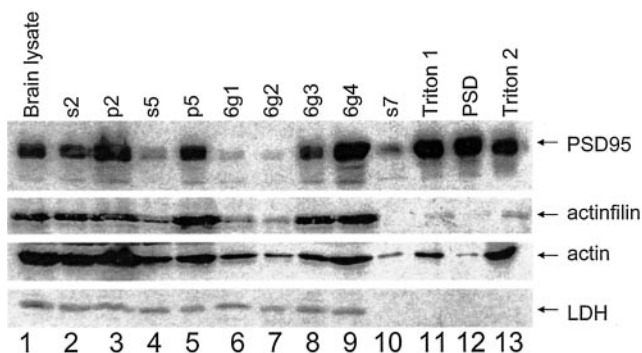


FIG. 5. Actinfilin in the postsynaptic density (PSD) preparation. Protein fractionation studies were performed as described under "Experimental Procedures." Samples from each step of the preparation were saved in proportion to the starting material and separated by SDS-PAGE as indicated on the top of the gel. Immunoblots were used to detect PSD95, actinfilin, actin, and lactate dehydrogenase (LDH) in each sample, as marked on the right. According to existing nomenclature, the brain lysate is called brain homogenate, also 6g3 is called synaptosomes, Triton 1 is called PSD (one Triton), and Triton 2 is called PSD (two Triton).

infilin in the brain increased gradually and reached the maximal level in the adult animal (Fig. 2b). The evidence of restricted tissue expression supports the idea that actinfilin plays a role in the neuronal function.

Domain Organization and Oligomerization of Actinfilin—POZ domains have been found to involve mediating protein-protein interaction to yield oligomers such as dimers (23, 24). A homologous POZ domain in Shaker potassium channels, also known as the NAB or T1 domain, mediates subunit interaction to specify compatible channel assembly among subunits of different subfamilies to form tetramers (20). To test for the homomeric interaction of actinfilin, we constructed differentially tagged, full-length and various truncated forms of actinfilin as shown in the Fig. 3a. These fusion constructs allow for the transient expression in cultured mammalian cells. To test for association, lysates from transiently transfected COS7 cells were prepared, immunoprecipitated, and detected by immunoblot using different combinations of plasmids. For the combi-

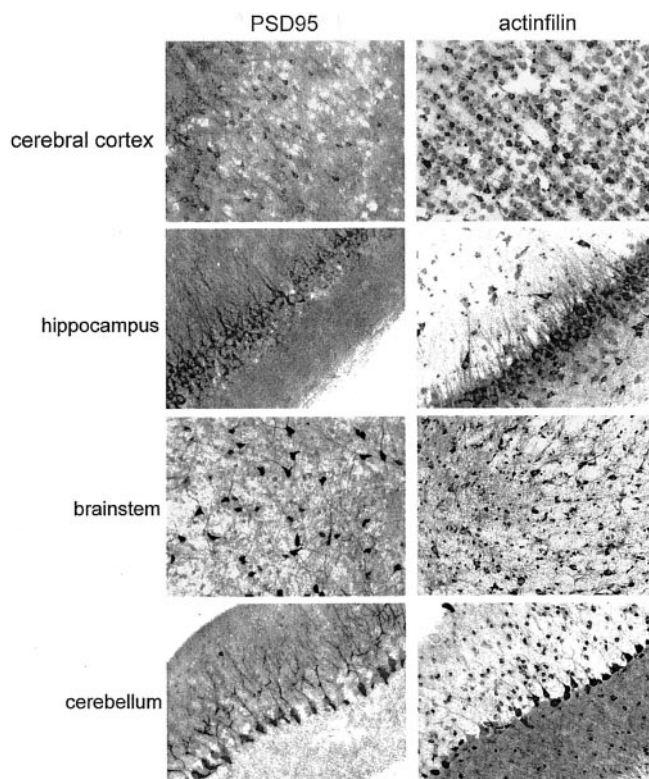


FIG. 6. Immunohistochemistry detection of the actinfilin protein. Sections of rat brain were stained with anti-actinfilin antibody and anti-PSD95 antibody ("Experimental Procedures"). The section regions, i.e. cerebral cortex, hippocampus, brainstem, and cerebellum, are indicated on the left. Antibodies used are shown on the top. The sections were viewed using a bright field light microscope with a $\times 20$ objective.

nation of Myc-POZ and FLAG-actinfilin, we found that a Myc-tagged POZ domain was precipitated by anti-FLAG antibody (Fig. 3b, lane 3). In contrast, a Myc fusion protein lacking the POZ domain was not precipitated (Fig. 3, lane 6). This result is consistent with the notion that actinfilin molecules interact with each other homomericly via association of the POZ domains.

