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Scapinin-induced Inhibition of Axon Elongation Is Attenuated by Phosphorylation and Translocation to the Cytoplasm[S]

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Scapinin is an actin- and PP1-binding protein that is exclusively expressed in the brain; however, its function in neurons has not been investigated. Here we show that expression of scapinin in primary rat cortical neurons inhibits axon elongation without affecting axon branching, dendritic outgrowth, or polarity. This inhibitory effect was dependent on its ability to bind actin because a mutant form that does not bind actin had no effect on axon elongation. Immunofluorescence analysis showed that scapinin is predominantly located in the distal axon shaft, cell body, and nucleus of neurons and displays a reciprocal staining pattern to phalloidin, consistent with previous reports that it binds actin monomers to inhibit polymerization. We show that scapinin is phosphorylated at a highly conserved site in the central region of the protein (Ser-277) by Cdk5 *in vitro*. Expression of a scapinin phospho-mimetic mutant (S277D) restored normal axon elongation without affecting actin binding. Instead, phosphorylated scapinin was sequestered in the cytoplasm of neurons and away from the axon. Because its expression is highest in relatively plastic regions of the adult brain (cortex, hippocampus), scapinin is a new regulator of neurite outgrowth and neuroplasticity in the brain.

The brain has a remarkable capacity to restructure its neuronal circuitry. This phenomenon is known as neuroplasticity and is critical for higher order functions, such as cognition, calculation, adaptation, and memory formation. It is especially active during development but persists in regions of the adult brain associated with higher order functions, especially the cortex and hippocampus. Neuroplasticity is achieved via two mechanisms: 1) altering the strength of existing synapses and circuits and 2) forming new synapses and circuits. The former mechanism primarily involves changes in the release of neurotransmitters at the pre-synapse or expression of neurotransmitter receptors at the post-synapse, whereas the latter mechanism involves neurite outgrowth and new synapse formation. Defects in neuroplasticity occur with aging and are dramatic in neurodegenerative diseases, such as Alzheimer disease. Conversely, enhancing neuroplasticity might be an effective therapy for improved mental function following brain insults, including stroke, dementia, and age-related cognitive decline. Therefore, it is important to understand the molecular mechanisms controlling neuroplasticity to discover new therapeutic targets for the maintenance of healthy brain function.

Scapinin[2] (scaffold-associated PP1-inhibiting protein) is a brain-specific member of the Phactr (phosphatase and actin regulator) family of proteins (also called Phactr3 (1–3)). There are three splice variants of scapinin in mammals encoding proteins of 559 (62.5 kDa), 518 (58.5 kDa), and 448 (51.5 kDa) amino acids (1), with isoform 2 (518 amino acids) being predominant in human brain (4). It was originally identified as a component of the nuclear matrix intermediate filament scaffold (1). Since then, it has been detected in both the nucleus and the cytoplasm of transformed cell lines (1, 2, 4, 5). In adult mouse brain, scapinin mRNA is enriched in the cortex and hippocampus (5), which also display relatively high neuroplasticity. Scapinin binds to actin monomers (G-actin) via RPEL motifs in the C-terminal region of the protein (5), inhibiting actin polymerization into filaments (4). It also binds to the abundant Ser/Thr protein phosphatase 1 (PP1) and inhibits its activity (5). Inducible expression of scapinin in HeLa cervical carcinoma cells modified their morphology, stimulated cell spreading, and promoted motility/migration, which was dependent on its ability to bind actin (4). Defects in cell adhesion were also noted, whereby scapinin-expressing cells exhibited increased (4) or decreased (2) adherence to tissue culture substratum.

Despite being exclusively expressed in the brain, the function of scapinin in neurons has not been investigated. Given its enrichment in relatively plastic regions (cortex, hippocampus (5)) and its ability to regulate the actin cytoskeleton, it is reasonable to predict that scapinin might regulate neuronal morphology and neuroplasticity. Here we demonstrate that scapinin inhibits axon elongation in primary rat cortical neurons via regulation of actin dynamics, and this function can be modified by Ser phosphorylation and subcellular localization. This sug-

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[2] The abbreviations used are: scapinin, scaffold-associated PP1-inhibiting protein; PP1, protein phosphatase 1; Cdk5, cyclin-dependent kinase 5; DYRK, dual specificity tyrosine phosphorylation-regulated kinase; MAL, megakaryocytic acute leukemia protein; E, embryonic day; DIV, days *in vitro*; DMSO, dimethyl sulfoxide.

gests that scapinin is a potential modulator of neuroplasticity in the brain.

EXPERIMENTAL PROCEDURES

Materials—The cDNA encoding full-length human scapinin isoform 2 (Swiss-Prot Q96KR7-2) was amplified by PCR from Image clone 4179728 (Open Biosystems, Thermo Scientific) using the primers 5′-GAATTCGCCACCATGGACTACAAGGACGACGATGACAAGGACCAAACGCCCCCG-3′ and 5′-GGCGAATTCCTATGGCCTGTGGAATCTTG-3′, including the introduction of a 5′ (N-terminal) FLAG tag. The PCR product was subcloned into pRK5 (CMV promoter) for mammalian expression. The S277A, S277D, R407A,P408A (RP-AA), and F515A mutants were generated using a QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. The pSer277 phospho-specific antibody was generated by injection of three rabbits with the peptide CRGpSPKKRM (where pS is phospho-serine) that was conjugated to keyhole limpet hemocyanin. Antisera were affinity-purified on a phospho-peptide antigen-agarose column. Immunoblotting and immunofluorescence analyses using purified pSer277 were routinely performed in the presence of dephospho-peptide to reduce nonspecific binding to dephosphorylated scapinin. Total scapinin goat polyclonal antibodies S-18 and N-20 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Tau-1 and MAP2 antibodies were obtained from Millipore (North Ryde, Australia). Alexa Fluor 568-phalloidin was from Invitrogen (Mount Waverly, Australia), whereas 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) and anti-FLAG monoclonal antibody were from Sigma (Castle Hill, Australia). All fluorescent secondary antibodies were supplied by Jackson ImmunoResearch Laboratories (West Grove, PA). Roscovitine was purchased from Merck (Kilsyth, Australia), and harmine was purchased from Cayman Chemical Company (Ann Arbor). Rats were supplied by the Animal Resources Centre (Perth, Australia).

Cell Culture—Primary cortical neurons were isolated from E17 or E18 Sprague-Dawley rats, plated onto glass coverslips coated with high molecular weight poly-D-lysine (Millipore), and incubated at 37 °C with 5% CO_2 for up to 2 weeks in Neurobasal media containing 2% (v/v) B27 serum replacement, 2 mM GlutaMAX, penicillin (50 units/ml), and streptomycin (100 units/ml) (Invitrogen). Media were replenished every 2–3 days. Neurons were transfected at the time of plating using the Amaxa electroporation kit according to the manufacturer's instructions (Lonza, Cologne, Germany) or at 1 days *in vitro* (DIV) using calcium phosphate precipitation. Neurons were treated with 20 μM roscovitine and 2 μM harmine for 48–72 h, as well as 2 μM latrunculin A and 1 μM cytochalasin D for 24 h. HEK293 cells were maintained in DMEM media supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM GlutaMAX, and penicillin/streptomycin at 37 °C with 5% CO_2. HEK293 cells were transfected using the DharmaFECT transfection reagent according to the manufacturer's instructions (Dharmacon, Lafayette, Colorado). Cells were lysed in buffer containing 1% (v/v) Triton X-100, 50 mM Tris-HCl, pH 7.5, 0.27 M sucrose, 1 mM EDTA, 0.1 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.1% (v/v) β-mercaptoethanol, and Complete protease inhibitor tablets (Roche Applied Science, Basel, Switzerland) (4 °C). Following centrifugation to remove insoluble material, supernatants were collected, and protein concentrations were determined using the Bradford method (Sigma (6)).

