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Research report

The identification of a second actin-binding region in spinophilin/neurabin II

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Abstract

Spinophilin/neurabin II is an actin-associated scaffolding protein enriched in the dendritic spines of neurons. Previously, the actin-binding domain (ABD) of spinophilin was localized to a domain between amino acids (aa) 1 and 154. In a mass spectrometry screen for spinophilin-binding proteins, we have identified an additional actin-binding region between aa 151 and 282. F-actin co-sedimentation and GST affinity chromotography experiments further substantiate this result. Phalloidin staining of Rat2 fibroblasts transiently expressing GFP-spinophilin deletion constructs indicates co-localization with a subset of actin. Regions of spinophilin that lack the revised ABD (aa 1–230) do not co-localize with phalloidin-labeled actin, suggesting that the actin-binding domain contributes to directing the subcellular distribution of spinophilin. Targeting experiments using primary hippocampal cultures indicate that only the first actin-binding site contributes to dendritic spine localization. The second ABD targets to spines inefficiently and thus may interact with and affect actin filaments in a different manner than the first ABD.
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1. Introduction

The actin cytoskeleton is capable of rapid remodeling in response to cues from the environment [17]. The reorganization of actin is regulated by a large number of actin-associated proteins that alter actin by stabilizing filaments, destabilizing them, or sequestering actin monomers from polymerization reactions [4]. One such protein is the actin bundling protein Spinophilin/Neurabin II. Spinophilin was originally identified based on its ability to bind F-actin [15] and protein phosphatase I (PP1) [1]. It contains three distinct protein–protein interaction modules: an actin-binding domain, a PDZ domain, and a region predicted to form a coiled-coil. Spinophilin shares domain organization and sequence homology with neurabin I, a brain-specific actin-binding protein. Spinophilin forms homomeric complexes that are thought to contribute to its actin-cross-linking activity [15]. In addition to homomeric interactions, spinophilin has also been found to interact with several other proteins including D2 dopamine receptor [20], alpha-adrenergic A2 receptor [14], neurabin 1 [9], trans-golgi protein 38 (TGN-38) [23], ryanodine receptor [10], p14ARF [24], and Tiam1 [2].

Spinophilin is enriched in the actin-rich dendritic spines of central nervous system (CNS) neurons that serve as the major site of glutamatergic synapses in the brain. Recently, Feng et al. [5] generated knock-out mice to better under-

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stand the role of spinophilin in normal physiology. Spinophilin-null mice display reduced taste aversion acquisition in a test of associative learning [21]. There is also a transient increase in dendritic spine number during brain maturation suggesting that spinophilin participates in regulating the actin cytoskeleton during CNS development [5]. Fischer et al. [6] also noted altered glutamate receptor function in the knock-out mice, a receptor system known to influence spine dynamics. The cause and effect relationship between spinophilin, neurotransmitter receptors, and spine alteration remains to be clarified, but this observation distinctly places spinophilin at the interface of cell signaling and cytoskeletal regulation. Consistent with this role, investigations of phosphorylation in the ABD of spinophilin indicate that the affinity of this region for actin can be altered by protein kinase A [8]. The dendritic spine phenotype in knock-out mice is, however, somewhat surprising since neurabin I is also known to bind actin [11] and PP1. Despite having a similar domain organization to spinophilin (Fig. 1A), neurabin I is unable to compensate for the loss of spinophilin. This may be explained in part by differences in binding partners.

In order to isolate proteins that associate only with spinophilin, we initiated a proteomic approach that employed affinity chromatography and mass spectroscopy. This resulted in the identification of several proteins, most of which are known to bind or associate with actin. Further experiments revealed that these proteins co-precipitated with F-actin and that an additional site exists within spinophilin that is capable of independently binding actin.

2. Methods and materials

2.1. Production of GST fusion proteins

Plasmids encoding various GST-rat spinophilin proteins were expressed in the bacterial strain BL21(DE3)pLysS (Stratagene) as described previously [20]. Pellets were resuspended in 0.1 volumes PBS + 5 mM EDTA, and bacterial suspensions were frozen on dry ice and stored at − 80 °C until purification. PMSF (170 μg/ml) and a protease inhibitor cocktail containing aprotinin (2 μg/ml, final), leupeptin (1 μg/ml), benzamidine (16 μg/ml), and

