

# Actin Filament Assembly by Myristoylated, Alanine-rich C Kinase Substrate–Phosphatidylinositol-4,5-diphosphate Signaling Is Critical for Dendrite Branching

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Dendrites undergo extensive growth and branching at early stages, but relatively little is known about the molecular mechanisms underlying these processes. Here, we show that increasing the level of myristoylated, alanine-rich C kinase substrate (MARCKS), a prominent substrate of protein kinase C and a phosphatidylinositol-4,5-diphosphate [PI(4,5)P<sub>2</sub>] sequestration protein highly expressed in the brain, enhanced branching and growth of dendrites both in vitro and in vivo. Conversely, knockdown of endogenous MARCKS by RNA interference reduced dendritic arborization. Results from expression of different mutants indicated that membrane binding is essential for MARCKS-induced dendritic morphogenesis. Furthermore, MARCKS increased the number and length of filamentous actin-based filopodia along neurites, as well as the motility of filopodia, in a PI(4,5)P<sub>2</sub>-dependent manner. Time-lapse imaging showed that MARCKS increased frequency of filopodia initiation but did not affect filopodia longevity, suggesting that MARCKS may increase dendritic branching through its action on filopodia initiation. These findings demonstrate a critical role for MARCKS–PI(4,5)P<sub>2</sub> signaling in regulating dendrite development.

## INTRODUCTION

The establishment of neural connections requires the proper navigation of axons to their targets and the elaboration of complex dendritic arbors to integrate synaptic inputs (Wong and Ghosh, 2002; Van and Cline, 2004; Ehlers, 2005). Because the dendritic branching pattern determines the number and type of inputs that a neuron can receive, it is important to know how neurons acquire their characteristic dendritic morphology during development.

The actin cytoskeleton plays a major role in dendritic morphogenesis (Luo, 2002). Multiple signals, such as extracellular guidance cues and growth factors, control dendritic morphology through changing the organization and dynamics of the actin cytoskeleton (Rosso *et al.*, 2005). The structure and dynamics of the actin cytoskeleton are regulated by a host of actin-binding proteins (ABPs). Phosphoinositides, especially phosphatidylinositol-4,5-diphosphate [PI(4,5)P<sub>2</sub>],

are the most effective and commonly encountered regulatory factors for ABPs. PI(4,5)P<sub>2</sub> can activate or inhibit the actin-binding activity of different ABPs, with the net effect that increasing the level of PI(4,5)P<sub>2</sub> tends to promote actin assembly, whereas hydrolyzing it leads to actin disassembly (Janmey and Lindberg, 2004). Currently, two mechanisms are proposed to be responsible for localized PI(4,5)P<sub>2</sub> accumulation: lateral sequestration and local synthesis (Sheetz *et al.*, 2006). The lateral sequestration model suggests that some proteins bind and passively concentrate PI(4,5)P<sub>2</sub> in lateral membrane domains. Myristoylated, alanine-rich C kinase substrate (MARCKS) is one of the major proteins that sequesters PI(4,5)P<sub>2</sub>.

MARCKS was originally identified as a prominent substrate of protein kinase C (PKC), and it has been implicated in the regulation of cellular adhesion and neurosecretion (Arbuzova *et al.*, 2002). MARCKS binds to membrane using two membrane anchors: an amino-terminal myristate inserts hydrophobically into the bilayer, and a basic effector domain interacts electrostatically with PI(4,5)P<sub>2</sub> on the membrane (McLaughlin and Aderem, 1995; Murray *et al.*, 1997). MARCKS binds PI(4,5)P<sub>2</sub> with high affinity and releases it in response to a local increase in Ca<sup>2+</sup>, or PKC activation (Raucher *et al.*, 2000; McLaughlin and Murray, 2005).

MARCKS is particularly enriched in brain (Patel and Kligman, 1987; McNamara *et al.*, 1998). Electron microscopic analysis revealed that MARCKS protein is distributed in axon terminals and small dendritic branches in adult rat brain (Ouimet *et al.*, 1990; McNamara and Lenox, 1997). Gene targeting experiments suggest that MARCKS is essential for CNS development. Homozygous mutant mice lacking MARCKS die before or within a few hours of birth and exhibit abnormal brain development characterized by de-

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Abbreviations used: 3'-UTR, 3'-untranslated region; ABP, actin-binding protein; Arp2/3, actin-related protein 2/3; BK, bradykinin; DIV, days in vitro; ED, effector domain; F-actin, filamentous actin; LTA, latrunculin A; MARCKS, myristoylated, alanine-rich C kinase substrate; PAO, phenylarsine oxide; PH, pleckstrin homology; PI(4,5)P<sub>2</sub>, phosphatidylinositol-4,5-diphosphate; PKC, protein kinase C; PLC, phospholipase C; PN, postnatal day; RNAi, RNA interference; shRNA, short hairpin RNA.

creased brain size, callosal and commissural agenesis, cortical lamination abnormalities, and exencephaly (Stumpo *et al.*, 1995). However, the connection between MARCKS and neuronal morphogenesis has yet to be established. In the present study, we found that overexpression of MARCKS led to increased dendritic arborization, whereas depletion of endogenous MARCKS by RNA interference (RNAi) decreased dendritic complexity. Binding of MARCKS to the plasma membrane was shown to be required for the morphogenetic effects of MARCKS. Further evidence indicated that PI(4,5)P<sub>2</sub>-mediated regulation of the actin cytoskeleton plays an essential role in MARCKS-induced dendritic morphogenesis.

## MATERIALS AND METHODS

Chemicals and media were from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. The use and care of animals followed the guidelines of the Shanghai Institutes for Biological Sciences Animal Research Advisory Committee.

### DNA Constructs and Short Hairpin RNAs (shRNAs)

pEGFP was from Clontech (Mountain View, CA), whereas full-length enhanced green fluorescent protein (EGFP)-tagged bovine wild-type MARCKS and three MARCKS mutants were gifts from P. Blackshear (National Institute of Environmental Health Sciences, Research Triangle Park, NC; Spizz and Blackshear, 2001). All MARCKS constructs were expressed under the promoter (cytomegalovirus), and their expression levels, quantified as mean pixel intensity, did not significantly differ.

pSUPER was a gift from Z. Luo (Institute of Neuroscience, Shanghai, China). The hairpin shRNA sequence (5'-GATCCCCCTGTACCAGTCAGTAATTATCAAGAGATAATTACTGACTGGTACAGTTTTGGAAA-3') against nucleotides 2036-2054 of rat MARCKS was inserted into the pSUPER vector. The nonsense hairpin shRNA sequence is 5'-GATCCCCGCGCTAIGTAGGATTCGTTCAAGAGACGAATCTAATAGCGCGCTTTTTGGAAA-3'. Monomeric red fluorescent protein (mRFP)-pleckstrin homology (PH)(PLC $\delta$ 1) was a gift from S. Grinstein (Hospital for Sick Children, Toronto, ON, Canada). Cyan fluorescent protein (CFP)-FKBP-Inp54p, Lyn11-FRB, and CFP-FKBP-Inp54p(D281A) were gifts from T. Meyer (Stanford University, Stanford, CA).