Biochemical and Functional Interaction with F-actin—The presence of six Kelch repeats suggests the possibility of interaction with F-actin. To directly test this hypothesis, the coding sequence for the entire six Kelch domains (amino acids 279–640) was expressed in *E. coli* and purified as glutathione *S*-transferase fusion (GST-Kelch). The resultant proteins were used for testing the interaction with F-actin using a standard co-sedimentation technique ("Experimental Procedures"). Fig. 4 shows that rabbit muscle F-actin was able to precipitate the GST-Kelch (lane 6). In contrast, the purified GST-Kelch was soluble and failed to be precipitated under the same conditions (lane 4). Control GST fusion proteins could not be precipitated, although a maltose-binding protein fusion of Kelch could also be precipitated.⁴ In addition, the amount of precipitated protein as judged by Coomassie staining was consistent, which is in agreement with the previous finding that the interaction of the GST-Kelch domain with F-actin apparently had no detectable effect on the amount of F-actin precipitated in this experiment (Fig. 4, lanes 5 and 6). This result is consistent with the proposed functional role in bundling actin filament with no direct effect on actin polymerization.

Actinfilin as Components of Postsynaptic Density—In brain lysates, both CAP70 and actinfilin behaved as insoluble pro-

⁴ Y. Chen and M. Li, unpublished data.

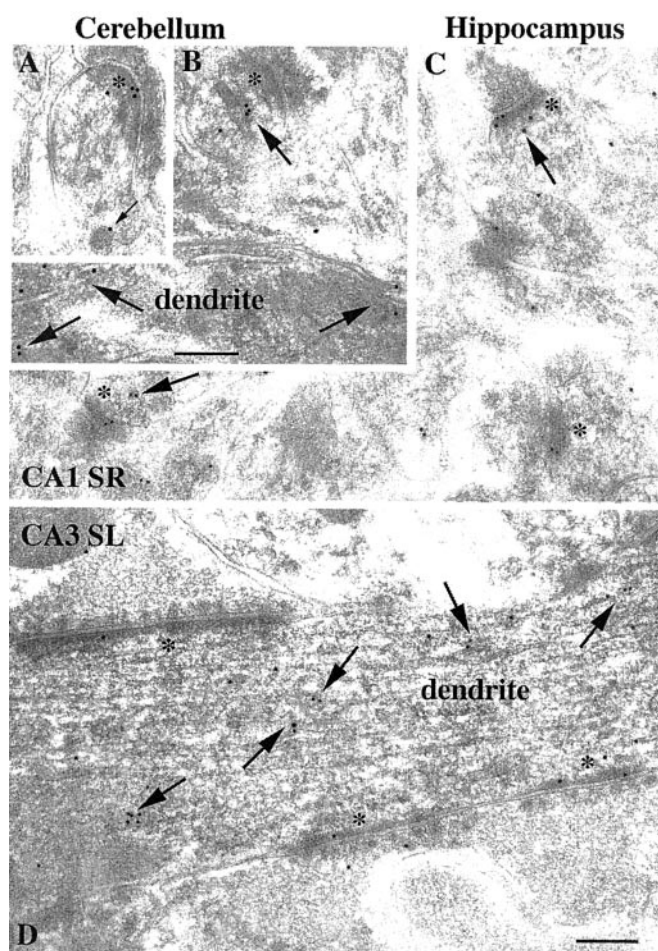


FIG. 7. Immunogold labeling for actinfilin in the cerebellum (A and B) and the hippocampus (C and D). Note the labeling associated with membranous cytoplasmic structures (arrows) in dendrites (B, Purkinje cell dendrite; D, interneuron dendrite) and postsynaptic spines. Asterisks indicate postsynaptic densities on spines (A–C: A and B are parallel fiber synapse spines) and the dendrite shaft of an interneuron (D). CA1 SR, CA1 stratum radiatum; CA3 SL, CA3 stratum lucidum. The small arrow indicates an oblique coated pit. The line scale is 0.2 μm (A and B and C and D).