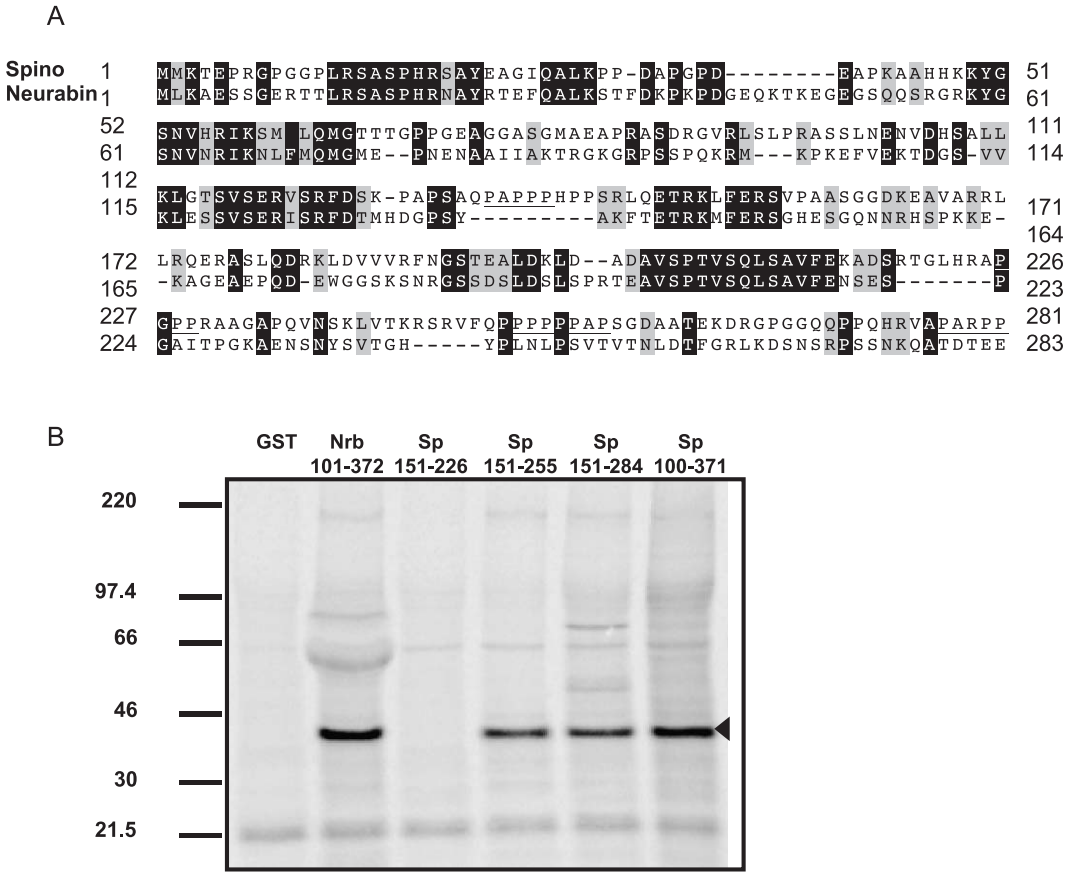

Fig. 1. A comparison of Rat Spinophilin/Neurabin II and Neurabin I. (A) Diagram of Rat Spinophilin and Rat Neurabin I indicating their sequence homology. (A) An alignment of Spinophilin(1–281) and Neurabin I(1–283) sequences. Identical residues are outlined in black. Conserved amino acids are indicated in gray. Proline rich regions of Spinophilin are underlined. (B) Autoradiogram of radiolabeled proteins purified from AtT-20 cells using various GST-Spinophilin and Neurabin fusion proteins. The closed arrowhead indicates a band interacting with GST-neurabin and several of the GST-Spino constructs. Molecular mass is indicated to the left (kDa).

lima bean trypsin inhibitor (LBTI) (50 μg/ml) was added during thawing. Lysed bacteria were sonicated, triton X-100 was added to 1%, and the lysate was incubated on ice for 10 min. Bacterial lysates were clarified by centrifugation at 30,000 × *g* for 30 min at 4 °C, followed by filtration through a 0.45-um filter. Fusion proteins were purified using glutathione sepharose (GSH) (Amershan Pharmacia Biotech). Fusion protein was eluted in 10 × 0.2 volume aliquots with 50 mM Tris–HCl, pH 8, 20 mM glutathione, 150 mM NaCl and 0.1% Triton X-100. Fractions containing fusion protein (as detected by SDS-PAGE and coomassie blue staining) were pooled and dialyzed against PBS + 10% glycerol overnight at 4 °C.

