Characterization of the Neuronal Targeting Protein Spinophilin and Its Interactions with Protein Phosphatase-1†

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ABSTRACT: Protein phosphatase-1 (PP1) plays an important role in a variety of cellular processes, including muscle contraction, cell-cycle progression, and neurotransmission. The localization and substrate specificity of PP1 are determined by a class of proteins known as targeting subunits. In the present study, the interaction between PP1 and spinophilin, a neuronal protein that targets PP1 to dendritic spines, has been characterized. Deletion analysis revealed that a high-affinity binding domain is located within residues 417−494 of spinophilin. This domain contains a pentapeptide motif (R/K-R/K-V/I-X-F) between amino acids 447 and 451 (R-K-I-H-F) that is conserved in other PP1 regulatory subunits. Mutation of phenylalanine-451 (F451A) or deletion of the conserved motif abolished the ability of spinophilin to bind PP1, as observed by coprecipitation, overlay, and competition binding assays. In addition, deletion of regions 417−442 or 474−494, either singly or in combination, impaired the ability of spinophilin to coprecipitate PP1. A comparison of the binding and inhibitory properties of spinophilin peptides suggested that distinct subdomains of spinophilin are responsible for binding and modulating PP1 activity. Mutational analysis of the modulatory subdomain revealed that spinophilin interacts with PP1 via a mechanism unlike those used by the cytosolic inhibitors DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, M_r 32 000) and inhibitor-1. Finally, characterization of the interactions between spinophilin and PP1 has facilitated the design of peptide antagonists capable of disrupting spinophilin−PP1 interactions. These studies support the notion that spinophilin functions in vivo as a neuronal PP1 targeting subunit by directing the enzyme to postsynaptic densities and regulating its activity toward physiological substrates.

Protein phosphatase-1 (PP1)[1] is a serine/threonine phosphatase that controls many aspects of cellular physiology, including muscle contraction, cell division, gene expression, neurotransmission, and glycogen metabolism (*1*−*3*). Increasing evidence suggests that the diverse actions of PP1 are regulated in vivo by inhibitory proteins such as DARPP-32 (*2*) and by a class of anchoring proteins known as targeting subunits (*4*, *5*). Targeting subunits restrict the otherwise broad specificity of the catalytic subunit ($PP1_C$) by directing the enzyme to discrete subcellular compartments and, in some cases, by modulating its activity toward particular substrates. Several mammalian targeting subunits have been isolated, including the G_M subunit that targets $PP1_C$ to glycogen particles and the sarcoplasmic reticulum of striated muscle (*6*, *7*), the G_L subunit that directs $PP1_C$ to liver glycogen (*8*, *9*), the M_{110} subunits that localize $PP1_C$ to myofibrils of skeletal and smooth muscle (*10*−*14*), and the nuclear proteins NIPP-1 and PNUTS that target $PP1_C$ to RNA (*15*−*18*). In each case, the interaction of $PP1_C$ with the targeting subunit couples the activity of PP1 to external signals and facilitates the coordination of downstream signaling pathways.

In the mammalian brain, PP1 plays an important role in the integration of postsynaptic neuronal inputs (*19*). Accordingly, PP1 is highly enriched in the dendritic spines of neurons, structures that receive a majority of the excitatory synaptic contacts (*20*). Several studies suggest that PP1 regulates certain ionic conductances by altering the phosphorylation state of channels. For instance, PP1 has been implicated in the dopamine-mediated regulation of Ca^{2+} currents (21), the neuropeptide-induced modulation of K^+ currents (*22*), and the PKA-mediated regulation of *N*-methyl-D-aspartate (NMDA) receptors (*23*). PP1 has also been proposed to be involved in both hippocampal long-term depression (*24*) and long-term potentiation (*25*), cellular models for learning and memory.

The specialized localization and coordinated activity of PP1 in the brain suggest the existence of one or more neuronal targeting subunits. Anchoring of PP1 to the postsynaptic membrane would not only place the phosphatase

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[1] Abbreviations: PP1, protein phosphatase-1; $PP1_c$, catalytic subunit of PP1; DARPP-32, dopamine- and cAMP-regulated phosphoprotein, M_r 32 000; G_M, glycogen-binding subunit of PP1 from striated muscle; G_L, glycogen-binding subunit of PP1 from liver; M_{110}, myofibrillar-binding subunits of PP1; NIPP-1, nuclear inhibitor of PP1; PNUTS, phosphatase 1 nuclear targeting subunit; MLC_{20}, 20-kDa regulatory light chain of myosin; NMDA, *N*-methyl-D-aspartate; AMPA, amino-3-hydroxy-5-methyl-4-isoxazole propionate; GST, glutathione *S*-transferase; PDZ, consensus sequence in PSD95/DLG/zo-1; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; IPTG, 0.1 mM isopropyl-1-thio-β-D-galactopyranoside; ELISA, enzyme-linked immunosorbent assay.

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where it could optimally respond to fluctuations in neurotransmitters, second messengers, and other signals, but also position the enzyme close to preferred substrates. This spatial organization could enable PP1 to coordinate responses to signals from a variety of neurotransmitters. However, the molecular mechanisms whereby PP1 regulates neuronal excitability and synaptic plasticity are not well understood.

Using the yeast two-hybrid system, Allen and co-workers recently identified spinophilin, a novel $PP1_C$-binding protein that is predominantly expressed in mammalian brain (*26*). Immunocytochemical analysis of hippocampal and striatal neurons revealed that spinophilin is highly localized to dendritic spines. A stable complex of spinophilin and $PP1_C$ can be coimmunoprecipitated from brain extracts, and spinophilin has been shown to suppress the phosphorylase phosphatase activity of PP1 in vitro. Spinophilin contains leucine zipper domains that are predicted to form trimeric or quaternary coiled-coils (*26*−*29*). In addition, it has an actin-binding domain in the amino terminus and a single PDZ domain. PDZ domains have been shown to bind the carboxy termini of ion channels and transmembrane receptors (*30*, *31*). Therefore, spinophilin may target $PP1_C$ to dendritic spines, which are rich in F-actin, and regulate the specificity of the phosphatase toward ion channels and other substrates. However, the mechanism by which spinophilin interacts with PP1 and modulates its activity remains to be elucidated.