tein⁵ in a typical nondetergent lysis buffer, similar to that of the postsynaptic density. To test whether actinfilin was associated with certain specific biochemical or subcellular structures in the brain, a postsynaptic density preparation was obtained using standard techniques (see “Experimental Procedures”) and the resultant proteins from the different preparation steps were collected and analyzed by immunoblot using specific antibodies against PSD95, actin, actinfilin, and lactose dehydrogenase. Consistent with previous results, PSD95 was enriched in the pellet after s5 and was resistant to detergent treatments (Triton 1 and Triton 2) (Fig. 5, lanes 11 and 13). Likewise, actin displayed a similar separation profile as PSD95. Actinfilin also had the same separation profile as PSD95 before the Triton X-100 treatment (Fig. 5, lanes 1–9) and was abundant in the synaptosome fraction (Fig. 5, lane 8). After Triton X-100 treatment, most of the actinfilin, however, was released from the PSD pellet by the detergent treatment that led to only a small fraction of actinfilin presented in the PSD preparation (Fig. 5, lanes 9–13). This result suggests that actinfilin was not the core component of the PSD protein complex. In contrast, a typical soluble protein, lactate dehydrogenase, did not show any enrichment in the insoluble preparations

nor did it display any resistance to detergent treatment (Fig. 5, lanes 8–13).

Tissue and Subcellular Localization of Actinfilin—Specific antibodies against actinfilin and PSD95 were used to determine the protein localization in different regions of the rat brain. Fig. 6 shows that PSD95 was localized primarily to the dendritic microdomains, which is consistent with the published reports. Actinfilin was specifically expressed in neurons but undetectable in glia (Fig. 6). Actinfilin was widely localized in various brain regions including the cortex, the pons, the hippocampus, and the cerebellum. In the cortex and hippocampus, the most prominent staining was observed in the pyramidal cells. In the cerebellum, Purkinje cells and granule cells were also positive. Generally, the staining was concentrated mostly in the soma and the dendritic processes (Fig. 6).

To determine the subcellular localization of actinfilin protein, a specific antibody was used to probe actinfilin localization by immunogold methods. In the cerebellum (Fig. 7, A and B) and the hippocampus (Fig. 7, C and D), immunogold labeling for actinfilin was associated most commonly with dendrite cytoplasm. Often the labeling decorated membranous structures on the cytoplasmic side of the plasma membrane or within the cytoplasm. Substantial labeling was seen in the dendrites of pyramidal cells of the hippocampus and Purkinje cells (Fig. 7B) of the cerebellum, as well as in interneuron dendrites (Fig. 7D). Labeling of synapses was variable. In many cases, a moderate postsynaptic labeling was observed, with gold particles associated with membranous cytoplasmic structures as in the dendrites (Fig. 7, B and D), as well as with the postsynaptic density and postsynaptic side of membrane (Fig. 7, C and D).

DISCUSSION

The identification of CAP70-binding proteins including synaptic RAS-GTPase activating protein, SAP90/PSD-95-associated proteins, and actinfilin suggests a role for CAP70 to nucleate a macromolecular complex. The biochemical interactions of CAP70 with these proteins were confirmed using direct binding assays of recombinant fusion proteins.⁴ Given that the actinfilin is found at least partially localized at the postsynaptic density, it would be interesting to test whether the COOH termini of these proteins are indeed responsible for the binding and which one(s) of the four PDZ domains is responsible for the interactions.