2.2. Metabolic labeling and GST affinity purification from labeled AtT-20 cells

Wild type AtT-20 cells were maintained in DMEM + 10% horse serum + 5% fetal bovine serum (FBS). Cells were rinsed once with PBS and incubated in serum and cysteine/methionine depleted DMEM (DMEM-cys/met) for 30 min at 37 °C, 5% CO_2. Media was replaced with DMEM lacking cys/met and containing 100 μCi/ml ^{35}S-labeled cysteine/methionine (Promix, Amersham). Cells were then incubated for 3 h at 37 °C, 5% CO_2. At the end of labeling, media were removed and cells were washed once with cold PBS. Cells were lysed in RIPA (50 mM NaPhosphate, pH 7.4, 150 mM NaCl, 1% IGEPAL CA-630, 0.5% NaDeoxycholate, 0.1% SDS, 10 mM NaF, 250 μM $NaVO_3$, and a protease inhibitor cocktail) and incubated on ice 10 min followed by eight passages through a 25-gauge needle. The lysates were centrifuged for 20 min at ~ 14,000 × *g*. The soluble fractions were pre-cleared by incubation with GST bound glutathione-sepharose for 30 min at 4 °C. After pelleting the GST bound sepharose and recovery of the soluble lysate, the lysate was split and used for affinity purification. Equal fractions of the lysate were added to various GST fusion proteins pre-bound to glutathione-sepharose. Reactions were tumbled for 3 h at 4 °C. Beads were washed extensively and bound proteins were eluted in 2X SDS sample buffer and separated by SDS-PAGE. Gels were stained with coomassie blue, dried and analyzed by PhosphorImager analysis.

2.3. Mass spectrometry of purified proteins from AtT-20 cells

GST affinity purifications were performed as above with unlabeled cells. Proportions were scaled up ~ 5-fold to enable visualization of bound proteins by coomassie blue staining. SDS-PAGE resolved bands of interest were excised and digested in-gel with trypsin (Boehringer Mannheim, Indianapolis, IN) in 10 mM Tris–HCl, pH 8.0, according to the procedure of Shevchenko et al. [19] except that alkylation of sulfhydryls was accomplished with 4-vinylpyridine. Liquid chromatography-tandem mass spectrometric (LC/MS/MS) analyses of in-gel digests were done using an Ultimate capillary LC system (LC Packings, San Francisco, CA) coupled to a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK) fitted with a Z-spray ion source as described previously [3]. Un-interpreted peptide product ion spectra generated by LC/MS/MS were searched against the non-redundant protein database for exact matches using the Mascot search program [13].

2.4. Actin co-sedimentation

Purified chicken skeletal muscle G-actin (provided by J. Berg and R. Cheney, UNC Chapel Hill) was incubated in polymerization buffer (10 mM Imidizole, pH 7.2, 75 mM KCl, 2.5 mM $MgCl_2$) on ice for 30 min in the presence of 80 μM phalloidin. GST fusion proteins were centrifuged at ~ 14,000 × *g* for 10 min prior to use. Polymerized F-actin was then mixed with either GST or GST-spinophilin 151–284 (5 μM) in F-actin buffer (10 mM Imidizole, pH 7.2, 75 mM KCl, 2.5 mM $MgCl_2$, 1 mM Tris–HCl, 0.1 mM ATP, 0.1 mM $CaCl_2$, 0.25 mM DTT) in a final volume of 300 μl. Control samples included F-actin alone or each individual GST fusion alone. The reactions were incubated for 30 min at room temperature, followed by centrifugation at 150,000 × *g* for 30 min at 4 °C. The supernatant was removed to a fresh tube and the pellet was resuspended in an equal volume of F-actin buffer. Samples were resuspended in laemmli buffer, equal fractions were separated by SDS-PAGE, and proteins were visualized by coomassie blue staining. In some experiments, actin binding was performed using the Actin-binding Protein Biochem Kit (Cytoskeleton) according to the manufacturer's instructions.

2.5. Transient transfections

Rat2 fibroblasts (generously provided by Dr. Frank Gertler) were maintained in DMEM/F12 media containing 10% FBS. Prior to transfection, cells were plated onto Costar six-well plates containing glass cover-slips and transfected the following day. Enhanced Green Fluorescence Protein (EGFP) vectors (Clontech) containing various regions of spinophilin fused to the carboxy terminus of GFP were transfected using either Effectene (Qiagen) or Fugene (Roche) following the manufacturer's suggested protocol. Cells were trypsinized, plated onto glass cover-slips and fixed 16 h later with 4% paraformaldehyde for 20 min. They were subsequently permeabilized in a solution of PBS/0.25% Triton X-100. The cells were then incubated for 30 min in a solution of 165 nM Alexa 594-conjugated phalloidin (Molecular Probes, Eugene OR). This was followed by three washes in PBS. Cells were mounted and images were captured using a Leica NT confocal microscope. The images were analyzed using Photoshop 5.0 software (Adobe). Primary hippocampal cultures were grown on poly-lysine coverslips for 18 to 23 days in vitro. Transfections were carried out with Lipofectamine2000 following manufacturer's protocol. Neurons were imaged using a Nikon Diaphot

micropscope equipped with a 1.3 n.a. 40× oil objective, epifluorescence attachment, Ludl filter wheels and a Hamamatsu ORCA CCD camera. Data acquisition and analysis were done by Openlab software (Improvision). Z-sections were obtained separately for EYFP (spinophilin) and the co-transfected ECFP and a maximal projection was used to remove out of focus fluorescence (OOFF). Alternatively, multi-neighbour deconvolution was employed to remove OOFF.