Immunofluorescence Microscopy—Neurons were fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.5% (v/v) Triton X-100, blocked with 2% (w/v) BSA, and incubated with primary antibodies in PBS for 2–4 h at room temperature at the following concentrations (anti-pSer277 at 0.1 μg/ml in the presence of 5 μM dephospho-peptide; anti-scapinin S-18 and N-20 at 2 μg/ml; anti-Tau-1 and anti-MAP2 at 1:1000; anti-FLAG at 2 μg/ml). Fluorescent secondary antibodies, phalloidin, and DAPI (Invitrogen) were diluted 1:500 and incubated on neurons for 1 h at room temperature. Image acquisition was performed on Olympus BX60 (Olympus Corp.) and Zeiss Axiocam mRm microscopes (Carl Zeiss, Inc.) using 20× and 40× objective lenses. Image analysis was performed using the National Institutes of Health ImageJ software. Axons were identified by their morphology (longest neurite, thin structure), positive staining for Tau-1, or negative staining for MAP2. Neurites and branches greater than 5 μm were included in statistical analyses. Growth cones greater than 10 μm in width were considered abnormally large. Statistical analyses were performed using paired Student's *t* test, and results were considered significant when $p < 0.05$.

Kinase Assays—FLAG-scapinin was isolated from 500 μg of HEK293 cell lysate using 10 μl of anti-FLAG-agarose (Sigma), washed in cold lysis buffer, and then left untreated or treated with 15 units of recombinant PP1α (New England Biolabs; 30 °C, 3 h). PP1 was removed by washing, and the untreated and PP1-treated scapinin proteins were separately subjected to *in vitro* kinase assays with recombinant Cdk5-p35 (50 milliunits; Millipore) in cell lysis buffer containing 50 mM Tris-HCl, pH 7.5, 0.03% (v/v) Brij-35, 0.1% (v/v) β-mercaptoethanol, 50 mM NaF, and radiolabeled [γ-^{32}P]ATP (30 °C, 0.5 h). Reactions were terminated by the addition of SDS loading buffer, subjected to SDS-PAGE, and stained with CBR-250. Radiolabeled bands were visualized by autoradiography and excised from the gels, and the amount of ^{32}P incorporated into scapinin wash was determined by liquid scintillation counting.

Western Blotting and Immunoprecipitation—Whole brains or frontal cortex from rats and mice were homogenized in cold cell lysis buffer using a glass Dounce homogenizer, whereas cultured cells were rinsed once in cold PBS and then scraped directly into cell lysis buffer. Insoluble material was removed by centrifugation and the protein concentration of the supernatant was determined using the Bradford assay. SDS loading buffer was added to cell lysates and subjected to SDS-PAGE and then transferred to nitrocellulose membrane using the XCell II blot module (120 V-h; Invitrogen). Membranes were blocked in 5% (w/v) skim milk powder in PBS and then incubated with primary antibody overnight at 4 °C (anti-pSer277 antibody 1 μg/ml with 1 μM dephospho-peptide; anti-FLAG antibody 1 μg/ml). Following extensive washing, membranes were incubated with fluorescent secondary antibodies (Li-Cor, Lincoln, NE), washed again, and visualized using a Li-Cor Odyssey infrared imaging system. FLAG-scapinin was immunoprecipitated

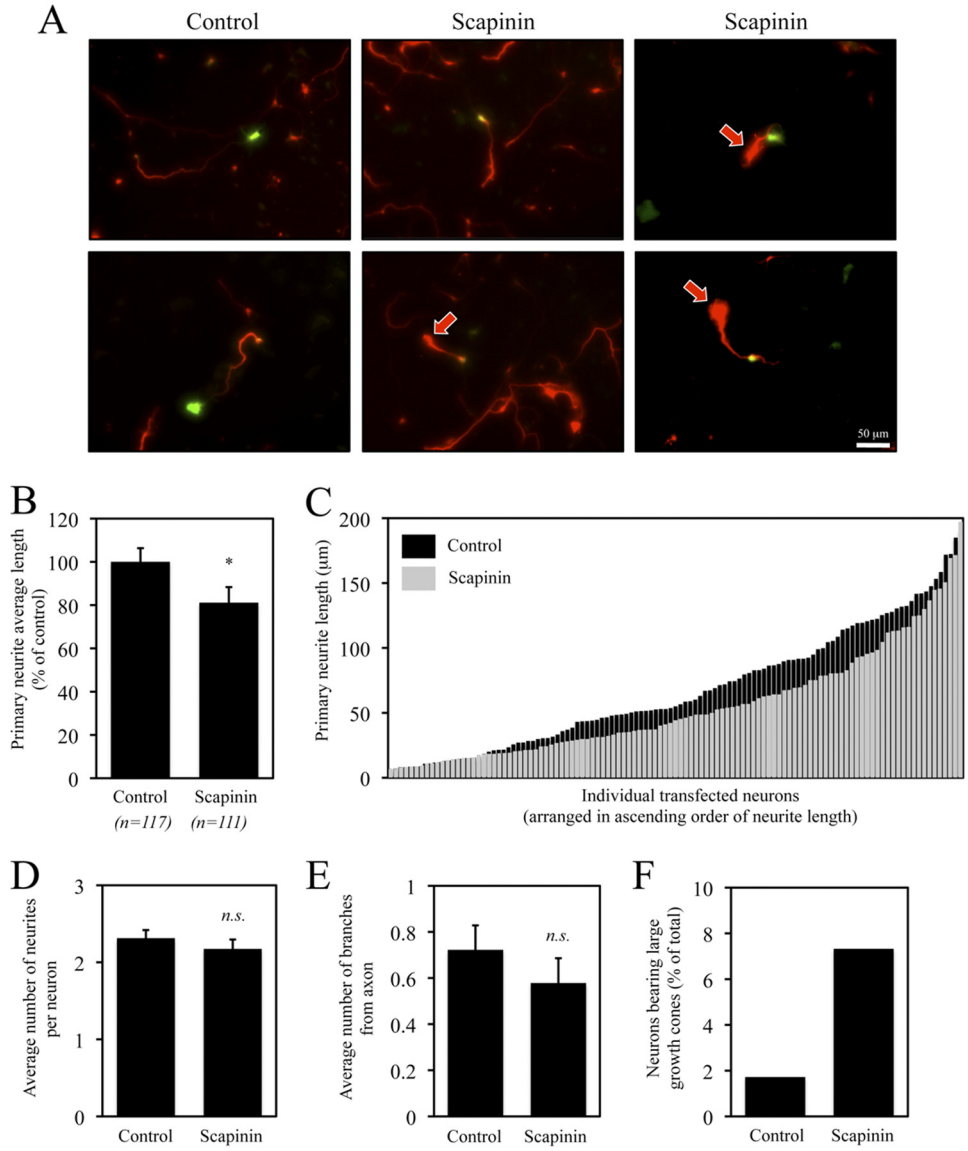


FIGURE 1. **Scapinin inhibits axon elongation in cortical neurons.** *A*, cortical neurons isolated from E18 rats were co-transfected using the Amaxa electroporation system at the time of plating with GFP and scapinin ($n = 111$) or GFP and empty vector (control; $n = 117$) and incubated for 3 days. Transfected neurons were identified by detection of GFP (*green*), whereas neuronal morphology was visualized using an antibody that recognizes the neuronal cytoskeleton-associated protein Tau-1 (*red*). *Arrows* indicate abnormally large growth cones on scapinin-transfected neurons. Neuronal morphology and neurite lengths were analyzed using the ImageJ software. *B–F*, results of analyses are presented as graphs, showing a comparison of averaged axon lengths (*B*), individual axon lengths (*C*), number of neurites per cell (*D*), branching from the axon (*E*), and percentage of neurons with abnormally large growth cones (*F*). *Error bars* = S.E., *, $p < 0.05$, *n.s.* = not significant, Student's *t* test.

from 2 mg of HEK293 cell lysates using 20 μl of anti-FLAG-agarose (4 °C, 3 h), washed once in cell lysis buffer containing 0.5 M NaCl, washed twice in cell lysis buffer, and then resuspended in SDS loading buffer and subjected to SDS-PAGE. Gels were stained with CBR-250 or transferred to nitrocellulose for Western blot analysis.