In this study, we have identified the primary PP1-binding domain of spinophilin and characterized the relative importance of structural elements within this domain for PP1 recognition. Our analysis of the molecular interactions between spinophilin and PP1 highlights the differences between spinophilin and the inhibitors DARPP-32 and inhibitor-1 and has led to the design of peptide antagonists capable of disrupting spinophilin−PP1 interactions. In combination, these studies provide insight into the role of spinophilin and support the notion that spinophilin functions as a neuronal targeting subunit in vivo.

MATERIALS AND METHODS

Materials. Restriction enzymes were obtained from Life Technologies (Gaithersburg, MD). The pcDNA3 vector was obtained from Invitrogen (Carlsbad, CA). The pGEX vector and glutathione−Sepharose were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The pET vectors were obtained from Novagen (Madison, WI). Protease inhibitor cocktail tablets were obtained from Boehringer Mannheim (Indianapolis, IN) and Ni−NTA agarose from Qiagen (Valencia, CA). Oligonucleotides were synthesized by Operon Technologies, Inc. (Berkeley, CA). DNA sequencing was performed on an Applied Biosystems 373 Stretch Sequencer (Protein/DNA Technology Center, The Rockefeller University). Immobilon-P membranes were obtained from Millipore (Bedford, MA). Horseradish peroxidase coupled to anti-rabbit IgG and reagents for chemiluminescent Western blotting were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The rabbit polyclonal antibodies against the PP1α catalytic subunit were prepared as described (*32*). Anti-FLAG M2 monoclonal antibodies coupled to agarose were purchased from IBI (New Haven, CT). The catalytic subunit of PP1, purified either from rabbit skeletal muscle (*33*) or from Sf-9 cells (*34*) as described, was kindly provided by Dr. Hsien-bin Huang. [^{32}P]Phosphorylase *a* was prepared from phosphorylase *b* as previously described (*33*).

Cloning of Spinophilin Deletion Mutants. A FLAG tag epitope was inserted in-frame with the carboxy terminus of spinophilin by ligating a synthetic double-stranded oligonucleotide encoding the FLAG tag into an *Eco*RI/*Xba*I-digested pcDNA3 plasmid containing full-length spinophilin cDNA. Deletion fragments of spinophilin were amplified by PCR using primers designed to contain a ribosomal RNA-binding site, an initiation codon, a *Not*I site, and 20−25 base pairs of the sequence to be amplified. For the amino-terminal deletion mutants, the reverse-strand primer 5′-GTGCTCATCCACAGCCTGGCA-3′ encompassing the *Pfl*MI site of spinophilin was also used. For the carboxy-terminal and internal deletion constructs, a reverse-strand primer containing 20−25 base pairs of the appropriate sequence and an additional *Eco*RI site was employed. PCR products were digested with *Not*I/*Pfl*MI or *Not*I/*Eco*RI, purified, and ligated into the corresponding sites of the spinophilin−FLAG pcDNA3 plasmid.

Expression in 293T Cells and Coimmunoprecipitation. Constructs were transiently expressed in 293T cells for 36 h following calcium phosphate transfection. The cells were lysed in 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/mL leupeptin, 20 μg/mL antipain, 5 μg/mL pepstatin, and 5 μg/mL chymostatin at 4 °C. Cell lysates were centrifuged at 19000*g* for 10 min, and the protein concentrations of the supernatants were determined by BCA assay (Pierce). Lysates (250 μg) were incubated with anti-FLAG monoclonal antibodies coupled to agarose at 4 °C for 2 h. The protein−agarose complexes were washed extensively with lysis buffer, washed once with 50 mM Tris-HCl, pH 7.0, separated by SDS−PAGE, and transferred to immobilon-P membranes for Western blotting.

Cloning of Full-Length Spinophilin and Purification of Bacterial-Expressed Protein. A histidine tag was inserted in-frame with the carboxy terminus of spinophilin by ligating an *Nde*I/*Eco*RI fragment of spinophilin and a synthetic double-stranded oligonucleotide containing *Eco*RI/*Xho*I sites into an *Nde*I/*Xho*I-digested pET-23a vector. The *Nde*I/*Eco*RI fragment of spinophilin was digested from a pET-15b plasmid containing spinophilin cDNA. The synthetic oligonucleotide encoded the last five amino acids of spinophilin, six histidine residues, a stop codon, and an additional *Xho*I site. Proteins were expressed in BL21-DE3 *E. coli* cells by induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 37 °C for 2 h. Cells were resuspended in ice-cold 50 mM Tris-HCl, 300 mM NaCl, and 10 mM imidazole, pH 8.0, containing protease inhibitors (inhibitor cocktail tablets, 1 mM PMSF) and lysed by French press. Triton X-100 was added to give a final concentration of 1%. After centrifugation at 12000*g* for 10 min, soluble proteins were purified from the supernatant by affinity chromatography using Ni−NTA agarose. Protein was eluted using a gradient of 10−250 mM imidazole, and fractions of high purity (∼95%) were combined for the assays.