2.6. Spinophilin serine-177 phosphorylation

Experiments were carried out essentially as described previously [8]. Briefly, human embryonic kidney 293 cells were transiently transfected using Effectene (Qiagen). Thirty six hours following transfection, cells were treated with either vehicle or 50 μM forskolin for 5 min. Cells were harvested in ice-cold RIPA buffer and GFP fusion proteins immunoprecipitated from the lysates. The precipitated protein was resolved by SDS-PAGE and blotted using either mouse anti-GFP (Covance) or rabbit anti-phosphoserine 177 (generously supplied by Drs. Angus Nairn and Paul Greengard).

3. Results

Spinophilin/neurabin II contains sequences beyond amino acids 1–154 [15], its previously defined N-terminal F-actin-binding domain (ABD), that are not found in a closely related protein neurabin I (Fig. 1A). In this study, we sought to identify proteins that could interact with this unique region in Spinophilin. Therefore, GST fusion proteins consisting of various portions of spinophilin/neurabin II were used to identify binding proteins from metabolically labeled AtT-20 cell lysates. AtT-20 cells were chosen because they express high levels of endogenous spinophilin/neurabin II. We reasoned that interacting proteins might be abundant in these cells as well. Lysates from cells labeled with ^{35}S-cysteine/methionine were incubated with glutathione-sepharose coupled to GST to reduce non-specific binding. The lysate was then incubated with GST, GST-neurabin (GST-Nrb) or the GST-spinophilin (GST-Spino) fusion proteins. Several labeled proteins bound specifically to the fusion proteins and not to GST (Fig. 1B). Among the most identifiable bands was a 43-kDa band that appeared to be associated with multiple GST-spino fusion proteins, as well as GST-Nrb 101–372 (Fig. 1B, closed arrowhead). Similarly, a ~220-kDa band appeared in these same reactions. Also of note were bands of ~55, ~70, and ~85 kDa that bound to GST-Spino(151–284), but not to GST-Spino(151–255) or GST-Spino(151–226). We were particularly interested in proteins that might bind to the proline-rich regions unique to spinophilin/neurabin II, specifically the motif FQPPPPPPAP (amino acid (aa) 244–253) (Fig. 1A). Therefore, our efforts focused on identifying proteins that bound to GST-Spino(151–284).

To identify these spinophilin-associated proteins, our chromatography protocol was scaled up ~5-fold and lysates were pre-cleared using a resin of GST alone, and incubated with GST-Spino(151–284). Bound proteins were analyzed by SDS-PAGE and colloidal coomassie blue staining (Fig. 2A). Several bands were identified that were specifically present in samples that had been incubated with AtT-20 lysate, but not in mock incubations. These bands were excised from the gel and processed for mass spectrometry by capillary liquid chromatography coupled quadrapole-time of flight tandem mass spectrometry/mass spectrometry, allowing both high accuracy peptide mass fingerprinting as well as direct peptide sequencing. The mass spectrometry data was used to search databases of known protein sequences. The identity of each protein identified by mass spectrometry is as follows (see Fig. 2A): 1, myosin II; 2,3,4 Gelsolin and Gelsolin degradation products; 5, bacterial proteins dnaK and heat shock-like protein 70; 6, cognate heat shock protein 70; 7, Arp3 and elongation factor 1; 8, GST-Spinophilin fusion protein; 9, GST fusion protein degradation product. The common characteristic shared by the majority of these proteins is known actin-binding activity. This result suggested that the intense 43-kDa band observed in the metabolic labeling experiments bound to several GST fusion proteins was actin. In experiments using Rat2 cell lysates, actin was detected by Western blot following affinity purification from cell lysates using GST-Spino(1–164) and GST-Spino(151–282), but not in those using GST alone (data not shown). While these data suggest that the proteins identified by mass spectroscopy with GST-Spino(151–284) may be the result of co-precipitation with actin, we cannot rule out the possibility that some of these proteins serve to link spinophilin with F-actin.