RESULTS

Scapinin Inhibits Axon Elongation in Cortical Neurons—Cultured neurons from E18 rats were co-transfected at the time of plating with GFP and either empty vector (control) or scapinin (full-length human isoform 2), and their morphologies were analyzed using immunofluorescence microscopy. As compared with control cells, scapinin-expressing neurons displayed significantly shorter primary neurites (nascent axons) in 3 DIV cortical neurons (Fig. 1, *A* and *B*). A similar reduction in axon length was observed in 5 DIV cultured rat hippocampal neurons (data not shown). A comparison of the individual axon lengths from control and scapinin-transfected neurons showed that both types of neurons had axon lengths ranging from 10 to 200 μm (Fig. 1*C*), including the same proportion of neurons with very short axons of less than 15 μm in length (~13%). However, there was a lower proportion of scapinin-transfected neurons with axons greater than 100 μm as compared with control (control 24%, scapinin 15%, $p < 0.005$). Also, the number of neurites/dendrites per cell was not significantly different between the groups (Fig. 1*D*), nor was there an increase in the number of axons per neuron. Together, these observations indicate that scapinin expression selectively reduces elongation

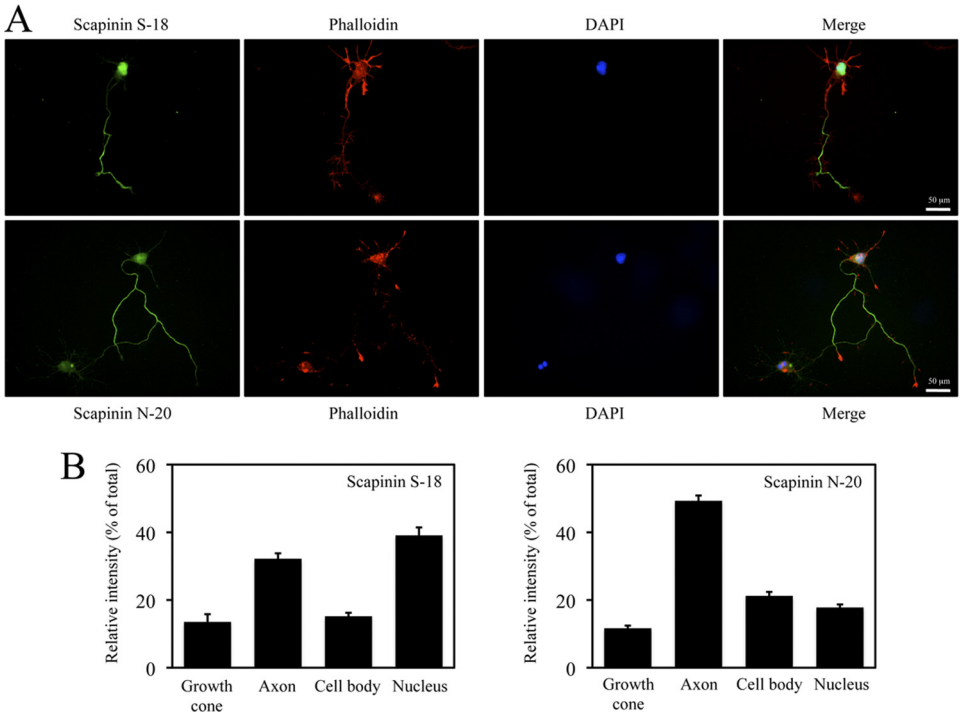


FIGURE 2. **Scapinin is predominantly located in the axon and nucleus.** *A*, immunofluorescence microscopy of endogenous scapinin in 3 DIV rat cortical neurons using two different polyclonal antibodies to scapinin (*green*). S-18 recognizes an epitope between amino acids 209 and 259 in both long and short isoforms of scapinin. The N-20 antibody recognizes 20 amino acids at the N terminus of the long form of scapinin that is not present in the short form. Neurons were also stained for phalloidin (*red*) and DAPI (*blue*). *B*, relative intensities of staining for the S-18 and N-20 antibodies between different subcellular locations were measured using the ImageJ software and are presented as percentage of total scapinin staining in graphs (*error bars* = S.E.).

of the developing axon. The number of branches extending from the axon was not significantly different between the groups (Fig. 1*E*), whereas there was an increase in the number of axons (not dendrites) with abnormally large growth cones (>10 μm in width; Fig. 1*F*). Neurons with abnormally large growth cones always had short axons, suggesting that defects in growth cone dynamics induced by scapinin expression might contribute to the inhibitory effect on axon elongation.

The localization of endogenous scapinin in cortical neurons was determined by immunofluorescence microscopy using two independent antibodies. The S-18 antibody recognizes an epitope between amino acids 209 and 259 that is present in all scapinin isoforms, whereas the N-20 antibody recognizes the N-terminal 20 amino acids of isoform 1 only. It has previously been demonstrated that isoform 2 is the predominant scapinin isoform in the brain (4). Fig. 2 shows that the majority of S-18 antibody staining in 3 DIV cortical neurons is in the axon and nucleus, whereas the N-20 antibody predominantly stained the axon. This suggests that scapinin in the nucleus is predominantly isoform 2 and/or 3, but not isoform 1, whereas it is not yet clear whether the axon contains isoform 1 alone or a mixture of scapinin isoforms. The intensity of staining for both antibodies was particularly high at the distal end of the axon. Scapinin expression is higher in the axon than in dendrites (see Figs. 7 and 8). Interestingly, staining for scapinin (both antibodies) was reciprocal to phalloidin staining, consistent with a previous report showing that scapinin binds to actin monomers and prevents its polymerization (4).

To confirm that the inhibitory effect of scapinin on axon elongation was due to its ability to bind to actin monomers and prevent its polymerization, a mutant form of scapinin that cannot bind actin (R407A,P408A (4)) was transfected into cortical neurons, and its effect on axon elongation was compared with wild type scapinin. Fig. 3 shows that although expression of wild type scapinin significantly inhibited axon elongation, expression of the R407A,P408A mutant had no effect and was similar to control neurons. An R407A,P408A/S277A double mutant also had no significant effect on axon elongation (supplemental Fig. 1). Scapinin is also able to bind to PP1 at its C terminus (1, 5). A point mutation (F515A) was introduced that inhibits the interaction between scapinin and PP1 (5). Expression of this mutant inhibited axon elongation in cortical neurons to the same extent as wild type scapinin. Together, these observations show that the ability of scapinin to inhibit axon elongation in cortical neurons is dependent upon binding to actin, but not PP1.

Scapinin Is Phosphorylated at Ser-277—Analysis of the primary amino acid sequence of scapinin revealed a Cdk5 phosphorylation consensus sequence surrounding Ser-277 (K*X*SP*X*K (7)). This sequence is very similar to other confirmed Cdk5 substrates (*e.g.* CRMP2 (8)) and is highly conserved from frogs and fish to mammals (Fig. 4*A*). We have recently developed a new approach for identifying physiological phosphorylation sites.[3] Briefly, scapinin was expressed in HEK293 cells and immunoprecipitated via its N-terminal FLAG tag. The scapinin was separated into two groups, untreated or treated with recombinant PP1 to remove any Ser/Thr phosphorylation that

[3] H. Farghaian, A. M. Turnley, C. Sutherland, and A. R. Cole, manuscript submitted.