Cloning and Expression of GST-Fusion Proteins. Spinophilin fragments flanked by *Sal*I and *Not*I restriction sites were amplified by PCR using appropriate primers and subcloned into *Sal*I/*Not*I-digested pGEX-6P-3. Replacement of phenylalanine-451 by alanine was performed using the

primers 5′-AAGATCCATGCCAGCACCGCA-3′ and 5′-TGCGGTGCTGGCATGGATCTT-3′. Glutamic acid-472 was mutated to alanine using the primers 5′-GACCGACG-CAATGCGGATGTGGACCCC-3′ and 5′-GGGGTCCA-CATCCGCATTGCGTCGGTC-3′. Mutation of aspartic acid-473 to alanine was accomplished using the primers 5′-CGACGCAATGAGGCTGTGGACCCCATG-3′ and 5′-CATGGGGTCCACAGCCTCATTGCGTCG-3′. Aspartic acid-475 was mutated to alanine using the primers 5′-AATGAGGATGTGGCCCCCATGGCGGCC-3′ and 5′-GGCCGCCATGGGGGGCCACATCCTCATT-3′. Mutation of both glutamic acid-472 and aspartic acid-473 to alanine was accomplished using the primers 5′-GACCGACGCAAT-GCGGCTGTGGACCCCATG-3′ and 5′-CATGGGGTCCA-CAGCCGCATTGCGTCGGTC-3′. The triple alanine mutant of glutamic acid-472, aspartic acid-473, and aspartic acid-475 was obtained using the primers 5′-ATGACCGACG-CAATGCGGCTGTGGCCCCCATGGC-3′ and 5′-GCCATG-GGGGCCACAGCCGCATTGCGTCGGTCAT-3′. Proteins were expressed in BL21 *E. coli* by the induction of log-phase cells with IPTG at 37 °C for 2 h. Cells were resuspended in ice-cold PBS containing protease inhibitors (1 mM PMSF, 20 μg/mL leupeptin, 20 μg/mL antipain, 5 μg/mL pepstatin, 5 μg/mL chymostatin, 1 mM benzamidine) and lysed by French press or sonication. Triton X-100 was added to give a final concentration of 1%, and the lysate was centrifuged at 12000*g* for 10 min at 4 °C. Soluble GST-fusion proteins were purified from the supernatant by affinity chromatography using glutathione−Sepharose.

Coprecipitation Assays Using GST-Fusion Proteins. GST-fusion constructs bound to glutathione−Sepharose were incubated with 293T cell supernatants for 1.5 h at 4 °C. The protein−agarose complexes were washed extensively with lysis buffer, followed by a single wash with PBS, resolved by SDS−PAGE, and transferred to immobilon-P membranes for Western blotting.

Western Blotting. Membranes were blocked in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 5% (w/v) nonfat milk, and then incubated with either anti-PP1α or anti-FLAG primary antibody (1 μg/mL). Proteins were detected using anti-rabbit horseradish peroxidase-conjugated secondary antibodies, followed by enhanced chemiluminescence development.

Overlay Assays. GST-fusion proteins were resolved by SDS−PAGE and transferred to immobilon-P membranes. Membranes were blocked in TBS containing 5% (w/v) nonfat milk, and then incubated with Sf-9 cell PP1α (0.5 μg/mL) in 50 mM Tris-HCl, pH 7.0, 0.1 mM EGTA, 15 mM β-mercaptoethanol, 0.01% (w/v) Brij-35, and 0.3 mg/mL BSA at 4 °C for 2−8 h. Following brief washes with TBS containing 0.05% Tween-20 and 0.25% (w/v) nonfat milk, blots were incubated with anti-PP1α rabbit polyclonal antibodies (1 μg/mL). Proteins were detected using anti-rabbit horseradish peroxidase-conjugated secondary antibodies, followed by enhanced chemiluminescence development.

PP1 Inhibition Assays. PP1 activity was assayed using the substrate [^{32}P]phosphorylase *a* essentially as described (*33*). Assay mixtures contained 50 mM Tris-HCl, 0.15 mM EGTA, 15 mM β-mercaptoethanol, 0.01% (w/v) Brij-35, 0.3 mg/mL BSA, 5 mM caffeine, 10 μM [^{32}P]phosphorylase *a*, various concentrations of the spinophilin mutants, and PP1 purified from rabbit muscle. PP1 and the spinophilin variants were preincubated at 30 °C for 10 min. The dephosphorylation reaction was initiated by the addition of substrate and stopped after 10 min by trichloroacetic acid precipitation. Radioactivity present in the supernatant was determined by Cerenkov counting. For the antagonist studies, peptides (100 μM or 17 μM), various concentrations of wild-type spinophilin (0−1 μM), and PP1 were preincubated at 0 °C for 30 min prior to the addition of substrate. All reactions were performed in duplicate.

Peptide Synthesis. Peptides were synthesized by the W. M. Keck Biotechnology Resource Center (Yale University, New Haven, CT). All peptides were purified by reversed-phase HPLC and had the expected amino acid compositions and mass spectra.

Competition Binding Experiments. Peptides (100 μM) were preincubated with 293T cell supernatants for 1 h at 4 °C. Spinophilin coupled to Ni−NTA agarose was subsequently added, and incubation was continued for 1 h at 4 °C. The protein−agarose complexes were washed extensively with lysis buffer, followed by a single wash with 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole, resolved by SDS−PAGE, and transferred to immobilon-P membranes for immunoblotting using anti-spinophilin and anti-PP1 antibodies.

RESULTS

Identification of the PP1-Binding Domain of Spinophilin. To elucidate the region of spinophilin involved in PP1 binding, various amino- and carboxy-terminal deletion mutants of spinophilin containing a FLAG tag were transiently expressed in 293T cells. A monoclonal antibody specific for the FLAG tag was used to coimmunoprecipitate the spinophilin variants with endogenously expressed PP1. Immunoprecipitated proteins were separated by SDS−PAGE, transferred to immobilon-P membranes, and blotted with anti-PP1α polyclonal antibodies. The amount of PP1 associated with each deletion mutant was assessed by enhanced chemiluminescence.

The analysis revealed that the actin-binding, PDZ, and coiled-coil domains of spinophilin were not required for interaction with PP1 (Figure 1). With regard to the actin-binding domain, region 1−393 failed to immunoprecipitate PP1 when expressed alone. Moreover, comparable amounts of PP1 were associated with mutant 394−817, mutant 417−817, and wild-type spinophilin (1−817). Further amino-terminal truncation severely impaired the ability of spinophilin to bind PP1, as indicated by the failure of PP1 to coimmunoprecipitate with both mutants 464−817 and 496−817.