### Cell Culture and Transfection

Hippocampal neurons were prepared as described previously, with some modifications (Shen *et al.*, 2006). Briefly, whole brains were isolated from embryonic day 18 Sprague-Dawley rats, and the hippocampus was dissected out and treated with 0.25% trypsin at 37°C for 12 min. Cells were suspended in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and 10% F-12 (Invitrogen), and then they were plated at a density of 65,000 cells/cm<sup>2</sup> on poly-D-lysine-coated 35-mm dishes (Corning Life Sciences, Lowell, MA). Twenty-four hours after plating, half of the medium was changed to serum-free neurobasal medium with 2% B27 supplement (Invitrogen) and 0.25% glutamate. Dissociated neurons were nucleofected with a primary neuron nucleofection kit (Amaxa Biosystems, Cologne, Germany) following the manufacturer's instructions before plating. In case of transfection with two plasmids, the DNAs of interest were mixed in a 1:1 ratio.

Human embryonic kidney (HEK) 293 cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and imaged 24–48 h after transfection using Lipofectamine 2000 (Invitrogen) on poly-L-lysine-coated coverslips.

### Immunocytochemistry and Actin Staining

The following antibodies were used: goat polyclonal anti-MARCKS (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-microtubule-associated protein (MAP2) (1:1000; Sigma-Aldrich), monoclonal anti- $\beta$ -tubulin III (1:1000; Promega, Madison, WI), and Alexa 488- and cyanine (Cy)3-coupled secondary antibody (1:1000; Millipore Bioscience Research Reagents, Temecula, CA).

For staining of endogenous proteins, neurons were fixed in ice-cold 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline (PBS) for 10–20 min. After fixation, cells were washed three times with PBS at room temperature, permeabilized at room temperature with 0.2% Triton X-100 in PBS for 10 min, and incubated with the primary antibody in PBS buffer overnight at 4°C. Cells were then washed three times in PBS for 10 min at room temperature. Secondary antibodies were applied in PBS for 1 h at room temperature and washed three times in PBS for 10 min. Secondary antibodies conjugated to Alexa 488 and Cy3 were used for double labeling.

For F-actin staining, cultures were fixed in 0.12–0.2% glutaraldehyde for 15 min, treated with 1 mg/ml sodium borohydride for 15 min, and then extracted with 0.1% Triton X-100 for 15 min. Actin filaments were stained with 8  $\mu$ M/ml phalloidin conjugated to Alexa 546 (Invitrogen) for 45 min.

### In Utero Electroporation

Plasmids were introduced into the lateral ventricle of E16 embryos and specifically targeted into forebrain pyramidal neurons by using in utero electroporation as described previously (Saito and Nakatsuji, 2001). Briefly, timed-pregnant rats (embryonic day [E]15.5–E16.5) were anesthetized with 100  $\mu$ l of 3% pentobarbital sodium per 100 g body weight (intraperitoneally). The abdominal cavity was opened, and the uterine horns were exposed. Approximately 3–5  $\mu$ l of DNA solution was injected through a glass micropipette into the lateral ventricle of embryos. Electroporation (50-ms square pulses of 60 V at 100-ms intervals; 5 times) was then carried out using an Electro Square Porator (ECM 830; BTX, San Diego, CA). The pups were perfused with 4% paraformaldehyde at postnatal day 12, and then the brains harvested and fixed in 4% paraformaldehyde for 2 h at 4°C, and cryoprotected in 30% sucrose overnight. Thereafter, the cerebrum was embedded in OCT compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and frozen in dry ice. Coronal cryosections (35  $\mu$ m) were made and examined by confocal microscopy.

### Confocal Imaging and Data Analysis

Fluorescence images were acquired with an Olympus confocal microscope (Fluoview 500; Olympus, Tokyo, Japan). Confocal images of neurons in culture were collected with sequential acquisition settings at the resolution of the microscope (512  $\times$  512 pixels) using the Argon laser (488 nm) and HeNe laser (543 nm). Sequential acquisition and the use of a bandpass filter (BA505/525) eliminated bleed-through between the 488 nm and the Cy3 channels. Each image was a z-series of five images, each averaged twice with a Kalman filter and taken at 0.50- $\mu$ m-depth intervals when a 60 $\times$  water-immersion objective (numerical aperture 1.2) was used. The resultant stacks were "flattened" into a single image by using maximal projection. The confocal settings were kept the same for all scans when fluorescence intensity was compared. Data analysis was performed as described previously, with some modification (Yu and Malenka, 2003). Protrusions (longer than 10  $\mu$ m) emerging from dendrites or axon were counted as branch tips. In some cases, the brightness and contrast of the images were adjusted to enhance the visibility of neurites. The branches were traced and analyzed with Image-Pro Plus (Media Cybernetics, Inc. Bethesda, MD). Imaging and quantification of the data shown in Figures 1–3 and Figure 9 were done blindly. Statistical significance was assessed using Student's *t* test.

For measurement of dendritic branching and length in vivo, coronal brain sections were prepared. Three-dimensional reconstructions of the dendritic processes of each green fluorescent protein (GFP)-positive neuron were made using z-series stacks of confocal images. The projection images were semiautomatically traced with ImageJ (National Institutes of Health, Bethesda, MD) by using the NeuronJ plugin. Total branch number and total dendritic length of each individual GFP pyramidal neuron were calculated. Statistical significance was assessed using Student's *t* test.

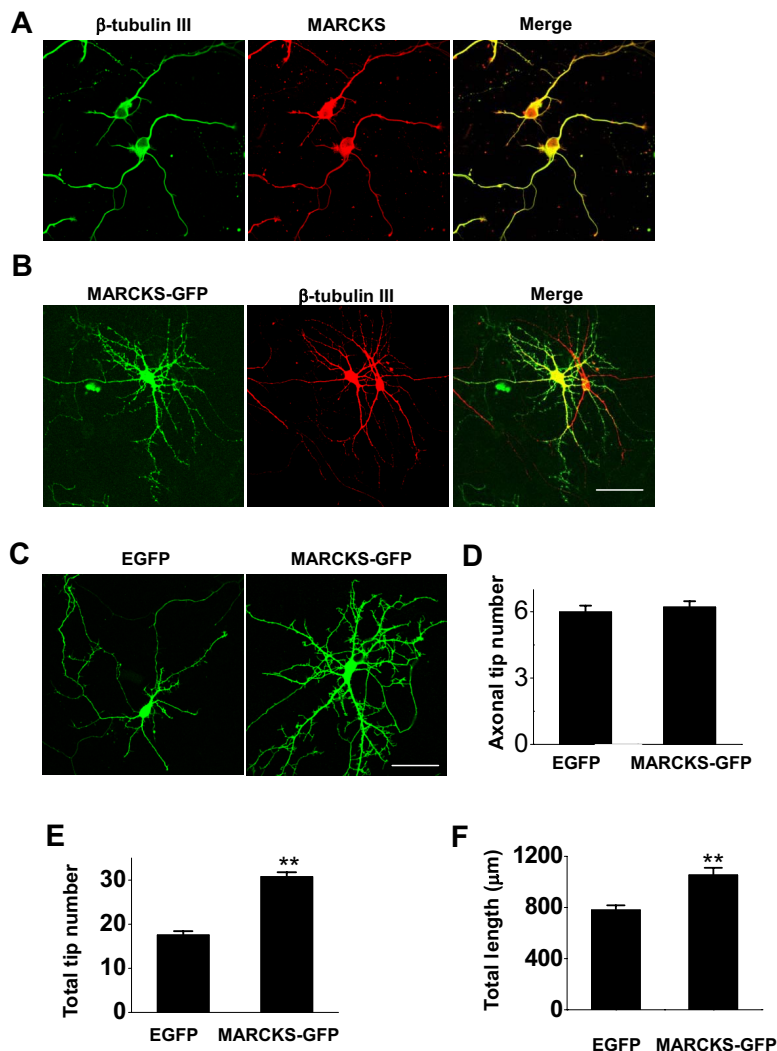
To evaluate motility of filopodia, confocal images were collected for 10 min at 5-s intervals. The velocity of the filopodia movement and distance of the filopodia from the start point were analyzed by Image-Pro Plus.