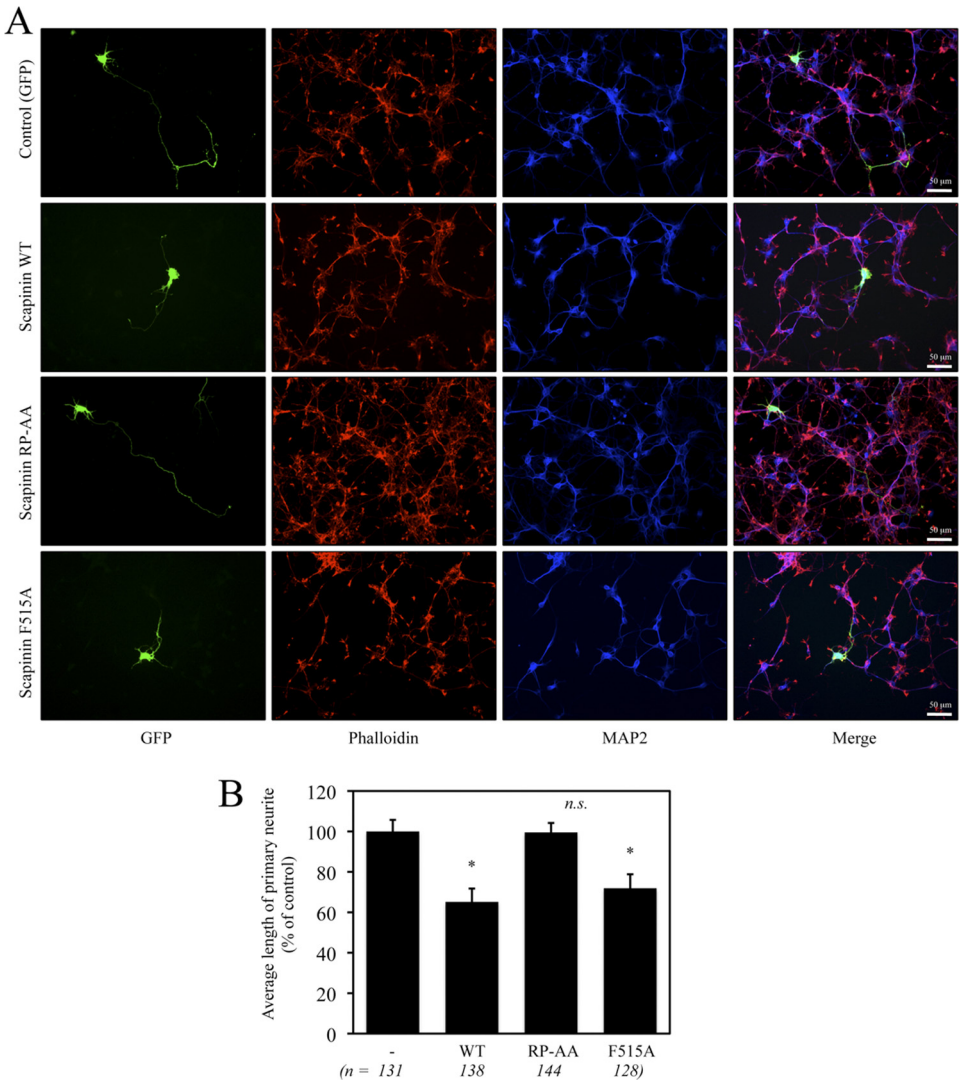


FIGURE 3. **Inhibition of axon elongation by scapinin is dependent on binding to actin.** *A*, cortical neurons isolated from E18 rats were co-transfected using the calcium phosphate method at 1 DIV with GFP and empty vector (control; $n = 131$), wild type scapinin ($n = 138$), S277A mutant ($n = 144$), or S277D ($n = 128$) mutant and incubated for 2 days. Transfected neurons were identified by detection of GFP (*green*), whereas neuronal morphology was visualized using an antibody that recognizes the neuronal somato-dendritic marker protein MAP2 (*blue*) and phalloidin (*red*). Neuronal morphology and neurite lengths were analyzed using the ImageJ software. *B*, average axon lengths are presented as a graph (*error bars* = S.E., *, $p < 0.05$, *n.s.* = not significant, Student's *t* test).

might be present on the protein. PP1 was removed by washing, and the scapinin proteins (untreated and PP1-treated) were separately subjected to *in vitro* kinase assays with recombinant Cdk5 in the presence of radiolabeled ATP. In theory, recombinant Cdk5 should be able to incorporate more radiolabeled phosphate into the PP1-treated scapinin as compared with control untreated scapinin. If so, this would indicate that scapinin is a good substrate for Cdk5 *in vitro* and that it was phosphorylated in the HEK293 cells (possibly by Cdk5). When scapinin was subjected to this assay, there was significantly more radiolabeled phosphate incorporated into the PP1-treated protein than untreated protein (Fig. 4*B*). In addition, when Ser-277 was mutated to a non-phosphorylatable alanine residue, phosphorylation of scapinin was completely inhibited. This indicates that Ser-277 of scapinin was phosphorylated when expressed in HEK293 cells, suggesting that it is a physiological phosphorylation site. The stoichiometry of scapinin phosphorylation by Cdk5 in the subsequent *in vitro* kinase assay approached 0.25 mol of phosphate/mol of scapinin. Considering that the substrate was isolated from cells and that co-precipitating proteins might interfere with the *in vitro* kinase assay or that dephosphorylation by PP1 might have been incomplete, 0.25 mol/mol incorporation indicates a reasonable efficiency of phosphorylation by Cdk5.

A rabbit polyclonal antibody was generated that specifically recognizes scapinin when phosphorylated at Ser-277 (pSer277 antibody). The specificity of this antibody was confirmed by Western blot, whereby it recognized wild type scapinin when expressed in HEK293 cells, but not the non-phosphorylated S277A or S277D mutants (Fig. 4*D*). To determine whether Cdk5 is the physiological kinase responsible for phosphorylating this site, phosphorylation of scapinin was compared in brain tissue from wild type and $Cdk5^{-/-}$ mice by Western blotting using the pSer277 antibody (Fig. 4*E*). Surprisingly, phosphorylation of Ser-277 was not altered in $Cdk5^{-/-}$ brain tissue, nor was it affected by treatment of cultured cortical neurons