The coiled-coil domain, when expressed alone (mutant 664−817) or in concert with the PDZ domain (mutant 496−817), failed to coimmunoprecipitate PP1 from 293T cell extracts. Furthermore, the amount of PP1 immunoprecipitated by mutant 1−494, which lacked both the PDZ and coiled-coil domains, was comparable to that of mutant 1−584 and of wild-type spinophilin. Notably, mutants 1−445 and 1−463 were unable to precipitate PP1, whereas mutant 1−494 exhibited strong PP1 binding, highlighting the importance of residues 464−494. Although the levels of expression of the spinophilin mutants varied, the results clearly implicated region 417−494 as the primary site of PP1 interaction.

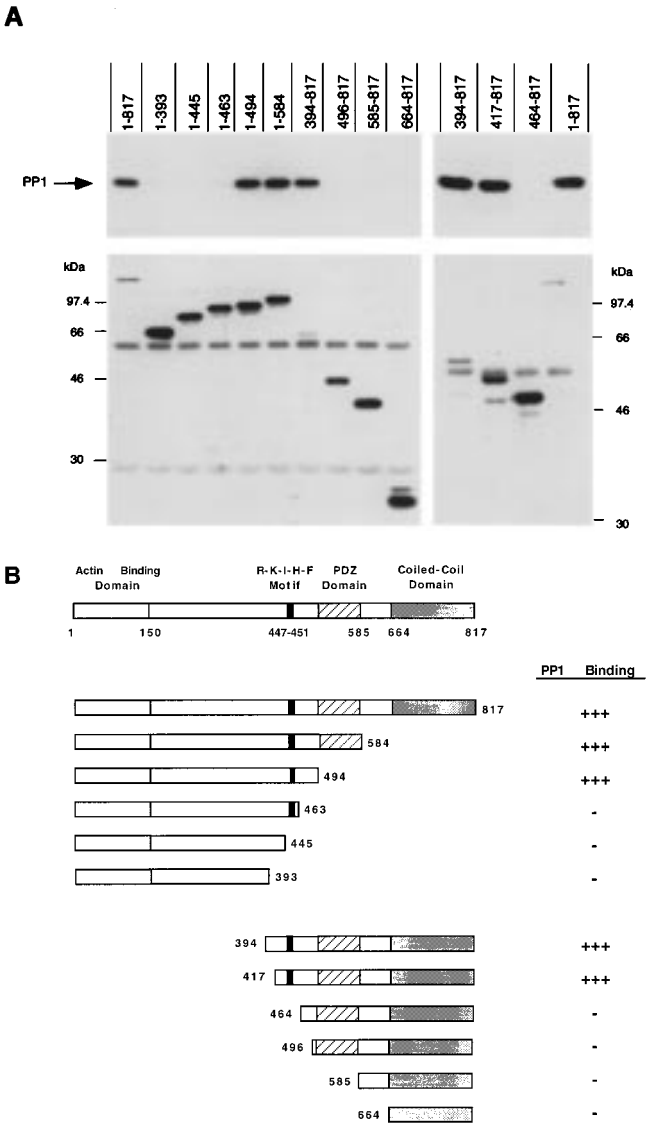


FIGURE 1: Binding of PP1 to amino- and carboxy-terminal deletion mutants of spinophilin. (A) Truncation mutants of spinophilin containing a FLAG tag epitope were transiently expressed in 293T cells. The spinophilin−PP1 complexes were coimmunoprecipitated with anti-FLAG monoclonal antibodies, and the amount of PP1 associated with each mutant was assessed by Western blotting. Blots were probed with anti-PP1α polyclonal antibodies (upper panel), then stripped and reprobed with anti-FLAG antibodies to determine the relative levels of expression of the mutants (lower panel). The protein band at approximately 50 kDa represents the anti-FLAG antibody. Representative data are shown; experiments were performed in triplicate. (B) Domain organization of spinophilin and schematic representation of the deletion analysis.

To determine whether residues 417−494 were sufficient for binding to PP1, several internal regions of spinophilin were expressed in 293T cells and assayed for their ability to associate with PP1 (Figure 2). The amount of PP1 precipitated by mutants 394−494, 417−494, and 394−584 was comparable to that of wild-type spinophilin, confirming that the primary region of interaction was located between residues 417 and 494. Further amino- and carboxy-terminal truncations of this core domain resulted in dramatic reductions in PP1 binding. Specifically, mutant 430−494 exhibited a decrease in binding, while mutant 464−494 showed no detectable PP1 binding. Similar reductions were observed upon truncation of the carboxy termini of regions 394−494

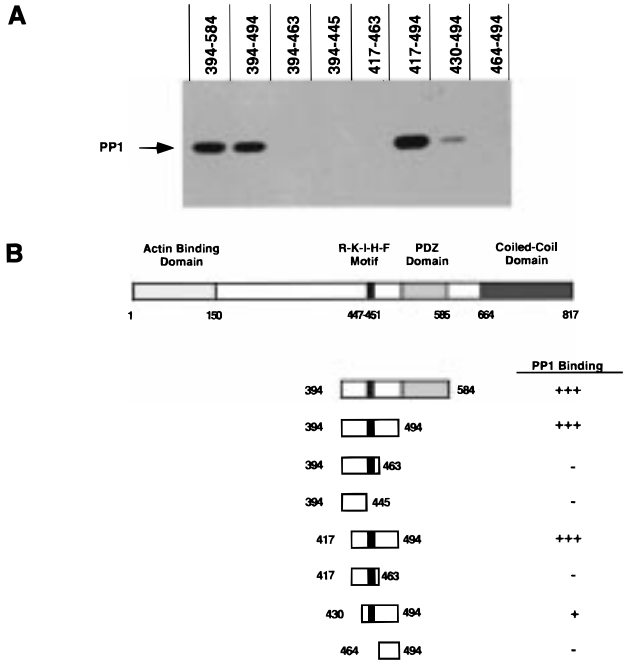


FIGURE 2: Binding of PP1 to internal regions of spinophilin. Various fragments of spinophilin containing a FLAG tag epitope were transiently expressed in 293T cells and coimmunoprecipitated with PP1 using anti-FLAG antibodies. The ability of each mutant to coprecipitate PP1 was assessed by Western blotting using both anti-PP1α (panel A) and anti-FLAG antibodies (not shown). Representative data are shown from experiments performed in duplicate. A schematic representation of the results is shown in panel B.

and 417−494: mutants 394−463, 394−445, and 417−463 all failed to precipitate PP1, suggesting that region 464−494, although insufficient by itself, contributed to the high-affinity interaction.