Actin co-sedimentation assays were performed to determine whether the interaction of GST-Spino(151–284) with F-actin is via direct protein–protein interaction. F-actin was incubated alone, and in the presence of GST or GST-Spino(151–284). The proteins were centrifuged at 150,000 × g for 30 min, and equal ratios of the soluble and particulate fractions were separated by SDS-PAGE and proteins were detected by coomassie staining (Fig. 2B). A significant fraction of GST-Spino(151–284) was recruited into the particulate fraction in the presence of F-actin, whereas it was completely soluble without F-actin. In contrast, GST was found in the soluble fraction in both the presence and absence of F-actin. These data clearly show that this fragment of spinophilin/neurabin II is able to directly bind F-actin. Previous analysis of the F-actin-binding region of spinophilin by Satoh et al. was performed using F-actin overlay analysis. They determined that the region between amino acids 1–154 was sufficient for F-actin binding, and that distal regions of spinophilin/neurabin II did not bind F-actin in this assay. However, our data suggest that there might be additional determinants of actin binding in the region between amino acids 151 and 284. Comparison of amino acids 1–281 in spinophilin with 1–

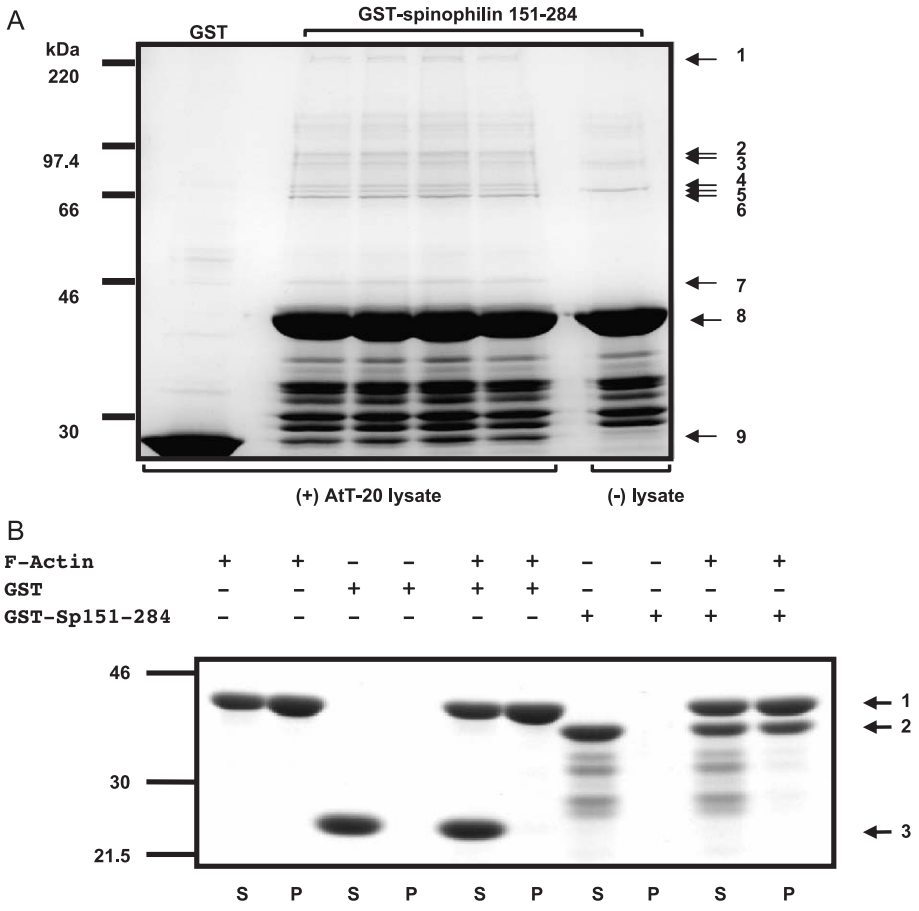

Fig. 2. Identification of GST-Spinophilin 151–284 binding proteins from AtT20 cells using mass spectrometry and F-actin Co-sedimentation Assay. (A) Coomassie-stained SDS-PAGE gel containing affinity purified proteins. Dots are adjacent to those bands that were excised for analysis. Numbers to the right correspond to each protein as described in the text. 1, myosin II; 2,3,4 Gelsolin and Gelsolin degradation products; 5, bacterial proteins dnaK and heat shock-like protein 70; 6, cognate heat shock protein 70; 7, Arp3 and elongation factor 1; 8, GST fusion protein degradation product. (+) Indicates that the GST protein was incubated with cell lysate, whereas (–) protein was mock incubated. (B) Actin co-sedimentation Assay. Following a 150,000 × *g* spin, equal amounts of the soluble and particulate fractions were separated by SDS-PAGE and visualized using Coomassie blue staining. Arrow 1 indicates the migration of actin, arrow 2 GST-Spino(151–282) and arrow 3 GST. Molecular mass is indicated to the left in kiloDaltons.