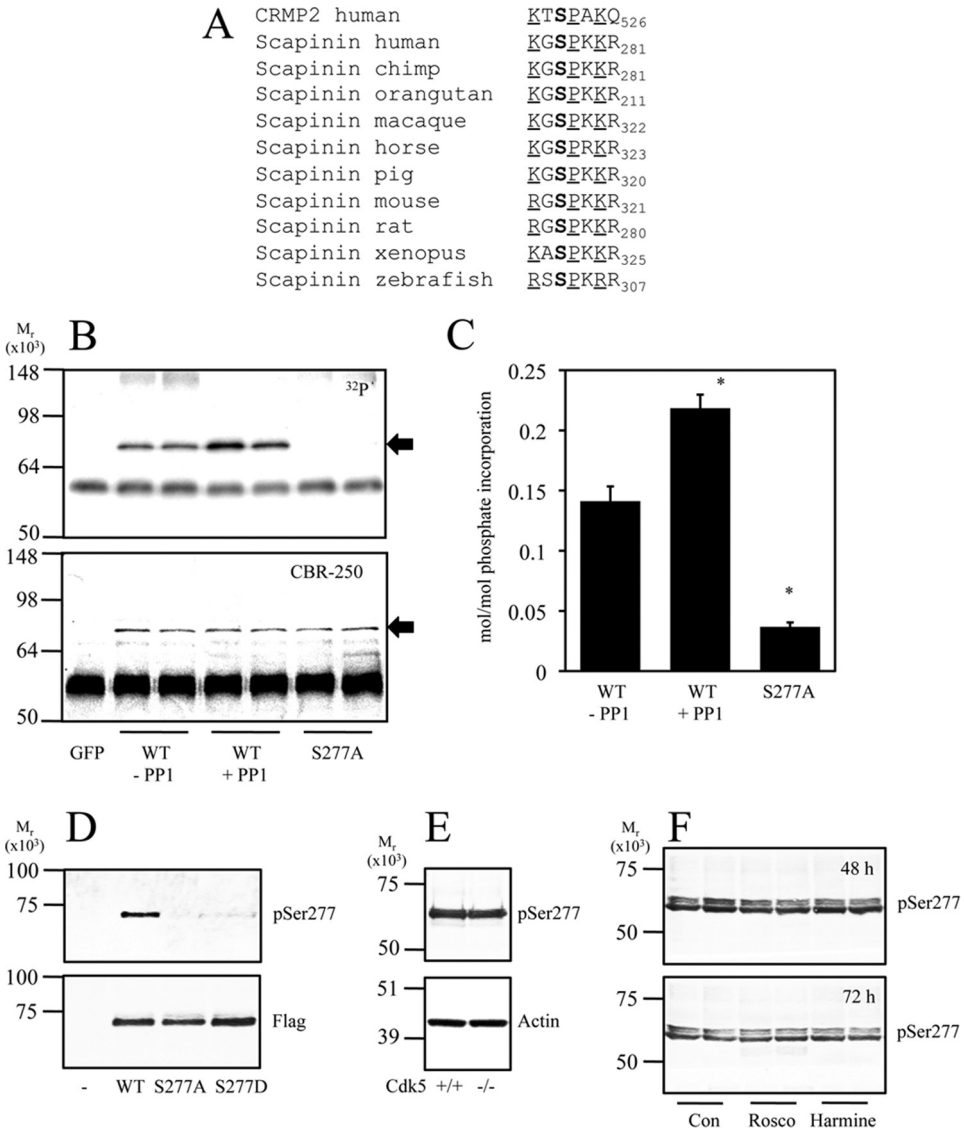


FIGURE 4. **Scapinin is phosphorylated at Ser-277.** *A*, sequence alignment of residues surrounding Ser-277 (*bold*) of scapinin from various species. Highly conserved proline and basic residues (lysine, arginine) that conform to a Cdk5 phosphorylation consensus sequence are *underlined*. For comparison, residues of human CRMP2 surrounding Ser-522, which is a confirmed physiological target of Cdk5, are shown. *B*, scapinin was cloned into a mammalian expression vector, expressed in HEK293 cells, and pulled down via an N-terminal FLAG tag. The isolated protein was separated into two groups; one was left untreated, whereas the other was dephosphorylated using PP1. Following removal of PP1 by washing, both treated and untreated scapinin proteins were subjected to an *in vitro* kinase assay with recombinant Cdk5/p35 in the presence of radiolabeled ATP. Assays were subjected to SDS-PAGE and visualized by exposure to autoradiography film (^{32}P incorporation; *upper panel*) or staining with CBR-250 (loading control; *lower panel*). In addition, the S277A mutant was subjected to the same assay. *Arrows* indicate the scapinin band. *C*, the stoichiometry of phosphorylation for untreated, PP1-treated, and S277A mutant forms of scapinin were determined and presented as a graph (*error bars* = S.E., *, $p < 0.05$, Student's *t* test). *D*, wild type and mutant forms of scapinin were expressed in HEK293 cells, pulled down via their N-terminal FLAG tags, subjected to SDS-PAGE, and transferred to nitrocellulose. Membranes were probed with antibodies that recognize pSer277 (*upper panel*) or FLAG as a loading control (*lower panel*). *E*, lysates from cortex of wild type and Cdk5$^{-/-}$ embryonic mice were subjected to Western blot analysis using antibodies that recognize pSer277 (*upper panel*) and actin as a loading control (*lower panel*). *F*, cultured primary rat cortical neurons were treated with DMSO (control (*Con*)), 20 μM roscovitine (*Rosco*), or 2 μM harmine for 48 (*upper panel*) and 72 h (*lower panel*). Lysates were prepared and subjected to Western blot analysis using an antibody that recognizes pSer277.

with the Cdk5 inhibitor roscovitine for 48 or 72 h (Fig. 4*F*). These observations indicate that Cdk5 is not the physiological kinase for this site (although the possibility of another kinase compensating for the lack of Cdk5 activity cannot be ruled out). The DYRKs (dual specificity tyrosine phosphorylation-regulated kinases) are a family of kinases that are closely related to Cdk5 and share a similar phosphorylation consensus sequence (9). To determine whether a DYRK might be the physiological kinase for Ser-277, neurons were treated with harmine, a highly specific inhibitor of members of this family (10). (Note that DYRKs are also inhibited by roscovitine (11)). In either case, phosphorylation at Ser-277 was unaltered. Although the kinase that catalyzes this reaction has not yet been identified, these experiments demonstrate that Ser-277 is a physiological phosphorylation site.

Phosphorylation of Ser-277 Regulates Axon Elongation—To examine the effect phosphorylation of Ser-277 has on axon elongation, cortical neurons were transfected with wild type, non-phosphorylated (S277A), or phospho-mimetic forms of scapinin, and the axon lengths were compared. Wild type and

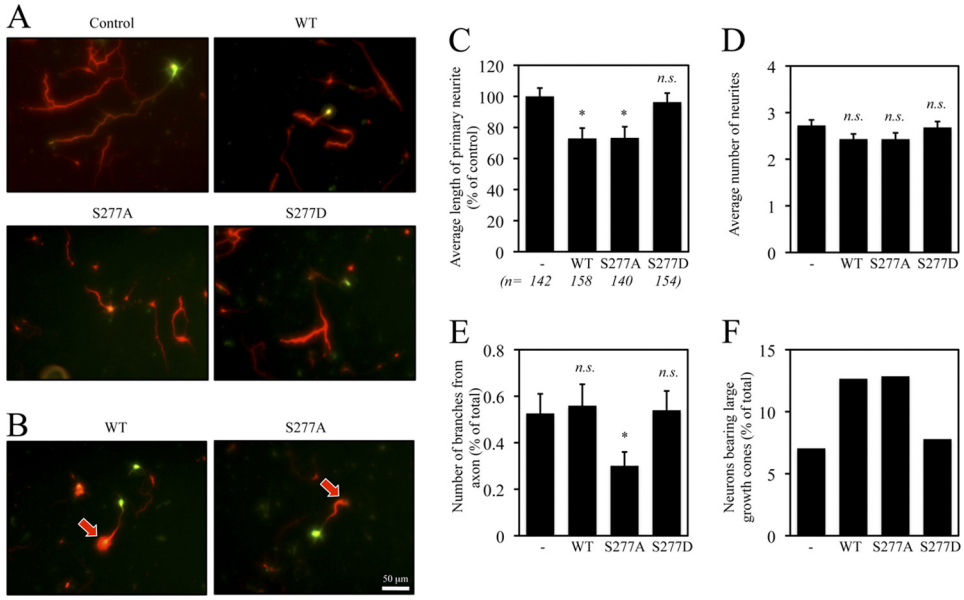


FIGURE 5. **Phosphorylation of Ser-277 on scapinin restores normal axon elongation.** *A*, cortical neurons isolated from E18 rats were co-transfected using the Amaxa electroporation system at the time of plating with GFP and empty vector (control; $n = 142$), wild type scapinin ($n = 158$), S277A mutant ($n = 140$), or S277D mutant ($n = 154$) and incubated for 3 days. Transfected neurons were identified by detection of GFP (*green*), whereas neuronal morphology was visualized using an antibody that recognizes the neuronal cytoskeleton-associated protein Tau-1 (*red*). *B*, neurons transfected with wild type or S277A scapinin that have abnormally large growth cones are shown by *arrows*. Neuronal morphology and neurite lengths were analyzed using the ImageJ software. *C–F*, results of analyses are presented as graphs, showing a comparison of averaged axon lengths (*C*), average number of neurites per cell (*D*), branching from the axon (*E*), and percentage of neurons with abnormally large growth cones (*F*). *Error bars* = S.E., *, $p < 0.05$, *n.s.* = not significant, Student's *t* test.

S277A forms of scapinin significantly reduced axon elongation; however, the S277D mutant had no effect (Fig. 5, *A* and *B*). Neither the wild type nor the mutant forms of scapinin significantly affected the number of neurites per cell (Fig. 5*C*). Wild type and S277D mutant forms of scapinin did not affect branching from the developing axon; however, it was significantly reduced by expression of the non-phosphorylatable S277A mutant (Fig. 5*D*). Also, the proportion of neurons with abnormally large growth cones was increased in neurons expressing wild type and S277A forms of scapinin, but not neurons expressing the S277D mutant (Fig. 5*E*), consistent with the effects of these isoforms on axon elongation. Together, these observations show that the ability of scapinin to inhibit axon elongation is most potent when it is not phosphorylated at Ser-277. In contrast, phosphorylation of Ser-277 (S277D mutant) relieves this inhibitory effect and is virtually indistinguishable from control neurons in terms of axon length, branching from the axon, total number of neurites, and growth cone size.

Because the ability of scapinin to inhibit axon elongation is dependent on binding to actin (Fig. 3) and phosphorylation at Ser-277 also regulates axon elongation (Fig. 5), it is reasonable to predict that phosphorylation at Ser-277 might affect the ability of scapinin to bind actin. Wild type and mutant forms of scapinin were expressed in HEK293 cells and pulled down via their N-terminal FLAG tags, and co-precipitating proteins were subjected to SDS-PAGE and visualized using CBR-250 staining and Western blotting for actin (Fig. 6 (Note that the major band at M_r 42,000 was confirmed as actin by Western blot analysis; data not shown)). Densitometric analysis of CBR-250-stained gels showed that wild type scapinin bound stoichiometrically to actin (*i.e.* one mole of actin per mole of scapinin), and mutation of Ser-277 to either alanine or aspartic acid did not affect actin

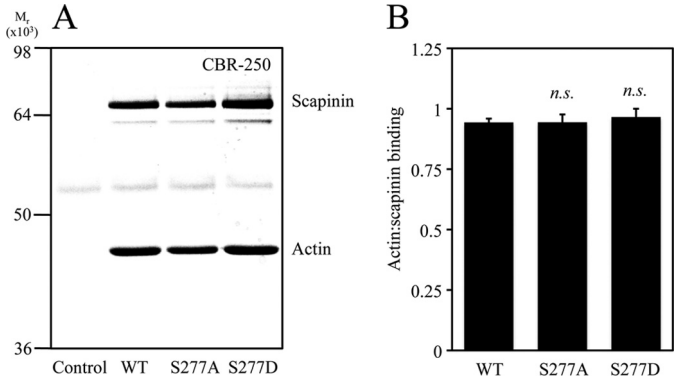


FIGURE 6. **Phosphorylation of scapinin at Ser-277 does not affect actin binding.** *A*, HEK293 cells were transfected with vector only (*Control*), wild type, S277A, or S277D forms of scapinin, and lysates were prepared. Scapinin was pulled down from the lysates using anti-FLAG-agarose, co-precipitating proteins were subjected to SDS-PAGE, and the gel was stained with CBR-250. The bands at M_r 42,000 were confirmed as actin by Western blot analysis (data not shown). *B*, the relative intensities of scapinin and actin bands were quantified using densitometry and are presented as a graph ($n = 4$, *error bars* = S.E., *n.s.* = not significant, Student's *t* test).

binding. This indicates that phosphorylation of Ser-277 does not affect actin binding by scapinin.