The domain 417−494, referred to as the "core domain", contains a pentapeptide motif (R/K-R/K-V/I-X-F) between residues 447 and 451 (R-K-I-H-F) that is conserved in other PP1 regulatory subunits (*34, 35*). Our data suggested that, in addition to this motif, other determinants such as region 464−494 might be critical for PP1 interaction. To dissect the relative contributions of these various structural elements, GST-fusion proteins encompassing residues within the core domain were constructed and their binding properties assessed using both coprecipitation and PP1 overlay assays. The importance of the conserved R/K-R/K-V/I-X-F motif was investigated by mutating phenylalanine-451 within GST-394−494 to alanine. This mutation abolished PP1 binding, as detected by overlay (Figure 3) and coprecipitation assays (not shown). Overlay analysis also revealed that short deletions within the core domain compromised the affinity of PP1 for spinophilin. For instance, PP1 binding decreased within the series G417−494, G417−484, and G417−474 (Figure 3). Similarly, less PP1 was associated with G429−484 and G429−474 compared to G429−494. This trend was accentuated upon further amino-terminal truncation, as illustrated by a significant reduction in PP1 binding for G442−474 relative to G442−494. Binding also decreased dramatically within the series G417−474, G429−474, and G442−474. Analogous results were obtained using the coprecipitation assay: deletion of regions 417−442 and 474−494, either singly or in combination, impaired the ability of spinophilin to precipitate PP1 (not shown). Thus,

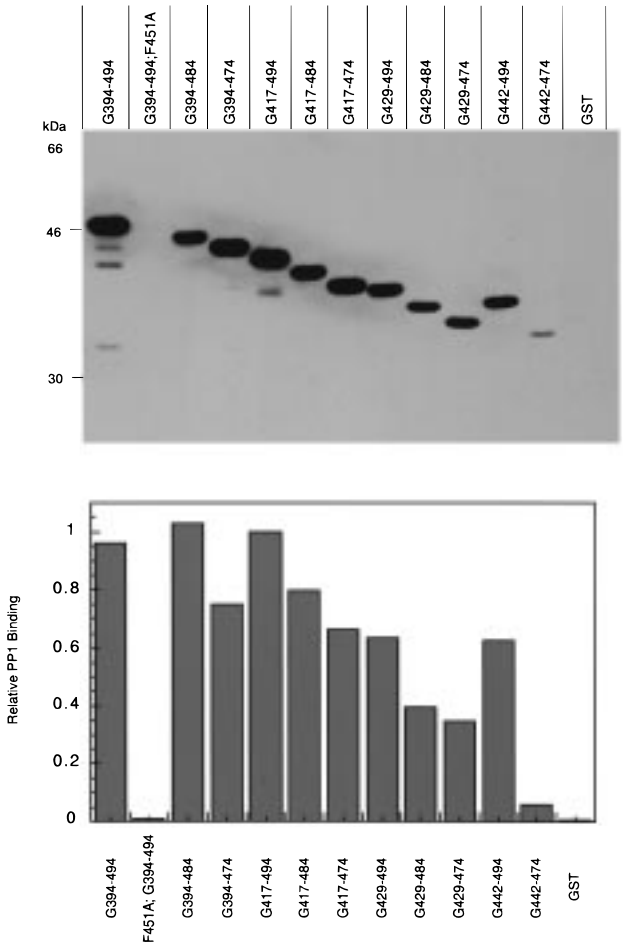
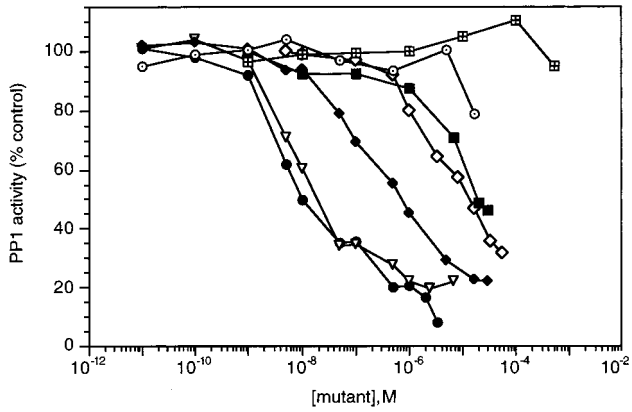


FIGURE 3: Overlay analysis of PP1 binding to deletion mutants within region 394−494. GST-fusion proteins encompassing residues within region 394−494 of spinophilin were purified by affinity chromatography, resolved by SDS−PAGE, transferred to immobilon-P membranes, and incubated with recombinant PP1α. (Upper panel) The amount of PP1 associated with each mutant was assessed using anti-PP1α polyclonal antibodies with enhanced chemiluminescence detection. (Lower panel) Relative PP1 binding to each mutant was corrected for the amount of mutant used in the assay, as determined by Coomassie staining, and was normalized with respect to G394−494. Approximately the same amount of each mutant was used except in the case of G394−484, where 44% less protein was present relative to G394−494.



	Mutant	IC_{50}
●	WT spinophilin	20.5 nM
▽	G298-817	22.9 nM
◆	G394-494	700 nM
◇	G442-494	11.1 μM
■	G429-484	38.6 μM
⊙	G394-494; F451A	> 0.1 mM
⊞	peptide 441-477	> 1 mM

FIGURE 4: Inhibition of PP1 activity by wild-type spinophilin and representative spinophilin mutants. PP1 was assayed using 10 μM [^{32}P]phosphorylase *a* as substrate. Phosphatase activity was expressed as a percentage of the activity measured in the absence of spinophilin. IC_{50} values, determined by a logarithmic fit of the kinetic data, are shown in the lower panel. Data represent average values obtained from at least two kinetic experiments.

while the conserved R-K-I-H-F motif comprised the primary site of PP1 recognition and was absolutely required for binding, regions adjacent to this motif played a critical role in stabilizing the interaction.