## RESULTS

### MARCKS Overexpression Increased Dendritic Arborization

Hippocampal cultures have been used widely for studying the molecular mechanisms of neuronal dendritic growth and branching. Pyramidal neurons constitute the majority neuronal population in the hippocampus. The hippocampus also contains a variety of interneurons, but they are comparatively few in number and are morphologically distinguishable from pyramidal neurons in culture (Kaech and Banker, 2006). We therefore focused our analysis on cultured hippocampal pyramidal neurons.

The MARCKS protein is abundant in the early postnatal rat brain. Immunostaining revealed that MARCKS distributes in the cell body and neuronal processes in cultured neurons (Figure 1A). Cultured hippocampal neurons undergo a highly stereotyped sequence of development, including initial specification of axon/dendrite identity followed by elaboration of axon and dendrites. To determine the roles of MARCKS in the development of neuronal morphology, we transfected neurons with a plasmid encoding wild-type MARCKS fused at its C terminus with GFP (MARCKS-GFP). The resulting fusion protein has been described previously (Spizz and Blackshear, 2001; Calabrese



**Figure 1.** MARCKS overexpression results in increased dendritic branching and length. (A) Expression of MARCKS protein in developing hippocampal neurons cultured at 4 DIV. (B) Hippocampal neurons transfected with MARCKS-GFP (green) were immunostained with the neuronal marker  $\beta$ -tubulin III (red). MARCKS-expressing neurons exhibited more complex morphology than nontransfected neurons. Bar, 50  $\mu\text{m}$ . (C) Confocal images of neurons nucleofected with EGFP or MARCKS-GFP before plating and analyzed at 6 DIV. MARCKS-expressing neurons elaborated complex arbors with some high-order branches. Bar, 50  $\mu\text{m}$ . (D) Averaged total axonal tips numbers per neuron.  $p > 0.05$ , compared with EGFP group. (E) Averaged total number of dendritic tips per neuron. \*\* $p < 0.001$ . (F) Averaged total dendrite length per neuron. \*\* $p < 0.001$ , compared with EGFP group. Measurements in D–F were taken from at least 100 neurons in five independent experiments for each group.

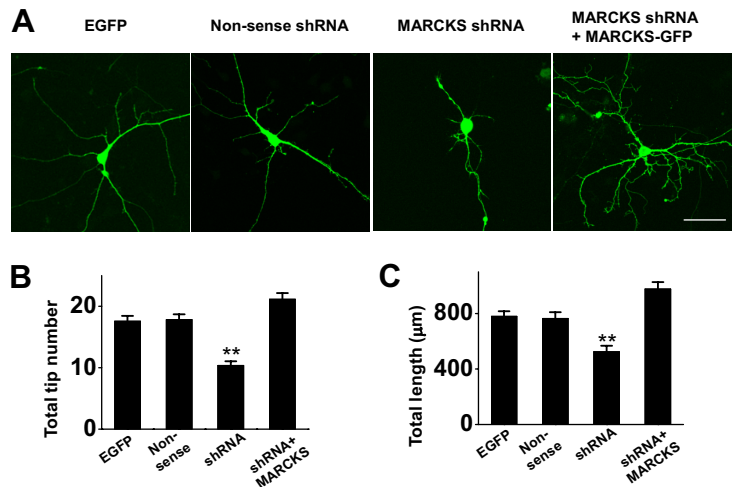
and Halpain, 2005). Neurons were nucleofected before plating and then cultured for 6 d. Neuronal morphology was visualized by immunostaining for  $\beta$ -tubulin III antibody (Tuj-1) (Figure 1B). Dendrites are distinguished from axons by their morphology and are immunopositive for MAP2 (Supplemental Figure S1). Dendrites emerge gradually from the cell body, taper with distance, generally have a radial orientation and terminate shortly from the cell body. Axons are thinner at their origin, show less taper, and follow a long and meandering course (Kaeck and Banker, 2006). A typical neuron has a single axon and several dendrites. Our data showed that neither overexpression nor knockdown of MARCKS affected the establishment of neuronal polarity (Figure 1C and Supplemental Figure S1). Dendritic arborization was strikingly increased in neurons transfected with MARCKS-GFP. In contrast, EGFP-transfected and nontransfected neurons displayed the same simple dendritic morphology. The result that neurons expressing MARCKS exhibited more short processes suggested that the effects of MARCKS were mainly on the development of fine architecture of dendritic arbors (Figure 1, B and C). To obtain a quantitative measurement of dendritic arborization, the total number of dendritic tip ends, which reflects branching in an individual neuron, was counted. Protrusions longer than 10  $\mu\text{m}$  and emerging from dendritic shafts or primary axons

were counted as branch tips. Analysis revealed that overexpressing MARCKS, although not affecting the number of axonal tip (Figure 1D), induced a 75% increase in total dendritic tip number and a 35% increase in total dendritic length (Figure 1, E and F). These results demonstrate that MARCKS is important for the regulation of dendritic branching at the early stages of neuronal development.

#### Down-Regulation of MARCKS Decreased Dendritic Arborization

To further investigate the role of endogenous MARCKS in dendritic development, we reduced MARCKS expression by using RNAi, an efficient approach used extensively to knockdown specific gene expression in neurons. We used a pSUPER vector to produce shRNA against MARCKS. The efficiency of the shRNA sequence used in this study has been verified in rat hippocampal neurons (Calabrese and Halpain, 2005). Neurons transfected with MARCKS shRNA before plating displayed a strong decrease in MARCKS immunoreactivity at 4 days in vitro (DIV), compared with untransfected cells (Supplemental Figure S2). Comparing with neurons transfected with EGFP, MARCKS shRNA-transfected neurons showed greatly shortened dendrites with severely reduced branching at 6 DIV (Figure 2A). Quantification revealed a 40% reduction of total dendritic tip

**Figure 2.** Depletion of endogenous MARCKS by shRNA decreases dendritic arborization. (A) Confocal images of 6 DIV neurons transfected with EGFP, nonsense shRNA, MARCKS shRNA, or MARCKS shRNA together with the rescue form of MARCKS-GFP. Depletion of endogenous MARCKS caused a defect in dendritic development. Coexpression of an shRNA-resistant form of MARCKS rescued the morphogenetic effect caused by MARCKS shRNA. Bar, 50  $\mu\text{m}$ . (B) Quantitative analysis of dendritic morphology. For MARCKS knockdown neurons, the total dendritic tip number was reduced by 41% of EGFP control.  $**p < 0.001$ . (C) A reduction of 33% in total length of dendrites was found in MARCKS down-regulated neurons.  $**p < 0.001$ , compared with EGFP group. Measurements were taken from 60 to 80 neurons in three independent experiments for each group.