Actin polymerization, stability, and subcellular anchoring are regulated by a number of factors including a large group of actin-binding proteins (25). The Kelch domain-containing actin-binding proteins were first identified from a *Drosophila* mutant, *kelch*, which encodes a protein that is involved in proper morphology of actin-rich intracellular canals (26, 27). A unitary cluster of six Kelch repeats usually forms a β -propeller structure and the interaction between the Kelch domain-containing protein and actin has been reported (for review, see Ref. 19). Current evidence is consistent with the model that this group of proteins interacts with F-actin via the conserved Kelch domain. Because many Kelch-containing proteins including actinfilin in this report contain homomeric protein-protein interaction domains, such as the POZ domain, the model further suggests that a Kelch-containing protein may play a role in actin bundling. Supporting evidence comes from studies of the *limulus* α -scurin and β -scurin, which are two proteins that lack POZ or other known protein-protein interactions but contain 12 Kelch domains that are separated into two clusters of six tandemly positioned Kelch domains, reminiscent of a dimeric form of actinfilin. Microscopic studies suggested that scruin serves as a bivalent interacting bridge that bundles the actin filaments (28).

The combination of the NH₂-terminal protein-protein inter-

⁵ N. Stricker, Y. Chen, and M. Li, unpublished data.

action POZ domain and the COOH-terminal positioned six Kelch domains makes actinfilin a member of the N-dimer/C-propeller subfamily (19). Truncated recombinant protein containing the six Kelch repeats, which presumably form blades of a propeller structure, is sufficient to mediate binding to F-actin. It should be noted that there is no experimental evidence that actinfilin or recombinant Kelch domain functions with dimeric stoichiometry, Fig. 2 shows that actinfilin is capable of interacting with itself via the POZ domain to form a homomeric oligomer(s). Similar to previous reports concerning several other Kelch proteins (22, 28, 29), the full-length recombinant protein of actinfilin tends to aggregate. We found this behavior to be more prominent when the full-length recombinant protein was overexpressed in transfected cells in which actinfilin forms aggregates. The appearance does not display morphological similarity to the known subcellular organelle structures.⁴ A better understanding of this highly aggregated structure and protein half-lives of the native actinfilin may provide the rationale to use various truncated forms of actinfilin to perform functional studies.

In addition to actinfilin, there are at least two other Kelch-containing actin-binding proteins that are localized in the brain, including mayven (29), NRP/B or ENC1 (30, 31). Except for NRP/B (or ENC1), which is reported to be a nuclear matrix protein, mayven is in the cytoplasmic compartments but may not be restricted to any specific subcellular domains. It is of great interest to determine their specific subcellular localization as several subcellular structures are abundant in actin filaments, such as the postsynaptic density and its adjacent spines of neurons. The biochemical fractionation and immunogold labeling studies reported in this paper show that actinfilin is partially localized in the postsynaptic density (Figs. 5 and 7). It is known that the actin-based membrane domain structures are critical for proper synaptic transmission in cultured neurons (32). The evidence presented in this report now associates the Kelch domain-mediated actin-binding proteins to the postsynaptic density. Thus, actinfilin and its related proteins are possibly involved in cytoskeleton-related neuronal functions. Supporting this notion is the recent identification of gigaxonin, a Kelch domain-containing protein with ubiquitous tissue distribution. Mutations of gigaxonin are found in patients with giant axonal neuropathy (33). Thus, the functional understanding of Kelch proteins such as actinfilin may provide further insights into the actin regulation and their roles in the nervous systems.