Inhibition of PP1 Activity. Previous studies had shown that spinophilin (G296−817) inhibited the phosphorylase phosphatase activity of PP1 with an IC_{50} value in the nanomolar range (*26*). Although it remains to be determined whether spinophilin acts as an inhibitor in vivo, the ability of spinophilin to modulate PP1 activity implies that spinophilin has the capacity to occlude the active site and/or to induce an active site conformational change upon binding to PP1. To examine whether the core domain of spinophilin was involved in modulating PP1 activity, we compared the ability of wild-type spinophilin, G394−494, and other representative mutants to inhibit the phosphorylase phosphatase activity of PP1. Wild-type spinophilin containing an amino-terminal histidine tag and GST fusion proteins of spinophilin were expressed in *E. coli* and purified by affinity chromatography.

The analysis revealed that mutant G298−817 was capable of mimicking the inhibitory potency of wild-type spinophilin (IC_{50} value ∼20 nM; Figure 4). In contrast, the IC_{50} value for G394−494 increased approximately 30-fold, indicating that additional regions outside of the high-affinity binding domain were required to modulate PP1 activity. Mutants G442−494 and G429−484 exhibited further reductions in inhibitory potency, with IC_{50} values of 11 and 39 μM, respectively. Peptide 441−477 failed to inhibit PP1 at concentrations up to 1 mM even though this region was capable of disrupting the binding of spinophilin to PP1 (data presented below), suggesting that residues 478−494 may comprise part of the inhibitory domain of spinophilin. No inhibition of PP1 activity was observed at concentrations up to 100 μM when phenylalanine-451 in G394−494 was mutated to alanine, consistent with the lack of binding observed for this mutant.

Comparison of Spinophilin with DARPP-32 and Inhibitor-1. Comparison of the core-binding domain of spinophilin with other PP1 regulatory subunits showed limited sequence homology beyond the R/K-R/K-V/I-X-F motif. However, one potential link to the phosphoproteins DARPP-32 and inhibitor-1 was observed in the region corresponding to threonine-34 of DARPP-32 and threonine-35 of inhibitor-1 (Figure 5A). DARPP-32 and inhibitor-1 are highly expressed in the brain and are found in many of the same neuronal cell types as spinophilin (*3*). Unlike spinophilin, however, both proteins are predominantly cytoplasmic and become potent inhibitors of PP1 upon phosphorylation of threonine-34/35. Mutation analysis strongly suggests that phosphothreonine-34/35 either occupies or binds close to the catalytic active site of PP1

A

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1   MEQDNSPRKIQFTVPLLEPHLDPEAAEQIRRRRPTPATLVLTSDQSSPEVD---EDREPNP   58   inhibitor-1
1   MDPKDR-KKIQFSVPAPPSQLDPRQVEMIRRRRPTPAMLFRLSEHSSPEEEASPHQRASGE   60   DARPP-32
441 EDPAPS-RKIHFST-APIQVFSTYSNEDYDRRNEDVDPM-----AASAEYELEKRVERLEL   494  spinophilin
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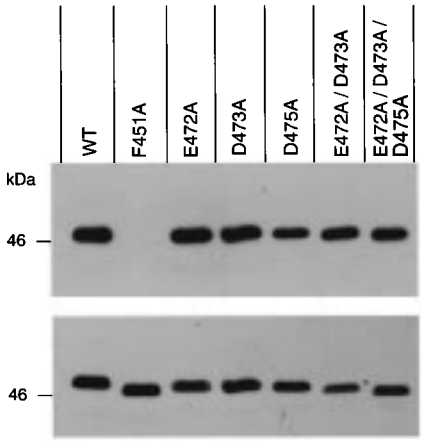
B



C

G394-494 Mutant	IC_{50} for PP1
WT	0.7 μM
E472A	0.3 μM
E472A / D473A	0.6 μM
D475A	2.0 μM
E472A / D473A / D475A	1.6 μM

FIGURE 5: Comparison of spinophilin with DARPP-32 and inhibitor-1. (A) Alignment of the PP1-binding domains of spinophilin, DARPP-32, and inhibitor-1. (B) Overlay analysis showing the ability of various alanine mutants of G394−494 to bind PP1. The amount of PP1 associated with each mutant (upper panel) and the amount of mutant used in the overlay analysis (lower panel) were assessed using anti-PP1α or anti-GST antibodies, respectively. (C) Inhibitory potency of representative alanine mutants of G394−494 toward PP1. PP1 was assayed using 10 μM [^{32}P]phosphorylase *a* as substrate. Phosphatase activity was expressed as a percentage of the activity measured in the absence of spinophilin. Kinetic analyses were performed in duplicate.

and that the preceding arginine residues play a role in recognition (*34*, *36*−*39*). Interestingly, spinophilin has three acidic residues preceded by two arginine residues in the region that aligns with threonine-34/35, and one or more of these acidic residues could mimic the negatively charged phosphate group of the phosphothreonine. The nuclear targeting subunit, NIPP-1, also contains a similar sequence (EDVD) within its PP1-binding domain (*15*). Mutation of the three acidic residues in spinophilin to alanine, singly and in combination, however, produced only modest perturbations in PP1 binding (Figure 5B) and inhibition (Figure 5C). These findings contrast with the 1000-fold lower potency of the inhibitors upon loss of phosphothreonine binding and suggest that the modulatory domain of spinophilin interacts with PP1 via a mechanism distinct from those of DARPP-32 and inhibitor-1.

Development of Peptide Antagonists To Disrupt Spinophilin−*PP1 Interactions*. Small-molecule antagonists that selectively disrupt spinophilin−PP1 interactions might be valuable as tools for understanding the cellular functions of spinophilin and PP1. Thus, short peptides (residues 414−437, 438−461, 462−494, and 441−477) corresponding to sequences within the identified core domain were synthesized and tested for their ability to modulate the spinophilin−PP1 interaction. The ability of each peptide to inhibit PP1, to antagonize spinophilin−PP1 binding, and to antagonize spinophilin-mediated PP1 inhibition was examined.