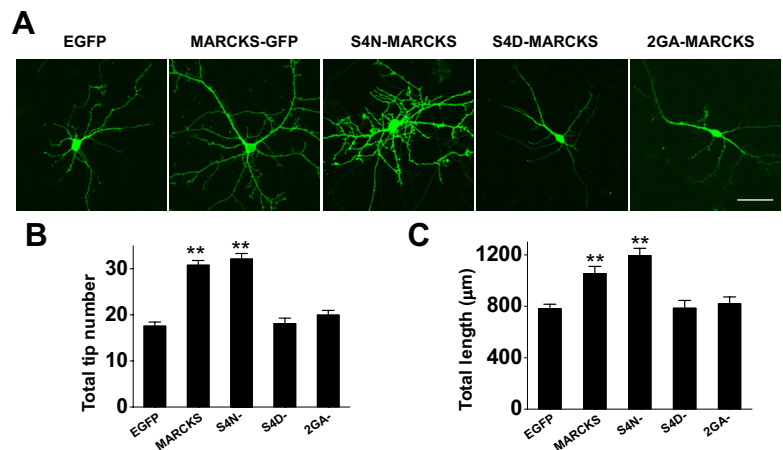
number caused by MARCKS shRNA (Figure 2B). Moreover, these neurons suffered a 33% decrease in total dendritic length (Figure 2C).

Although shRNA is thought to display a high degree of specificity for target sequence, it may also nonspecifically activate the Janus tyrosine kinase–signal transducer and activator of transcription pathway, leading to dendritic retraction and inhibition of dendritic growth in neurons. Therefore, we designed two control experiments to determine whether the observed phenotype is specifically due to knockdown of MARCKS. First, nonsense shRNA was made by scrambling the target shRNA sequence. After transfection of this shRNA, neurons showed morphology similar to the EGFP-transfected control (Figure 2). Second, we carried out a rescue experiment. Because the MARCKS shRNA sequence used here is located at the 3'-untranslated region (3'-UTR), rescue of expression can be achieved by simply coexpressing a MARCKS plasmid lacking this 3'-UTR. Results showed that the effect of MARCKS shRNA on dendrite morphology could be rescued by coexpression of an shRNA-resistant MARCKS lacking the 3'-UTR (Figure 2) but not by coexpression of an unrelated cDNA (DsRed; data not shown). Together, these controls validated the idea that the effects of MARCKS shRNA transfection are indeed caused by specific knockdown of MARCKS expression. Thus, endogenous MARCKS is critical for dendritic development.

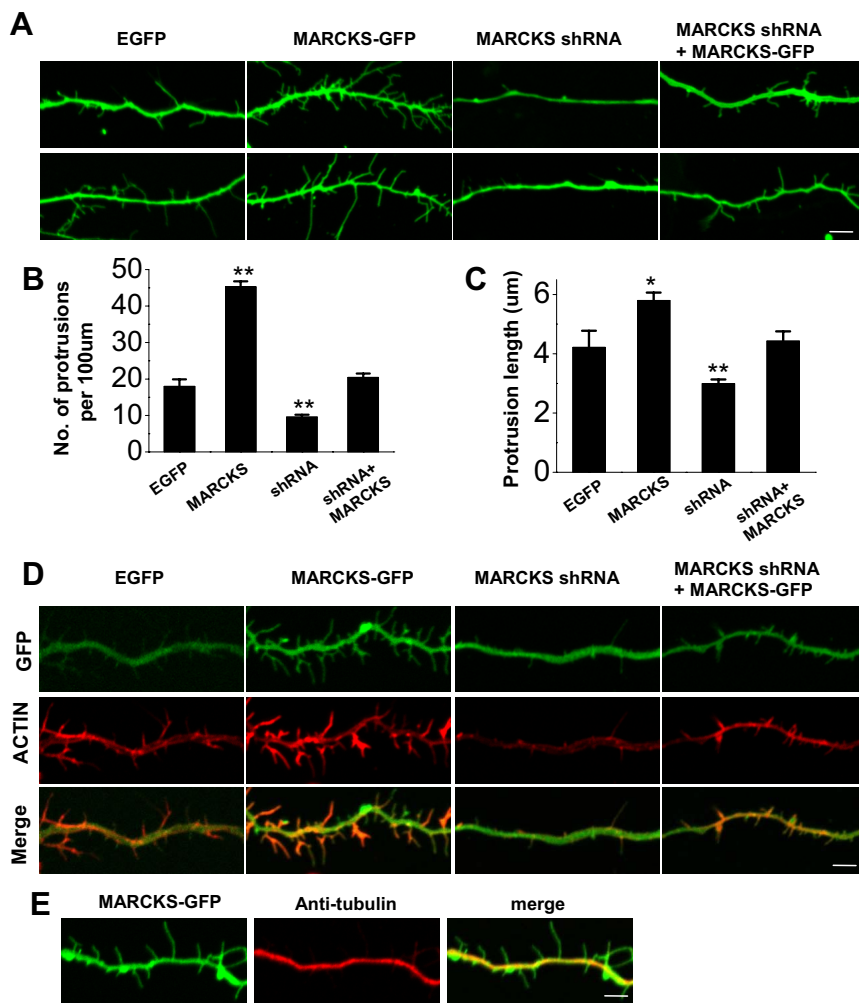
*Expression of Nonphosphorylatable, but Not Pseudophosphorylated or Nonmyristoylatable, Forms of MARCKS Increased Dendritic Arborization*

MARCKS has two conserved regions involved in membrane binding: a myristoylated N terminus and a basic effector domain (ED) (Arbuzova *et al.*, 2002). To determine whether membrane binding is required for dendritic arborization, we transfected neurons with the following constructs, which have been defined previously (Spizz and Blackshear, 2001; Calabrese and Halpain, 2005): 1) S4N-MARCKS, in which four serines in the ED are replaced by asparagines to make a mutant that is nonphosphorylatable by PKC and binds strongly to PI(4,5)P<sub>2</sub>; 2) S4D-MARCKS, in which the same four serines are replaced by aspartates to mimic constitutively phosphorylated MARCKS; and 3) 2GA-MARCKS, in which glycine at position 2 is replaced by alanine to create a nonmyristoylatable form of MARCKS. MARCKS binds to membrane through electrostatic interaction of the ED with the plasma membrane as well as myristate insertion into the bilayer. Neither of these interactions alone is sufficient for membrane binding, thus neither the S4D- nor the 2GA-MARCKS mutant can associate with the membrane.

Expression of S4N-MARCKS resulted in a marked increase in dendritic branching (Figure 3, A–C), similar to the effects of MARCKS-GFP (Figure 1). In agreement with previous studies



**Figure 3.** Expression of S4N-, but not S4D- or 2GA-MARCKS mutants, increases dendritic branching. (A) Confocal images of 6 DIV neurons transfected with EGFP, MARCKS-GFP, S4N-MARCKS (nonphosphorylatable), S4D-MARCKS (pseudophosphorylated), or 2GA-MARCKS (nonmyristoylatable). Bar, 50  $\mu\text{m}$ . (B and C) Quantitative analysis of dendritic morphology. S4N-MARCKS transfection led to increased branching (B) and length (C) of dendrites, whereas S4D-MARCKS and 2GA-MARCKS were unable to affect neuronal morphology.  $**p < 0.001$ , compared with EGFP group. Measurements were taken from 60 to 80 neurons in three independent experiments for each group.