Immunofluorescence analysis of three DIV cortical neurons revealed that although total scapinin (S-18 antibody) was predominantly localized to the axon and nucleus, the phosphorylated form of scapinin was predominantly localized to the cytoplasm and nucleus (Fig. 7). The intensity of staining for pSer277 was particularly low in the axon and high in the cell body, whereas relative staining of the growth cone and nucleus was unaltered. This suggests that phosphorylation of Ser-277 induces translocation of scapinin from the axon to the cytoplasm. To confirm this, cortical neurons were transfected with wild type and phospho-mutant forms of scapinin, and their

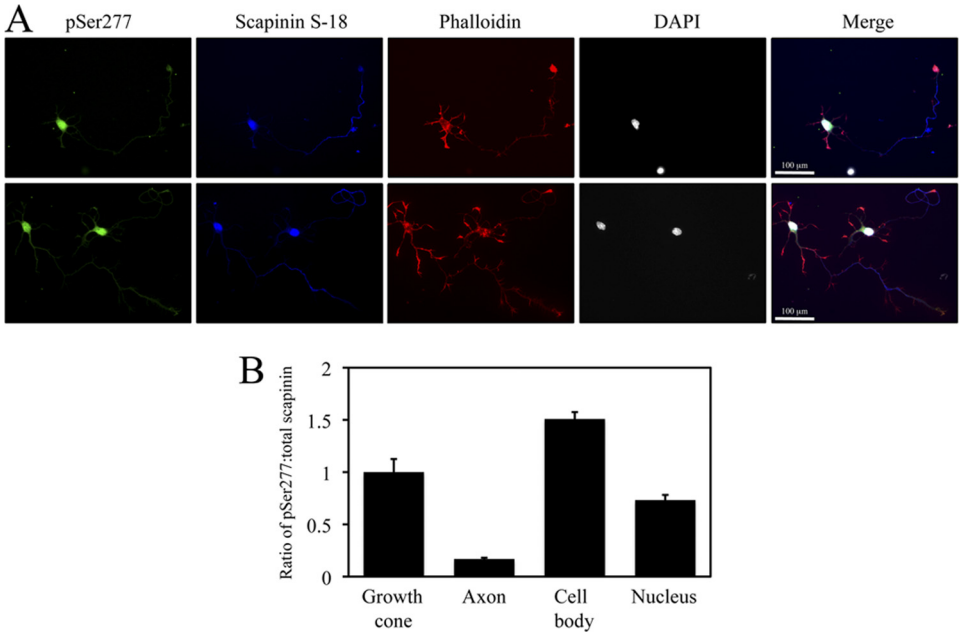


FIGURE 7. **Phospho-Ser-277 localizes to the cell body and nucleus.** *A*, immunofluorescence microscopy of endogenous scapinin in 3 DIV rat cortical neurons using polyclonal antibodies that recognize pSer277 (*green*) or total scapinin (S-18, *blue*). Neurons were also stained for phalloidin (*red*) and DAPI (*white*). *B*, the ratio of pSer277:total scapinin (S-18) in different subcellular locations is presented as a graph.

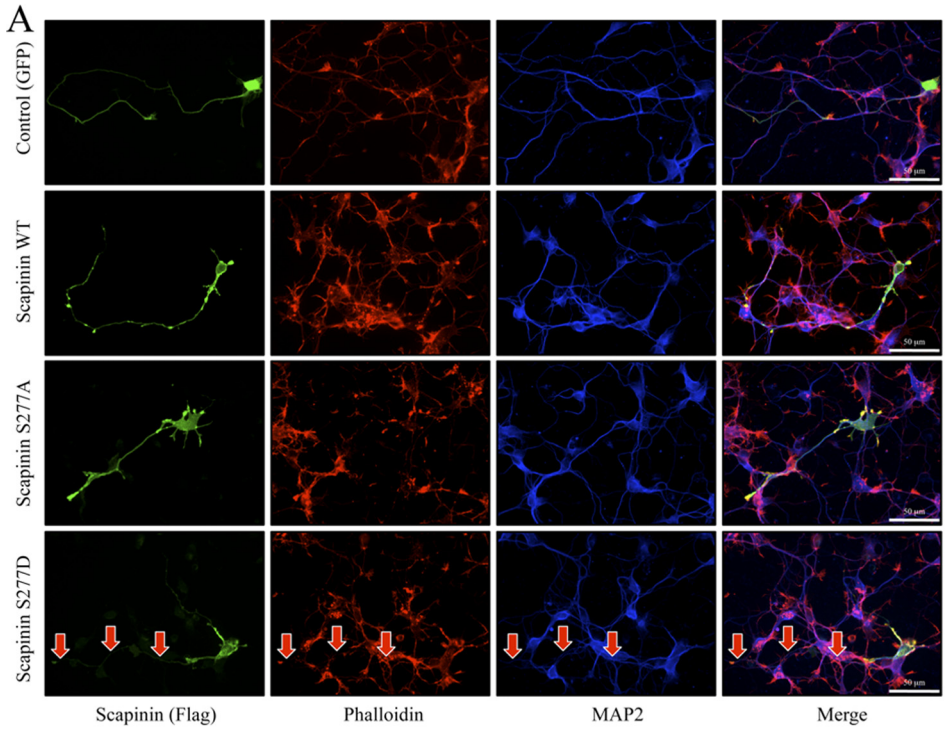
subcellular localization was determined by immunofluorescence microscopy using an anti-FLAG antibody. Fig. 8 shows that wild type and S277A scapinin were more evenly distributed between the axon, cell body, and nucleus of neurons, whereas the S277D mutant was relatively lower in the axon but increased in the cell body. Together, these observations demonstrate that phosphorylation of Ser-277 directs the localization of scapinin from the axon to the cytoplasm. Interestingly, the phosphorylated form of scapinin is more dramatically restricted to the nucleus of neurons that have been cultured for longer periods (14 DIV; Fig. 8, *C* and *D*). This is accompanied by a redistribution of a significant proportion of total cellular scapinin from the axon to the cytoplasm and nucleus. Biochemical fractionation of adult rat brain detected phosphorylated scapinin in cytoplasmic, membrane, and nuclear fractions, but not the cytoskeletal fraction (supplemental Fig. 2).

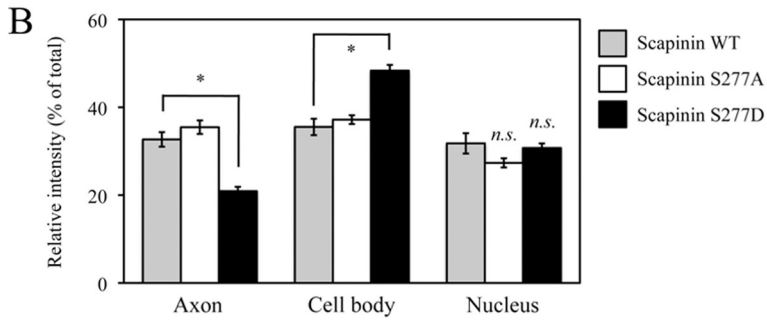
Scapinin shares structural and functional similarities to another actin-binding protein called megakaryocytic acute leukemia protein (MAL). The RPEL motif of MAL binds to free actin monomers in the cytoplasm, but under stress conditions when actin is polymerized to form stress fibers and the concentration of actin monomers is reduced, free MAL translocates to the cell nucleus and regulates gene transcription (12, 13). Because scapinin also binds to actin monomers via an RPEL motif and is distributed in the cytoplasm and nucleus, we investigated whether changes in the intracellular concentration of actin monomers induce scapinin enrichment in the nucleus. Neurons were treated with two drugs that inhibit actin polymerization; latrunculin A binds directly to actin monomers and prevents their polymerization, whereas cytochalasin D binds to the barbed ends of actin filaments to inhibit polymerization and causes dissociation of the actin subunits. Treatment of cortical neurons (3 DIV) with these drugs for up to 24 h effectively inhibited actin polymerization, as evidenced by decreased staining with phalloidin, but did not affect the subcellular localization of either the phosphorylated form (pSer277 antibody) or total scapinin (S-18 antibody; Fig. 9). Therefore, scapinin localization is upstream of actin polymerization, unlike MAL.