Direct inhibition of PP1 activity was measured in the presence of various concentrations of peptide using radiolabeled phosphorylase *a* as substrate. None of the peptides inhibited PP1 activity at concentrations up to 1 mM (Figure 4; not shown). Competition binding experiments were performed by incubating 293T cell extracts with peptide and recombinant spinophilin containing a histidine tag. Spinophilin−PP1 complexes were coprecipitated using Ni−NTA agarose, and the amount of PP1 in the complex was assessed by immunoblot analysis. The presence of the pentapeptide motif was necessary to confer antagonistic potency: peptides 438−461 and 441−477, but not peptides 414−437 and 462−494, competed with wild-type spinophilin for binding to PP1 (Figure 6A; *40*). The same two peptides antagonized the spinophilin-mediated inhibition of PP1 activity toward phosphorylase *a*. Peptide 438−461 caused a 6.5-fold increase in the IC_{50} value of wild-type spinophilin (Figure 6B). The longer peptide 441−477 was slightly more effective, increasing the IC_{50} value by a factor of 9. Mutation of phenylalanine-451 to alanine abolished the ability of peptide 438−461 to compete with spinophilin in both the binding and inhibition assays.

DISCUSSION

In this study, we have identified the primary PP1-binding domain of spinophilin and characterized the relative impor-

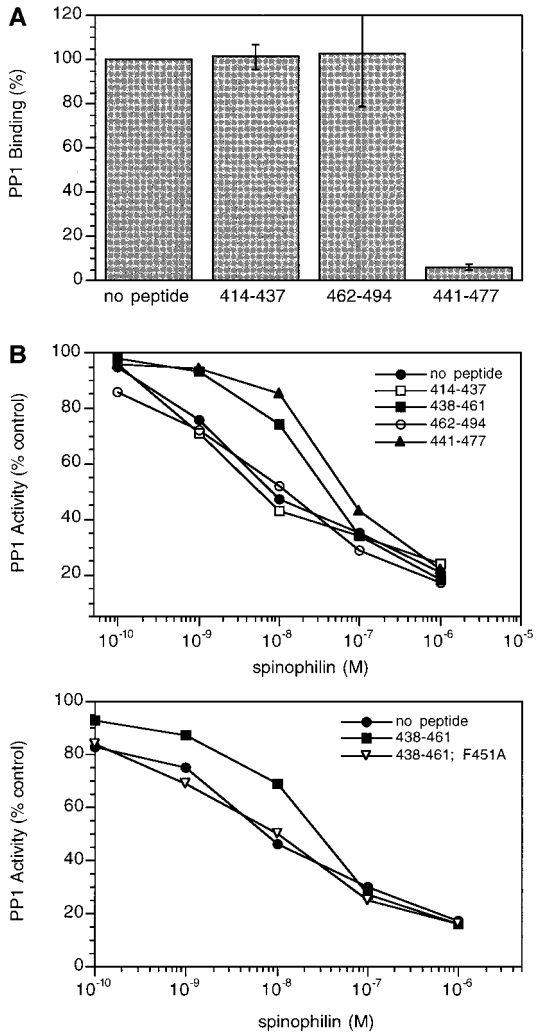


FIGURE 6: Ability of peptides corresponding to sequences within the core domain to disrupt spinophilin−PP1 interactions. Spinophilin peptides were assayed for their ability to disrupt both binding of spinophilin to PP1 and inhibition by spinophilin of PP1 activity. (A) Competition binding analysis. Peptides (100 μM) and recombinant spinophilin containing a histidine tag were incubated with 293T cell extracts. Spinophilin−PP1 complexes were coprecipitated using Ni−NTA agarose, and the amount of PP1 in the complex was determined by immunoblot analysis. Data were expressed as a percentage of the PP1 bound relative to that observed in the absence of peptide. Each bar represents the mean ± SEM of 6, 3, or 9 experiments using peptide 414−437, 462−494, or 441−477, respectively. (B) Ability of the peptides to antagonize the inhibition of PP1 by wild-type spinophilin. The inhibition of PP1 activity toward phosphorylase *a* was measured in the presence of wild-type spinophilin and a fixed concentration of peptide (100 μM, upper panel; 17 μM, lower panel).

tance of structural elements within this domain for PP1 recognition. Our analysis indicates that residues 417−494 are responsible for mediating the high-affinity interaction with PP1. This region is both necessary and sufficient for binding to PP1. No interaction was detected in coprecipitation assays when other regions of spinophilin, including the actin-binding, PDZ, and coiled-coil domains, were tested individually. The most important determinant for PP1 recognition is the R/K-R/K-V/I-X-F pentapeptide between residues 447 and 451 (R-K-I-H-F) of spinophilin, a motif that is conserved in other PP1 regulatory subunits. Deletion of these residues or mutation of phenylalanine-451 to alanine abolished the PP1 binding observed by coprecipitation and overlay analysis. In addition to the conserved motif, regions 417−442 and 463−494, although themselves insufficient for PP1 binding, appear to stabilize the interaction, thereby enhancing the affinity of PP1 for spinophilin.

The present results suggest that at least two distinct subdomains are involved in PP1 recognition: a high-affinity binding domain which contains the R-K-I-H-F motif and a second domain for modulating PP1 activity. In support of this idea, both peptides 441−477 and 438−461 could compete with wild-type spinophilin for binding to and inhibition of PP1. However, neither peptide inhibited the PP1-catalyzed dephosphorylation of phosphorylase *a* at concentrations up to 1 mM. In contrast, G442−494 inhibited PP1 activity toward phosphorylase *a* with an IC_{50} value of 11 μM. The difference in the potencies of G442−494 and peptide 441−477 implicates region 478−494 as part of the inhibitory domain of spinophilin. The inhibitory domain also appears to encompass sequences beyond the identified core domain. For example, wild-type spinophilin was much more potent than G394−494 as an inhibitor of PP1.