**Figure 4.** Effects of MARCKS on filopodia-like protrusion formation and F-actin distribution. (A) Selected neuritic regions from 2 DIV neurons transfected with EGFP, non-sense shRNA, MARCKS shRNA, or MARCKS shRNA together with the rescue form of MARCKS-GFP constructs. Depletion of endogenous MARCKS caused a defect in filopodia development. Coexpression of an shRNA-resistant form of MARCKS rescued the morphogenetic effect caused by MARCKS shRNA. Bar, 5  $\mu$ m. (B and C) Protrusion density (B) and length (C) in neurons that were transfected with indicated constructs. (D) Selected neuritic regions from 2 DIV neurons transfected with the indicated constructs and stained with phalloidin (red) to detect F-actin. (E) Example images showing that filopodia in MARCKS-transfected neurons did not contain tubulin.

(Calabrese and Halpain, 2005), we found that both MARCKS-GFP and S4N-MARCKS were largely localized at the plasma membrane (data not shown). Therefore, it is likely that plasma membrane enrichment of both constructs is responsible for their effects on dendritic development. In contrast, overexpression of either the S4D- or the 2GA-MARCKS mutant had no effect on dendritic branching (Figure 3, A–C). These results indicate that the association with plasma membrane via the myristoylated N terminus and the ED domain is important for MARCKS-induced dendritic branching.

#### MARCKS Is Critical for Filopodia Formation

Filopodia formation is a key prerequisite for neuritogenesis (Dent *et al.*, 2007). Neurite branching is generally initiated by motile filopodia and involves reorganization of the cytoskeleton (Gallo and Letourneau, 2004; Lalli and Hall, 2005). Live imaging of developing pyramidal neurons in hippocampal slices revealed that dendritic shafts constantly extend and retract filopodia during early stages of development; some of these filopodia are transformed into nascent dendritic branches (Dailey and Smith, 1996; Niell *et al.*, 2004). Thus, regulation of filopodia is critical for outgrowth and arborization of neuronal processes.

Because we have demonstrated that MARCKS increased the number of short branches, it is likely that overexpression of MARCKS in cultured hippocampal neurons first induces filopodia formation and then promotes dendritic branching. In-

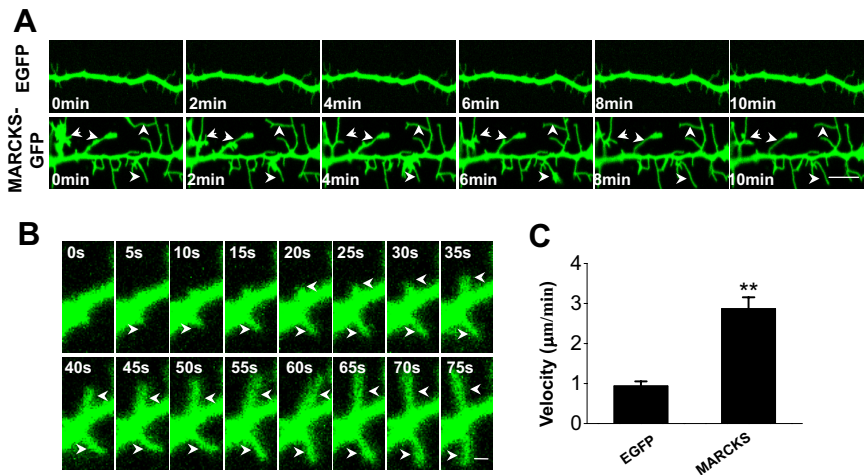
deed, the number and length of filopodia in neurons transfected with MARCKS were clearly increased at 2 DIV (Figure 4, A–C). In contrast, in the presence of MARCKS shRNA, few filopodia emerging from neurites were observed (Figure 4, A–C). The specificity of the MARCKS knockdown phenotype was confirmed by rescue experiments. MARCKS refractory to shRNA restored filopodia formation in neurons expressing MARCKS shRNA (Figure 4, A–C). These results indicated that MARCKS is critical for filopodia formation in hippocampal neurons.

To investigate the cytoskeletal organization of filopodia, we stained the transfected neurons with Alexa 546-conjugated phalloidin to reveal actin filaments, and with anti-tubulin antibody to visualize microtubules. Filopodia were labeled with phalloidin but did not contain microtubules (Figure 4, D and E). MARCKS-overexpressing neurons had more actin-based filopodia sprouting from neurites (Figure 4D). This morphological change was sensitive to latrunculin A (LTA)-induced actin depolymerization, because LTA treatment almost abolished the formation of filopodia (data not shown). These observations suggest that MARCKS may regulate the number and length of filopodia by affecting the actin cytoskeleton.

#### MARCKS Increased the Spontaneous Motility of Filopodia

Time-lapse imaging revealed two types of filopodia: stable filopodia that did not change in length, and dynamic filop-

**Figure 5.** MARCKS increases filopodia motility. (A) Representative images of 2 DIV hippocampal neurons, showing multiple filopodia (arrowhead) extending and retracting along the shaft in neurons transfected with MARCKS-GFP but not in neurons transfected with EGFP. Images were collected for 10 min at 5-s intervals. Bar, 5  $\mu$ m. (B) Higher magnification image of a shaft region, showing two filopodia (arrowhead) with sprouting from the shaft of MARCKS transfected neuron. (C) Elongation/withdrawal rate of filopodia. Transfected hippocampal neurons were subjected to time-lapse analysis. Dynamics of filopodia were observed by GFP signals. Images were collected for 10 min at 5-s intervals. \*\* $p < 0.001$ .



odia that extended and retracted frequently during the 10-min recording period (Figure 5A). Filopodial motion from 15 EGFP-transfected neurons and 16 MARCKS-transfected neurons was quantified. In EGFP-transfected neurons, most filopodia were stable and only a small portion (87 of 228) were dynamic (Supplemental Video 1.mov), with a mean velocity of  $0.94 \pm 0.013 \mu\text{m}/\text{min}$ . Transfection with MARCKS greatly increased the population of dynamic filopodia (169 of 216) (Supplemental Video 2.mov), as well as the velocity of movement ( $2.87 \pm 0.26 \mu\text{m}/\text{min}$ ) (Figure 4C).

The positive regulation of filopodia density by MARCKS could be due to either an increased frequency of new filopodium initiation or an increased stability of filopodia. The number of filopodia formed de novo from a stable neurite in MARCKS-transfected neurons ( $16.95 \pm 1.32/100 \mu\text{m}$ ;  $n = 15$ ) was significantly higher than that in EGFP-transfected controls ( $4.89 \pm 0.79/100 \mu\text{m}$ ;  $n = 15$ ) during the 10-min recording period. Most of these new filopodial protrusions extended and then retracted quickly (Figure 5B). The stability of new filopodia was analyzed as the duration of filopodial persistence along neurites. There was no significant difference in the average duration of filopodial persistence between EGFP ( $2.89 \pm 0.12 \text{ min}$ ;  $n = 20$ ) and MARCKS-transfected neurons ( $3.07 \pm 0.18 \text{ min}$ ;  $n = 20$ ). Thus, it is the increased frequency of new filopodium initiation that contributes to the increased number of filopodia along neurites in MARCKS-transfected neurons.