283 in neurabin shows that there are several stretches with high sequence identity (Fig. 1A). Sequence alignment also shows that there is a region of high sequence homology between the two proteins distal to the currently accepted F-actin-binding domains. We believe that this region also contributes to the F-actin-binding properties of these two proteins, and that GST-Spino(151–284) binds F-actin within this region. Competition experiments between Spino(1–164) and Spino(151–284) also suggests that these binding domains may recognize distinct sites on F-actin (unpublished observation). We next sought to determine if the interaction between this second actin-binding site and actin occurs in a physiological context. To address this question, we created spinophilin fusion proteins with enhanced green (EGFP) and enhanced yellow fluorescent protein (EYFP).

Rat2 fibroblasts were transiently transfected with EGFP fused to various regions of spinophilin, and stained with Texas Red- or Alexa 594 phalloidin in order to assay co-localization with polymerized actin in vivo. EGFP alone demonstrated no co-localization with phalloidin-labeled F-actin (Fig. 3A). GFP-Spino (1–164) was sufficient to target F-actin (Fig. 3B), consistent with the previous observations and similar experiments using tagged neurabin I [11]. To test the ability of Spino(164–282) to independently associate with actin in a cellular context, we transfected a construct spanning aa 164–282 fused to GFP. In this case, the fusion protein localized to lamellapodia-like structures in the cell periphery (Fig. 3C). These peripheral structures were enriched with F-actin. A construct containing a longer stretch of the amino terminus of spinophilin (aa1–230) is also able to target to F-actin (Fig. 3D).

We further characterized the properties of Spino(164–282) in the context of the full-length protein. Full-length spinophilin predominately, but not completely, co-localized with F-actin (Fig. 3E), however, it did not alter the cytoskeleton significantly. EGFP fused to Spino(164–817) fails to interact with the actin cytoskeleton to any appreciable degree (Fig. 3F). This is surprising considering that Spino(164–282) is capable of binding actin and perhaps suggests that the C-terminal region of Spinophilin may