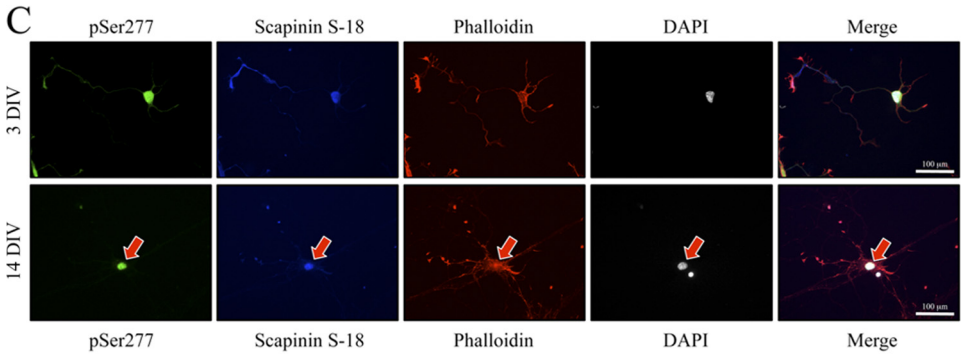
DISCUSSION

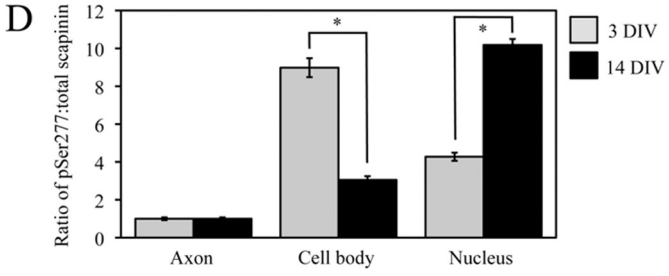
Scapinin is an actin- and PP1-binding brain protein that has been shown to regulate cell adhesion and migration in Cos7 kidney carcinoma cells (4). However, its function in neurons had not been investigated. Here we show that scapinin inhibits elongation of axons in primary rat cortical neurons. This is dependent on its ability to bind actin monomers via RPEL repeats in its C-terminal region but is independent of its ability to bind PP1. Scapinin had previously been shown to preferentially bind to actin monomers and inhibit polymerization (4). Accordingly, actin polymerization is low in regions where scapinin expression is high, particularly the distal axon shaft. The concentration of monomeric actin (G-actin) in the axon is 2–3 times higher than filamentous actin (F-actin (14)). Because scapinin binds actin monomers with high affinity (stoichiometrically, Fig. 6), its ability to inhibit actin polymerization is most likely achieved by sequestering actin monomers, thereby reducing its local concentration and decreasing the kinetics of filament formation.

Scapinin expression is high in the distal axon shaft, and it is likely that its inhibitory effect on axon elongation is mediated by scapinin in this region. This is supported by the observation that the S277D phospho-mimetic mutant of scapinin caused a shift in scapinin from the distal axon to the cytoplasm and that this mutant did not inhibit axon elongation. It is clear that actin dynamics in filopodia and lamellipodia at the leading edge of the growth cone are important for outgrowth and navigation of the extending axon (15, 16). Scapinin is present in the growth cone, albeit at comparatively lower levels than the adjacent dis-


A
Control (GFP)
Scapinin WT
Scapinin S277A
Scapinin S277D
Scapinin (Flag)
Phalloidin
MAP2
Merge


B
Relative intensity (% of total)
60
40
20
0
*
*
n.s.
n.s.
Axon
Cell body
Nucleus
Scapinin WT
Scapinin S277A
Scapinin S277D


C
pSer277
Scapinin S-18
Phalloidin
DAPI
Merge
3 DIV
14 DIV
100 μm
pSer277
Scapinin S-18
Phalloidin
DAPI
Merge


D
Ratio of pSer277:total scapinin
12
10
8
6
4
2
0
*
*
Axon
Cell body
Nucleus
3 DIV
14 DIV

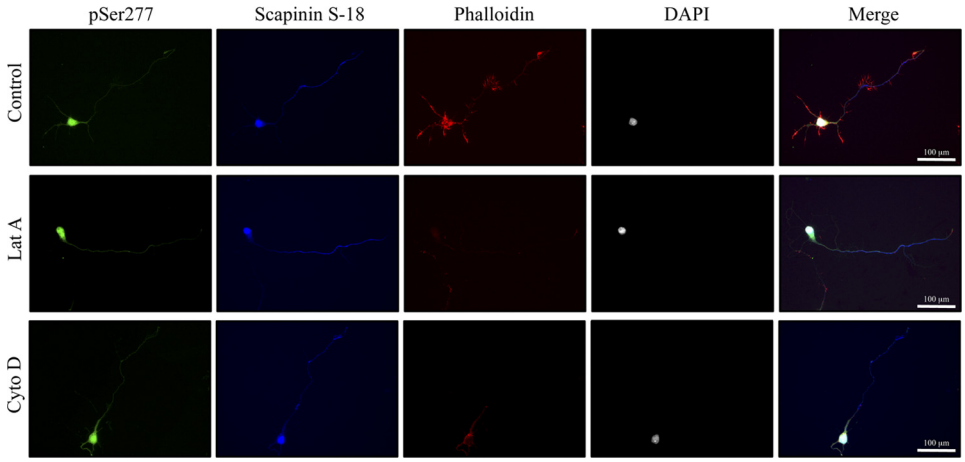


FIGURE 9. **Actin depolymerization does not change scapinin localization.** Rat cortical neurons (3 DIV) were treated with DMSO (*Control*), 2 μM latrunculin A (*LatA*), or 1 μM cytochalasin D (*CytoD*) for 24 h. The subcellular localization of endogenous scapinin was analyzed using immunofluorescence microscopy using polyclonal antibodies that recognize pSer277 (*green*) or total scapinin (S-18, *blue*). Neurons were also stained for phalloidin (*red*) and DAPI (*white*).

tal axon shaft. It is possible that scapinin levels in the growth cone and/or the distal axon shaft are sufficient to regulate actin dynamics and filopodia/lamellipodia formation at the leading edge of the growth cone, thereby affecting axon elongation. Consistent with this, abnormally large growth cones were more frequently observed in scapinin-transfected neurons, indicating defects in growth cone function. Also, these neurons were always accompanied with particularly short axons. This supports the hypothesis that scapinin-induced inhibition of axon elongation is caused by changes in actin dynamics in the growth cone. However, the effects of scapinin on neuronal morphology are likely to be more complicated than its ability to regulate actin polymerization alone because other actin-binding RPEL motif proteins (*e.g.* MAL) do not have the same effect (see Ref. 4) and treatment of neurons with cytochalasin to depolymerize actin does not inhibit axon elongation (17, 18). Therefore, it will be important to also identify non-actin-related roles of scapinin in future investigations.

Differing reports have described nuclear or cytoplasmic localization of scapinin in various transformed cell lines (1, 4). Here we have shown that endogenous scapinin is present in the cytoplasm, nucleus, axon shaft, and growth cone of primary cortical neurons. This demonstrates that scapinin is resident at both the cytoplasm and the nucleus, and the differences between previous reports are probably due to abnormal expression levels of transfected protein and the use of transformed non-neuronal cell types. Also, it is possible that different isoforms of scapinin might display preferences for different subcellular locations, although similar patterns of expression in cortical neurons were observed here using two independent antibodies that recognize isoform 1 only (N-20) or all isoforms (S-18; Fig. 2). The presence of scapinin in the axon clearly has functional effects on actin polymerization and elongation; however, it is currently unclear whether it has specific nuclear functions as well. It was initially identified as a component of the nuclear matrix intermediate filament scaffold complex (1), suggesting an additional structural function in the nucleus. Alternatively, scapinin shares the RPEL actin-binding motif with MAL, a transcriptional coactivator protein for serum response factor, suggesting that nuclear scapinin might regulate gene transcription. Translocation of MAL from the cytoplasm to the nucleus is regulated by actin polymerization (12, 13). Here treatment of neurons with actin depolymerizing agents did not alter the subcellular localization of scapinin and did not increase the proportion of scapinin in the nucleus. Similarly, serum stimulation does not affect scapinin translocation to the nucleus (4), and overexpression of scapinin does not affect serum response factor activity (2). These observations indicate that unlike MAL, scapinin localization is not regulated by actin dynamics/availability of monomeric actin and that it is not a cofactor for serum response factor. However, these observations do not exclude the possibility that scapinin regulates the transcription of other genes in the nucleus.