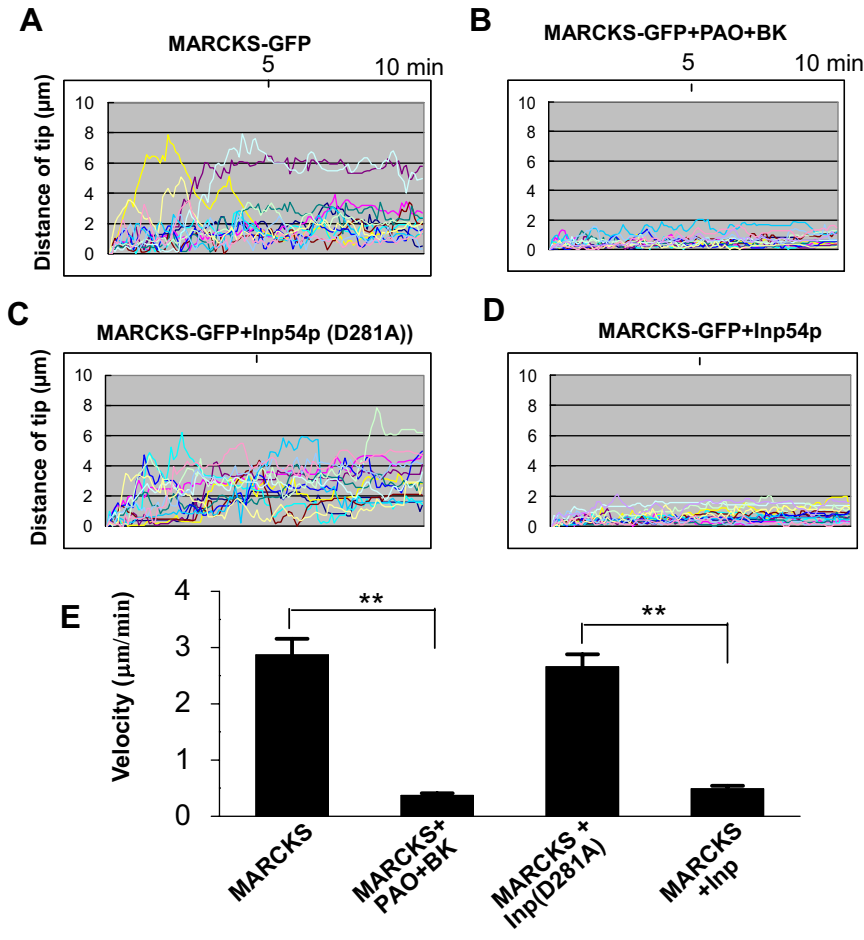
#### MARCKS Increased Filopodia Motility in a PI(4,5)P2-dependent Manner

The extension and withdrawal of filopodia is determined by the dynamics of the underlying actin cytoskeleton. Because MARCKS binds and passively concentrates PI(4,5)P2 in the lateral membrane domains (McLaughlin *et al.*, 2002) and PI(4,5)P2 interacts with a large number of actin-binding proteins (Janmey and Lindberg, 2004), it is likely that the increased PI(4,5)P2 level at the plasma membrane is responsible for actin remodeling within MARCKS-induced filopodia.

To investigate the potential involvement of PI(4,5)P2 in the MARCKS-induced filopodia motility, we used two approaches to reduce PI(4,5)P2 availability. The first approach was to manipulate membrane PI(4,5)P2 by using phospholipase C (PLC)-mediated PI(4,5)P2 breakdown. Bradykinin (BK) activates PLC to induce the breakdown of PI(4,5)P2 into diacylglycerol and 3,4,5-trisphosphate (InsP3) (Van and Jalink, 2002). Phenylarsine oxide (PAO) is an inhibitor of PI

4-kinase, which is involved in the production of phosphatidylinositol monophosphate, the precursor for PI(4,5)P2 (Wiedemann *et al.*, 1996). Pretreatment of the culture with a low dose of PAO (1  $\mu\text{m}$ ) prevented the recovery of the decreased PI(4,5)P2 back to the basal levels following the BK-induced PLC activation (van and Jalink, 2002). To determine the dynamic changes in PI(4,5)P2 signal, we transfected neurons with mRFP-PH(PLC $\delta$ ), the PI(4,5)P2 reporter. We found that treatment with PAO and BK for 5 min induced a redistribution of mRFP-PH(PLC $\delta$ ) from the plasma membrane to the cytoplasm in neurons expressing MARCKS and mRFP-PH(PLC $\delta$ ) (Supplemental Figure S3), suggesting a rapid hydrolysis of PI(4,5)P2 at the plasma membrane. Furthermore, when the same treatment was applied to hippocampal neurons expressing MARCKS at 2 DIV, filopodia movement was immediately immobilized (Figure 6, B and E, and Supplemental Video 3.mov).

BK effectively hydrolyzes PI(4,5)P2 at the plasma membrane, but it also increases cytosolic InsP3, which may induce  $\text{Ca}^{2+}$ -dependent processes. Thus, we used a more specific method to deplete PI(4,5)P2: rapamycin-induced membrane translocation of Inp54p, a yeast inositol polyphosphate 5-phosphatase that specifically cleaves the phosphate at the 5-position of PI(4,5)P2 (Suh *et al.*, 2006). HEK293 cells were transfected with mRFP-PH(PLC $\delta$ ), Lyn11-FRB, together with either CFP-FKBP-Inp54p(Inp54p) or the phosphatase-dead mutant Inp54p(D281A). Addition of rapamycin induced a rapid translocation of mRFP-PH(PLC $\delta$ ) from the plasma membrane to the cytosol within 3 min in cells transfected with Inp54p (Supplemental Figure S4) as described previously (Suh *et al.*, 2006), confirming the PI(4,5)P2 depletion. In contrast, mRFP-PH(PLC $\delta$ ) did not detach from the plasma membrane upon the addition of rapamycin in cells expressing Inp54p(D281A). The effect of Inp54p on PI(4,5)P2 in hippocampal neurons was also examined. The expression of Inp54p had no apparent toxic effect on the development of hippocampal neurons (Supplemental Figure S4C). Quantitative analysis showed that mutant Inp54p(D281A) did not induce significant translocation of fluorescence signal from the plasma membrane to the cytoplasm (Supplemental Figure S4, D and E) in response to rapamycin application. In contrast, cells expressing Inp54p showed a significant translocation of the fluorescence signal 3 min after rapamycin application (Supplemental Figure S4, D and E), indicating rapid hydrolysis of PI(4,5)P2.