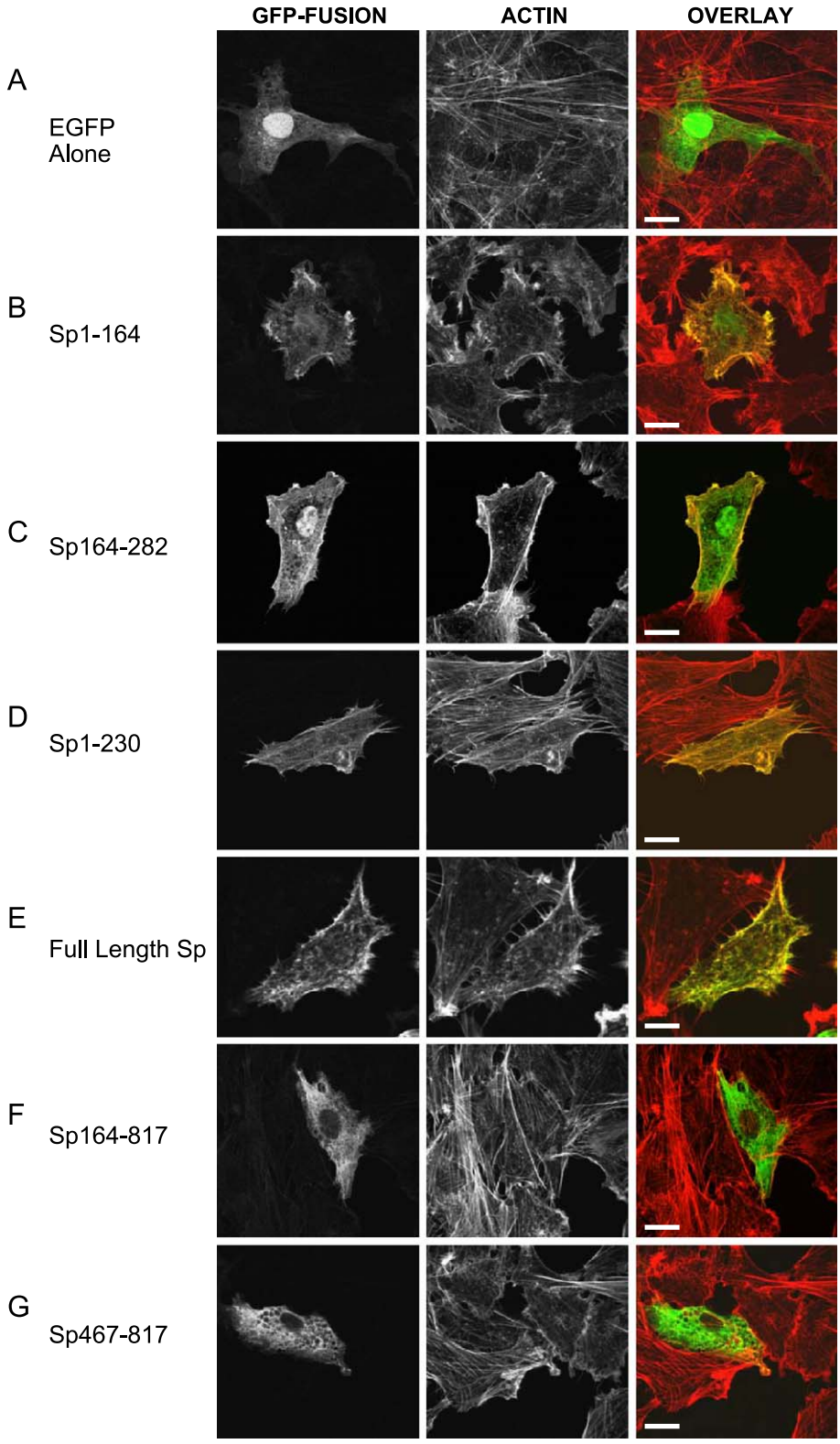

Fig. 3. (A) Actin association by putative actin-binding fragments of spinophilin fused to EGFP. Representative images from confocal micrographs of EGFP fusion proteins transiently expressed in Rat2 cells. F-actin is counter-stained using TRITC-phalloidin (scale bar = 10 μm). (A) EGFP transfected alone does not associate with actin. (B) EGFP-Spino(1–164) is capable of interacting with actin. (C) EGFP-Spino(164–282) localizes with a subset of F-actin. (D) EGFP-Spino(1–230) demonstrates complete co-localization with actin. (E) EGFP-Spino full-length co-localizes with the majority of F-actin with the cell. (F) EGFP-Spino(164–817) does not associate with F-actin. (G) EGFP-Spino(467–817) also fails to interact with F-actin. -actin is counter-stained using TRITC-phalloidin (scale bar = 10 μm).

regulate actin binding via intra or intermolecular interactions. This regulation would be in contrast to the observed pattern for a similar construct of neurabin I that effectively targets actin in COS7 cells, and may reflect either a regulatory difference in the proteins or phenotypic differences in the cell lines used [11]. Experiments using phosphor-specific antibodies against serine-177 indicate that in Sp(164–817) this site is basally unphosphorylated (data not shown) and therefore should render the protein more able to bind actin based on previous studies [8]. This suggests another mechanism may be responsible for the loss of actin-binding in this construct. Similar to Sp(164–817), GFP-Spino(467–817) failed to associate with actin in Rat2 cells (Fig. 3G), consistent with the lack of any remaining F-actin-binding properties, but contrasting previously reported data for the human ortholog of Spinophilin using a similar construct that indicated nuclear localization [24]. This discrepancy may reflect cell line differences or proteolysis leaving only EFGP. Western blot analysis of our constructs demonstrates only a single band larger than GFP alone for each construct (data not shown). Taken together these results are consistent with the in vitro binding results discussed previously. These data indicate that Spino(164–282) displays actin-binding activity, but suggest it may have subtle differences from the original ABD (aa 1–154) such as a distinct pool of actin bound.

We next transiently transfected primary hippocampal neurons to address the role aa 164–282 may play in targeting spinophilin to the actin-rich dendritic spines. Consistent with the previous reports [7], full-length spinophilin was able to target appropriately (Fig. 4A). A similar pattern was observed for a construct containing aa 1–164 (data not shown). In contrast, a short amino terminal region of spinophilin (aa 1–43) was not routed to dendritic spines (Fig. 4B) nor does it co-localize with actin in Rat2 cells (unpublished observation). The remainder of the first ABD (aa 44–164) was sufficient to effectively target EYFP to spines (Fig. 4C). This construct is also able to target actin in Rat2 cells (unpublished observation). However, fusion proteins consisting of only the second ABD (aa 164–282) were weakly able to route to spines (Fig. 4D). This result suggests that while actin-binding may be necessary for spine local-