The involvement of at least two distinct subdomains in PP1 recognition is emerging as a common theme among PP1 regulatory subunits. Two binding sites have been identified in the phosphoproteins DARPP-32 and inhibitor-1: the conserved R/K-R/K-V/I-X-F motif (residues 7−11 of DARPP-32; residues 8−12 of inhibitor-1) and the region surrounding the phosphorylated threonine (residue 34 of DARPP-32; residue 35 of inhibitor-1) (*34*, *36*, *38*, *39*, *41*). These two, relatively low-affinity binding interactions act in concert to inhibit PP1 with nanomolar potency. Dephosphorylation, leading to loss of phosphothreonine binding, reduces the inhibitory potency of both DARPP-32 and inhibitor-1 by at least 1000-fold. Deletion analysis of the G_M, G_L, and M_{110} subunits, inhibitor-2, and NIPP-1 also suggests distinct PP1 binding and modulatory subdomains (*6*, *17*, *37*, *42*, *43*). For instance, the G_M peptide 63−75, encompassing the pentapeptide motif R-R-V-S-F, prevented G_L from suppressing the dephosphorylation of phosphorylase *a*, but failed to dissociate G_L from PP1 (*6*). Moreover, the M_{110} peptide 1−38, which contains the conserved binding motif, was not sufficient to mimic the properties of PP1−M_{110} toward the substrate MLC_{20} or phosphorylase *a* (*6*, *43*). In combination, the biochemical studies of spinophilin, DARPP-32, and the M_{110} subunit suggest that the conserved motif may not be directly involved in modulating PP1 activity. Consistent with this notion, crystallographic studies have shown that the motif in G_M binds a hydrophobic channel in the carboxy-terminal region of PP1, away from the catalytic active site (*35*). In the case of spinophilin, the R-K-I-H-F motif is absolutely required for association with PP1 and presumably binds the phosphatase in a manner similar to the G_M peptide. The recognition of PP1 by spinophilin may therefore involve an ordered mechanism whereby docking of the pentapeptide must occur prior to binding of the modulatory subdomain.

The identified PP1-binding domain of spinophilin shares extensive homology with the corresponding region of neurabin I (*44*), a recently reported protein that has 48% amino acid identity and a domain organization similar to spinophilin. The conserved motifs of spinophilin (R-K-I-H-F) and neurabin I (R-K-I-K-F) differ by only a single substitution. Moreover, residues 446−494 of spinophilin, which may include part of its inhibitory domain, show 84%

identity to the corresponding region of neurabin I. The adjacent PDZ (residues 496−585 of spinophilin) and coiled-coil (residues 664−805 of spinophilin) domains also share a high degree of homology (85% and 61%, respectively). Thus, spinophilin and neurabin I are likely to interact with PP1 via similar mechanisms. It will be interesting to examine whether the single amino acid substitution and sequence variations amino-terminal to the conserved motif result in distinct PP1-binding affinities or modes of regulation for these closely related proteins.

The present study provides support for the notion that spinophilin functions as a neuronal targeting subunit and underscores the differences between spinophilin and the cytosolic inhibitors DARPP-32 and inhibitor-1. First, the subcellular localization of spinophilin is consistent with a targeting role for this protein, and our studies indicate that spinophilin, PP1, and F-actin can form a trimeric complex in vitro (not shown). In contrast to the diffuse, cytosolic distribution of DARPP-32 and inhibitor-1, spinophilin is highly localized to dendritic spines, sites of excitatory synaptic transmission where proteins, receptors, and ion channels are assembled with an actin−microfilament network (*20*, *26*). Second, the biochemical properties of spinophilin and the mechanism by which it interacts with PP1 are distinct from those of the cytosolic inhibitors, supporting a targeting subunit role for spinophilin. Spinophilin is a weaker inhibitor of PP1, and the minimum domain required to mimic the potency of wild-type spinophilin is much larger than that of DARPP-32 and inhibitor-1. While the inhibitory properties of DARPP-32 and inhibitor-1 can be mimicked by a 38 amino acid peptide, greater than 100 residues are required in the case of spinophilin. Moreover, the PP1 interaction with DARPP-32 and inhibitor-1 cannot be demonstrated by coprecipitation, overlay, or yeast two-hybrid analysis (*37*), suggesting that spinophilin forms a more stable complex with PP1. The association constant for the binding of spinophilin to PP1 has been estimated to be $\sim 10^8$ M by ELISA (not shown), approximately 100-fold higher affinity than that for DARPP-32 (*41*). Third, the ability of spinophilin peptides to form a stable complex with PP1 while leaving the PP1 active site unobstructed is consistent with the notion that spinophilin functions as a targeting protein. The observation that PP1 can bind both the active site inhibitor microcystin and spinophilin simultaneously (*45*) further supports this idea. In fact, the purification of PP1-binding proteins via microcystin affinity chromatography may inherently select for targeting subunits rather than inhibitors.

The ability of spinophilin to inhibit PP1 activity toward phosphorylase *a* may seem contrary to its function as a targeting subunit. However, the effects of targeting subunits have been shown to be substrate-dependent. For example, the G-subunit endows PP1 with glycogen synthase phosphatase activity, but decreases its phosphorylase *a* phosphatase activity (*4*). The peptides developed in this study, as well as other antagonists capable of disrupting PP1 interactions at the postsynaptic membrane, should help to elucidate novel neuronal substrates for PP1 and signaling pathways in the brain. In fact, studies in vivo using the spinophilin peptide 438−461 indicate that spinophilin-mediated anchoring of PP1 to postsynaptic densities facilitates the dephosphorylation of AMPA channels (*40*). Disruption of the interaction between spinophilin and PP1 using peptide 438−461 or inhibition of PP1 activity using okadaic acid had the same effect of stabilizing AMPA conductances in neostriatal neurons. In addition to the AMPA receptor, potential candidates for PP1 regulation include Ca^{2+} channels, K^+ channels, NMDA receptors, the Na^+/K^+ pump, and the actin cytoskeleton. Regulation of the phosphorylation state of ion channels and receptors through spinophilin−PP1 interactions in dendritic spines may contribute to the coordination of neuronal inputs and explain the role of PP1 in long-term synaptic plasticity.

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