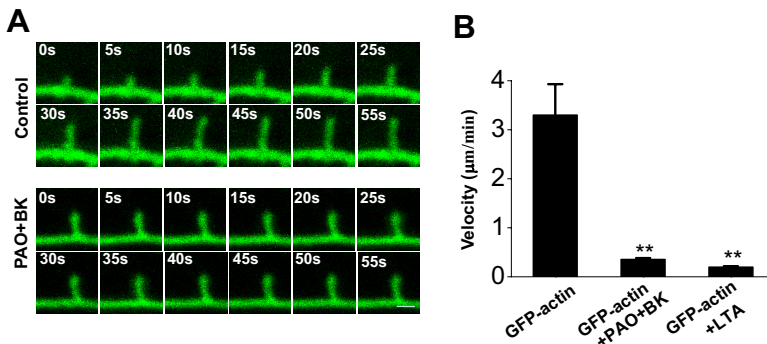


**Figure 6.** Effect of PI(4,5)P2 depletion on filopodia dynamics. (A–D) Time-lapse analysis of filopodia dynamics in neurons transfected with MARCKS-GFP (A) or pretreated with PAO for 5 min and then stimulated with BK (B). Time-lapse analysis of filopodia dynamics in neurons expressing MARCKS-GFP, Lyn11-FRB, and either CFP-FKBP-Inp54p (D281A) (C) or CFP-FKBP-Inp54p(Inp) (D) after treatment with rapamycin. Images were taken every 5 s, and the relative positions of filopodia tip were measured during the 10-min imaging period. In the graphs, the x-axis represents time, whereas the y-axis represents the elongation/withdrawal length of filopodia tip from the start point over time. (E) Elongation/withdrawal rate of filopodia. \*\* $p < 0.001$ . PAO, 1  $\mu\text{M}$ ; BK, bradykinin, 1  $\mu\text{M}$ ; Rapa, rapamycin, 100 nM.

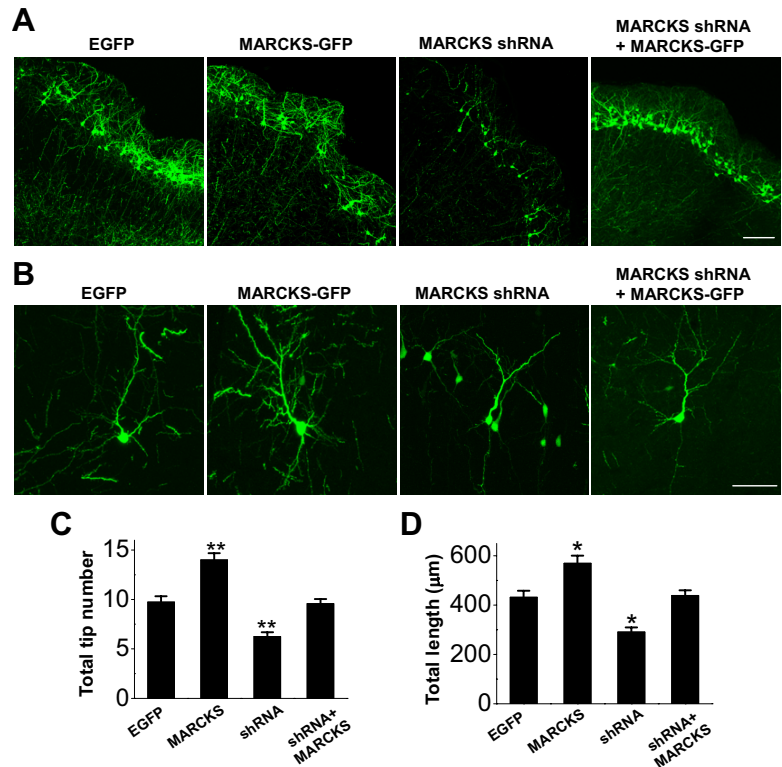
We then determined whether PI(4,5)P2 depletion suppressed filopodia dynamics in MARCKS-transfected neurons. Addition of rapamycin had no effect on the filopodia dynamics in cells expressing the mutant Inp54p(D281A) (Figure 6C and Supplemental Video 4.mov). In contrast, upon rapamycin-mediated recruitment of Inp54p to the plasma membrane, the motility of filopodia in MARCKS-expressing neurons largely ceased (Figure 6D and Supplemental Video 5.mov), indicating that PI(4,5)P2 is required for MARCKS-induced actin remodeling.

Filopodia are slender, actin-based protrusions and regulation of assembly of the actin cytoskeleton at filopodium tips controls their extension and retraction (Mallavarapu and

Mitchison, 1999). We further examined the behavior of the actin cytoskeleton by time-lapse imaging of neurons coexpressing GFP-actin and MARCKS. The actin clusters in MARCKS-induced filopodia were highly motile with an average speed of  $3.29 \pm 0.6 \mu\text{m}/\text{min}$ . Strikingly, PI(4,5)P2 hydrolysis evoked by PAO and BK caused prolonged inhibition of actin extension within filopodia (Figure 7A and Supplemental Video 6.mov). These results show that filopodial actin assembly correlate well with membrane PI(4,5)P2 content. Treatment with the actin-depolymerizing drug LTA (2  $\mu\text{M}$ ; 15 min) also abolished actin extension. Collectively, these results suggest that regulation of actin assembly within filopodia by MARCKS is mediated by PI(4,5)P2 at the plasma membrane.



**Figure 7.** Influence of PI(4,5)P2 depletion on actin dynamics within filopodia. (A) Time-lapse series of confocal images of GFP-actin expression in MARCKS-induced filopodia. Neurons were pretreated with 1  $\mu\text{M}$  PAO for 5 min and then stimulated with 1  $\mu\text{M}$  BK. BK induced PI(4,5)P2 breakdown, resulting in the inhibition of actin movement. Bar, 1  $\mu\text{m}$ . LTA (2  $\mu\text{M}$ ; 15 min) treatment blocked F-actin assembly. Bar, 1  $\mu\text{m}$ . (B) The elongation/withdrawal rate of filopodia. \*\* $p < 0.001$ .



**Figure 8.** MARCKS regulates dendritic branching in vivo. (A) Coronal sections of neocortex showing EGFP-positive neurons transfected by in utero electroporation. Bar, 100  $\mu\text{m}$ . (B) Higher magnification images of neocortical slices from the same animal as in A, showing the morphology of individual pyramidal neurons. The apical dendrites are oriented toward the top of the figure. Dendritic branching and growth were significantly increased in neurons overexpressing MARCKS. In contrast, neurons transfected with MARCKS shRNA developed simpler arbors. Coexpression of a shRNA-resistant form of MARCKS rescued the morphogenetic effect caused by MARCKS shRNA. Each image consists of a z-stack of 20–30 individual pictures taken at depth interval of 1  $\mu\text{m}$  and then projected into one image. Bar, 50  $\mu\text{m}$ . (C and D) Averaged total dendritic tip number and total dendrite length in neurons transfected with various constructs as shown in A. \* $p < 0.005$ ; \*\* $p < 0.001$ , compared with EGFP group. Measurements were taken from 30 neurons in five animals for each group.

#### Effect of MARCKS on Neuronal Morphological Development in Vivo

To extend the results obtained in cultured neurons to a more physiological system, we addressed the role of MARCKS in the developing brain by in utero electroporation, an efficient approach for delivering DNA into developing neural cells to visualize neuronal morphology (Saito and Nakatsuji, 2001; Borrell *et al.*, 2005). The morphology of cortical pyramidal neurons is comparable with that of hippocampal pyramidal neurons, characterized by their distinct apical and basal dendritic trees and the pyramidal shape of their soma (Spruston, 2008). Thus, for technical convenience, we limited our analysis to cortical pyramidal neurons for the in utero electroporation studies.