Interestingly, three putative nuclear localization sequences have been proposed by Sagara *et al.* (1), with the second of these located adjacent to the Ser-277 phosphorylation site (^{277}pSPKKR^{281}; putative nuclear localization sequence is underlined). The relationship between phosphorylation and this particular nuclear localization sequence is difficult to investigate directly because mutation of the proline or basic residues to disrupt the putative nuclear localization sequence would almost certainly interfere with phosphorylation by the Ser-277 kinase. However, it seems unlikely that this putative nuclear localization sequence and/or Ser-277 phosphorylation

FIGURE 8. **Phospho-mimetic mutant S277D localizes to the cytoplasm and nucleus.** *A*, cortical neurons were transfected using the calcium phosphate method at 1 DIV with GFP only (*Control (GFP)*), wild type scapinin ($n = 21$), S277A mutant ($n = 21$), or S277D mutant ($n = 20$) and incubated for 2 days. Transfected neurons were identified by detection of GFP (*green*), whereas neuronal morphology was visualized using an antibody that recognizes the somato-dendritic marker protein MAP2 (*blue*) and phalloidin (*red*). *Arrows* indicate the location of axon. *B*, relative intensities of wild type and mutant scapinin in different subcellular locations were analyzed using the ImageJ software and are presented as a graph (*error bars* = S.E., *, $p < 0.05$, *n.s.* = not significant, Student's *t* test). *C*, immunofluorescence microscopy of endogenous scapinin in 3 DIV and 14 DIV rat cortical neurons using polyclonal antibodies that recognize pSer277 (*green*) or total scapinin (S-18, *blue*). Neurons were also stained for phalloidin (*red*) and DAPI (*white*). *Arrows* indicate the location of the nucleus. *D*, the ratio of pSer277:total scapinin (S-18) in different subcellular locations is presented as a graph.

controls nuclear localization because the major effect of phosphorylation at Ser-277 is relocalization of scapinin from the axon to the cytoplasm in preference to the nucleus, as evidenced by enrichment of endogenous phospho-scapinin (Fig. 7) and the phospho-mimetic S277D form of scapinin (Fig. 8) in the cytoplasm. It should be noted that the amount of phosphorylated and total scapinin in the nucleus is significantly increased in older cortical neurons (14 DIV) as compared with control younger neurons (3 DIV; Fig. 8*C*). This suggests that other unknown factors are likely to contribute to nuclear localization of scapinin. How phosphorylation of Ser-277 influences the subcellular distribution of scapinin is not yet known. It is possible that phosphorylation regulates its interaction with axonal transport motor proteins, such as kinesin and dynein, or regulates its interaction with other binding proteins that localize it to a particular compartment. Efforts are underway to identify scapinin-binding proteins that are regulated by phosphorylation.

At present, the physiological kinase targeting Ser-277 remains unknown. This residue is located within a perfect Cdk5 consensus sequence ((R/K)*X*(S/T)P*X*(R/K) (7)) and is efficiently phosphorylated by recombinant Cdk5 *in vitro* (Fig. 4). However, phosphorylation was not altered in Cdk5 knock-out brain tissue or treatment of primary neurons with a Cdk5 inhibitor (Fig. 4), indicating that Cdk5 is unlikely to be the physiological kinase for this site, although compensation for the loss of Cdk5 activity by another kinase cannot be ruled out. The sequence surrounding Ser-277 also conforms to a DYRK consensus site (9); however, phosphorylation was not altered by treatment of neurons with DYRK inhibitors. Therefore, the identity of the Ser-277 kinase remains unknown. The region surrounding Ser-277 is highly conserved throughout mammals, reptiles, and fish. In particular, conservation of the proline residue at the +1 position is invariant. This indicates that the kinase is almost certainly a member of the CMGC family of kinases, the majority of which are proline-directed kinases, and no other kinase subtypes are able to phosphorylate SP or TP sites as efficiently. Because treatment with roscovitine and harmine had no effect on phosphorylation, it is unlikely to be any member of the Cdk or DYRK families, nor is it likely to be targeted by GSK3 because this kinase requires a priming phosphorylation site located 4 or 5 residues downstream of the target site, which is not present in scapinin. This leaves the stress-activated kinases (MAPK, JNK, p38) and several groups of poorly characterized kinase families, including HIPK, CdkL, PCTAIRE, and CKL, as possible physiological kinases for the Ser-277 site. Efforts to identify this kinase are continuing.

Scapinin expression is enriched in adult brains in regions with relatively high neuroplasticity, namely the cortex and hippocampus (5). Coupled with our observations that it regulates axon elongation, it is tempting to speculate that scapinin regulates neuroplasticity in adult brains. It will be interesting to determine whether it also affects dendritic spine growth and synapse formation in the cortex and hippocampus. Ultimately, the effect scapinin and various mutants have on cortical and hippocampal function, such as learning and memory, will need to be investigated using behavioral tests of scapinin knock-out and transgenic mice.

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REFERENCES

1. Sagara, J., Higuchi, T., Hattori, Y., Moriya, M., Sarvotham, H., Shima, H., Shirato, H., Kikuchi, K., and Taniguchi, S. (2003) *J. Biol. Chem.* **278,** 45611–45619
2. Favot, L., Gillingwater, M., Scott, C., and Kemp, P. R. (2005) *FEBS Lett.* **579,** 100–104
3. Worch, S., Fiedler, E., Hansmann, I., and Schlote, D. (2006) *Cytogenet. Genome Res.* **115,** 23–29
4. Sagara, J., Arata, T., and Taniguchi, S. (2009) *PLoS One* **4,** e4247
5. Allen, P. B., Greenfield, A. T., Svenningsson, P., Haspeslagh, D. C., and Greengard, P. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 7187–7192
6. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
7. Songyang, Z., Lu, K. P., Kwon, Y. T., Tsai, L. H., Filhol, O., Cochet, C., Brickey, D. A., Soderling, T. R., Bartleson, C., Graves, D. J., DeMaggio, A. J., Hoekstra, M. F., Blenis, J., Hunter, T., and Cantley, L. C. (1996) *Mol. Cell. Biol.* **16,** 6486–6493
8. Cole, A. R., Causeret, F., Yadirgi, G., Hastie, C. J., McLauchlan, H., McManus, E. J., Hernández, F., Eickholt, B. J., Nikolic, M., and Sutherland, C. (2006) *J. Biol. Chem.* **281,** 16591–16598
9. Campbell, L. E., and Proud, C. G. (2002) *FEBS Lett.* **510,** 31–36
10. Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C. J., McLauchlan, H., Klevernic, I., Arthur, J. S., Alessi, D. R., and Cohen, P. (2007) *Biochem. J.* **408,** 297–315
11. Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003) *Biochem. J.* **371,** 199–204
12. Miralles, F., Posern, G., Zaromytidou, A. I., and Treisman, R. (2003) *Cell* **113,** 329–342
13. Vartiainen, M. K., Guettler, S., Larijani, B., and Treisman, R. (2007) *Science* **316,** 1749–1752
14. Morris, J. R., and Lasek, R. J. (1984) *J. Cell Biol.* **98,** 2064–2076
15. Lowery, L. A., and Van Vactor, D. (2009) *Nat. Rev. Mol. Cell Biol.* **10,** 332–343
16. Pak, C. W., Flynn, K. C., and Bamburg, J. R. (2008) *Nat. Rev. Neurosci.* **9,** 136–147
17. Marsh, L., and Letourneau, P. C. (1984) *J. Cell Biol.* **99,** 2041–2047
18. Letourneau, P. C., Shattuck, T. A., and Ressler, A. H. (1987) *Cell Motil. Cytoskeleton* **8,** 193–209