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REFERENCES

- Bailey, C. H., and Kandel, E. R. (1993) *Annu. Rev. Physiol.* **55**, 397–426
- Chang, F. L., and Greenough, W. T. (1984) *Brain Res.* **309**, 35–46
- Crick, F. (1982) *Trends Neurosci.* **5**, 44–46
- Desmond, N. L., and Levy, W. B. (1988) *Brain Res.* **453**, 308–314
- Fifkova, E., and Van Harreveld, A. (1977) *J. Neurocytol.* **6**, 211–230
- Toni, N., Buchs, P. A., Nikonenko, I., Bron, C. R., and Muller, D. (1999) *Nature* **402**, 421–425
- Wang, S., Yue, H., Derin, R. B., Guggino, W. B., and Li, M. (2000) *Cell* **103**, 169–179
- James, P., Halladay, J., and Craig, E. A. (1996) *Genetics* **144**, 1425–1436
- Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H. (1992) *Nucleic Acids Res.* **20**, 1425
- Carlin, R. K., Grab, D. J., Cohen, R. S., and Siekevitz, P. (1980) *J. Cell Biol.* **86**, 831–845
- Chen, H. J., Rojas-Soto, M., Oguni, A., and Kennedy, M. B. (1998) *Neuron* **20**, 895–904
- Spudich, J. A., and Watt, S. (1971) *J. Biol. Chem.* **246**, 4866–4871
- Bailly, M., Ichetovkin, I., Grant, W., Zebda, N., Machesky, L. M., Segall, J. E., and Condeelis, J. (2001) *Curr. Biol.* **11**, 620–625
- Petralia, R. S., Wang, Y. X., Sans, N., Worley, P. F., Hammer, J. A., 3rd, and Wenthold, R. J. (2001) *Eur. J. Neurosci.* **13**, 1722–1732
- Petralia, R. S., and Wenthold, R. J. (1999) in *Methods in Molecular Biology: NMDA Receptor Protocols* (Li, M., ed) pp. 113–120, Humana Press, Totowa, NJ
- Kim, J. H., Liao, D., Lau, L. F., and Haganir, R. L. (1998) *Neuron* **20**, 683–691
- Takeuchi, M., Hata, Y., Hirao, K., Toyoda, A., Irie, M., and Takai, Y. (1997) *J. Biol. Chem.* **272**, 11943–11951
- Aravind, L., and Koonin, E. V. (1999) *J. Mol. Biol.* **285**, 1353–1361
- Adams, J., Kelso, R., and Cooley, L. (2000) *Trends Cell Biol.* **10**, 17–24
- Li, M., Jan, Y. N., and Jan, L. Y. (1992) *Science* **257**, 1225–1230
- Dong, S., Zhu, J., Reid, A., Strutt, P., Guidez, F., Zhong, H. J., Wang, Z. Y., Licht, J., Waxman, S., Chomienne, C., Chen, Z., Zelen, A., and Chen, S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 3624–3629
- Robinson, D. N., and Cooley, L. (1997) *J. Cell Biol.* **138**, 799–810
- Ahmad, K. F., Engel, C. K., and Prive, G. G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 12123–12128
- Chen, W., Zollman, S., Couderc, J. L., and Laski, F. A. (1995) *Mol. Cell. Biol.* **15**, 3424–3429
- Pollard, T. D., Blanchoin, L., and Mullins, R. D. (2000) *Annu. Rev. Biophys. Biomol. Struct.* **29**, 545–576
- Xue, F., and Cooley, L. (1993) *Cell* **72**, 681–693
- Robinson, D. N., Cant, K., and Cooley, L. (1994) *Development* **120**, 2015–2025
- Way, M., Sanders, M., Garcia, C., Sakai, J., and Matsudaira, P. (1995) *J. Cell Biol.* **128**, 51–60
- Soltysik-Espanola, M., Rogers, R. A., Jiang, S., Kim, T. A., Gaedigk, R., White, R. A., Avraham, H., and Avraham, S. (1999) *Mol. Biol. Cell* **10**, 2361–2375
- Kim, T. A., Lim, J., Ota, S., Raja, S., Rogers, R., Rivnay, B., Avraham, H., and Avraham, S. (1998) *J. Cell Biol.* **141**, 553–566
- Hernandez, M. C., Andres-Barquin, P. J., Martinez, S., Bulfone, A., Rubenstein, J. L., and Israel, M. A. (1997) *J. Neurosci.* **17**, 3038–3051
- Allison, D. W., Gelfand, V. I., Spector, I., and Craig, A. M. (1998) *J. Neurosci.* **18**, 2423–2436
- Bomont, P., Cavalier, L., Blondeau, F., Ben Hamida, C., Belal, S., Tazir, M., Demir, E., Topaloglu, H., Korinthenberg, R., Tuysuz, B., Landrieu, P., Hentati, F., and Koenig, M. (2000) *Nat. Genet.* **26**, 370–374