Plasmids encoding EGFP, MARCKS, or MARCKS shRNA were injected together with a GFP construct (pCAGGS-GFP) into the lateral ventricle of E15.5–E16.5 embryos for electroporation. Because neurons destined for each region of the neocortex are known to arise in an “inside-to-outside” sequence from ventricular epithelium, and the layer 2/3 neurons are generated from E15 through E17 (Takahashi *et al.*, 1999), the majority of the GFP-transfected cells in our experiment were layer 2/3 pyramidal neurons. Two weeks after electroporation, coronal sections of the cerebral cortex were prepared to allow assessment of the morphology of transfected neurons. A band of GFP-positive neurons was found in the cortical layer, and no aberrant layer patterning was observed (Figure 8A). Essentially, all of the GFP-labeled cells had a pyramidal-like morphology characteristic of layer 2/3 neurons, with apical dendrites oriented toward the pia and axons extending into the white matter. Compared with the EGFP-transfected control neurons, dendritic growth and branching were greatly compromised in the MARCKS shRNA-transfected neurons; in contrast, a marked increase in dendritic branching was seen in MARCKS-transfected

neurons (Figure 8B). In both cases, however, the differentiation of axons was not apparently affected (data not shown). The effect of MARCKS shRNA on dendrite morphology was rescued by coexpression of an shRNA-resistant MARCKS (Figure 8, A and B) but not by coexpression of an unrelated cDNA (DsRed; data not shown). To quantify the number of branches from the dendrites of pyramidal neurons, we used confocal microscopy with a z-stack to reconstruct the arborization of GFP neurons. Consistent with the results from cultured neurons, we found that neurons expressing MARCKS shRNA had marked defects in dendritic arborization. Although expression of MARCKS significantly increased the complexity of the dendritic arbor (Figure 8, C and D). Thus, MARCKS is important for the regulation of dendritic arbor complexity in vivo as well as in cultured neurons.

#### DISCUSSION

##### MARCKS Is Critical for Dendritic Arborization

Our results indicate that overexpression of MARCKS alone is sufficient to enhance dendritic arborization, whereas knockdown of endogenous MARCKS reduces dendritic complexity. MARCKS has two conserved domains for membrane binding: a myristoylated N-terminal domain and a basic effector domain. Interestingly, we found that overexpression of MARCKS or S4N-MARCKS in neurons enhanced branching morphogenesis, whereas S4D- or 2GA-MARCKS did not have any effect on neuronal morphology. Thus, membrane binding of MARCKS is essential for its effect on neuronal morphogenesis.

A previous study showed that MARCKS is critical for the maintenance and morphology of dendritic spines. Knockdown of endogenous MARCKS reduces spine density and size, whereas its overexpression dramatically increases the



length of the protrusions along the dendrites (Calabrese and Halpain, 2005). Both dendritic branching and spine formation may have positive correlation with filopodia initiation. It has been suggested that the dendritic shafts of pyramidal neurons in hippocampal slices constantly extend and retract filopodia during early development, with some of these filopodia transformed into nascent dendritic branches, whereas later in development these dynamic filopodial protrusions can develop into dendritic spines (Dailey and Smith, 1996). The dendritic filopodia and the putative precursors of stable branches may also act as spine precursors (Yuste and Bonhoeffer, 2004). Thus, MARCKS is necessary for both dendritic branching and spine formation, although they occur at different developmental stages.

#### **Regulation of the Actin Cytoskeleton by MARCKS via PI(4,5)P2**

The actin cytoskeleton is fundamental for morphogenetic processes in neurons. Our results confirmed that MARCKS is a regulator of the actin cytoskeleton in developing neurons. Filopodia are cell surface extensions filled with bundles of actin filaments. Regulation of filopodia in neurons is important for neurite branch formation, which determines the ultimate arborization and connectivity in the nervous system (Gallo and Letourneau, 1999). We found that MARCKS increased the number and length of filopodia. Time-lapse imaging showed that expression of MARCKS dramatically increased the motility of filopodia and engendered more filopodia initiation from the shaft, probably through its effects on F-actin dynamics.

The mechanisms involved in the regulation of the actin cytoskeleton by MARCKS remain unclear. MARCKS has the ability to bind and cross-link with F-actin *in vitro* (Hartwig *et al.*, 1992); however, evidence for a direct interaction between MARCKS and actin in cells is still lacking. More recent studies suggest an alternative hypothesis for the regulation of the actin cytoskeleton by MARCKS through PI(4,5)P2 (McLaughlin *et al.*, 2002). MARCKS sequesters a significant fraction of PI(4,5)P2 at the plasma membrane and releases this lipid in response to local signals such as increased calcium or activation of protein kinase C. Increasing the level of cellular PI(4,5)P2 tends to promote actin assembly, whereas removing or hydrolyzing it leads to actin disassembly (Janmey and Lindberg, 2004). The effect of PI(4,5)P2 on actin assembly is strong and specific. Colocalization of MARCKS and PI(4,5)P2 is observed in fixed cells. In addition, overexpression of MARCKS leads to a concomitant increase in the level of PI(4,5)P2 in the cell (Laux *et al.*, 2000). Our results also suggest that MARCKS regulates actin cytoskeleton assembly through sequestering and increasing the local concentration of PI(4,5)P2 at the plasma membrane. We found that membrane binding was necessary for MARCKS-induced dendritic morphogenesis, consistent with a previous report that only membrane-bound MARCKS sequesters and increases the local concentration of PI(4,5)P2 at the plasma membrane (McLaughlin *et al.*, 2002). Furthermore, we found that MARCKS accumulated and modulated the PI(4,5)P2 pool at the plasma membrane in hippocampal neurons. In addition, depletion of PI(4,5)P2 inhibited the motility of filopodia in neurons overexpressing MARCKS. These results demonstrate that PI(4,5)P2 is required for MARCKS-induced neuronal morphogenesis.

#### **Initiation of Filopodia and Dendritic Branches through MARCKS-PI(4,5)P2 Signaling**

The mechanisms underlying the initiation of filopodia and dendritic branches are not clear, but filopodia dynamics and

dendritic branching have been reported to depend on calcium signaling (Konur and Ghosh, 2005). Neonatal hippocampal CA1 neurons generate spontaneous elevations in intracellular calcium that spread through short lengths of dendrites, occurring more frequently at future branch points (Yuste *et al.*, 1995; Koizumi *et al.*, 1999). Under resting conditions (low free cytoplasmic  $Ca^{2+}$ ), MARCKS binds PI(4,5)P2 and attaches to the plasma membrane of developing neuronal processes, leading to an increased local pool of PI(4,5)P2 (McLaughlin and Murray, 2005). Recent studies showed that activation of actin-related protein2/3 (Arp2/3) by neuronal Wiskott-Aldrich syndrome protein (N-WASP) is ultrasensitive to PI(4,5)P2 concentration and may be coupled with the MARCKS protein (Murray and Honig, 2005; Papayannopoulos *et al.*, 2005). When the free cytoplasmic  $Ca^{2+}$  concentration increases in the future branch point,  $Ca^{2+}$ /calmodulin may bind to MARCKS with high affinity and pull it off the membrane, releasing the laterally sequestered pool of PI(4,5)P2. This may result in local bursts of free PI(4,5)P2 that produces a local potential strong enough to activate N-WASP, which in turn activates and localizes the Arp2/3 complex to nucleate actin filaments near the plasma membrane (Murray and Honig, 2005), leading to the formation of branched actin filaments and causing filopodia formation, some filopodia transforming into new branches.

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