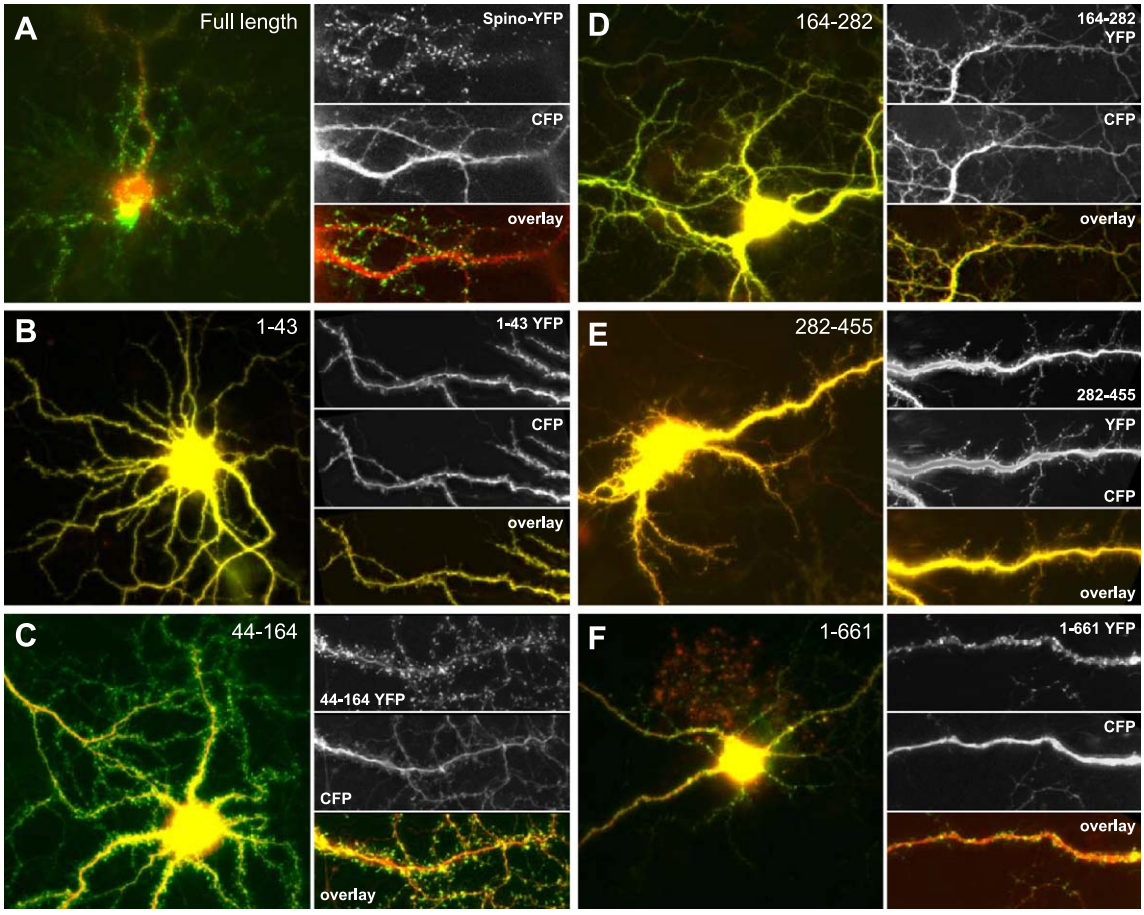

Fig. 4. Deconvolution Microscopy of EYFP fusion proteins in primary hippocampal neurons. Cells were co-transfected with ECFP to delineate cell morphology. Enlargements of individual dendrites are shown to the right of each figure with each signal shown individually and in overlay. (A) EYFP-Spino full-length targets accurately to dendritic spines. (B) Spino(1–43) is unable to localize EYFP. (C) EYFP-Spino(44–164) effectively route EYFP to spines. (D) EYFP-Spino(164–282) only weakly targets EYFP. (E) EYFP-Spino(284–455) demonstrates no targeting. (F) EYFP-Spino(1–661) targets similarly to full length.

ization of spinophilin, in this case, it is not sufficient. The regions of spinophilin that have little homology to neurabin I (aa 282–455) show no spine labeling (Fig. 4E). A larger fragment of the protein that included both the PP-1 binding site and PDZ domain (aa 1–661) recapitulated the spine targeting seen with full-length spinophilin (Fig. 4F) and in Rat2 cells is observed to associate with actin filaments (unpublished observation).

4. Discussion

In this study, eight bands were identified by affinity chromatography and chosen for mass spectrometry analysis, the majority of which were proteins known to associate with actin. However, we were unable to detect bands between ~ 30 and 42 kDa since the partially degraded GST fusion proteins also masked this region of the gel, thus limiting a subset of smaller proteins that may have bound GST-Spino(151–284).

The result that the major proteins isolated were actin-associated proteins was somewhat surprising. We had predicted GST-Spino(151–284) would not bind actin, as this fusion protein only contains three amino acids that overlap the F-actin-binding domain previously defined [15]. In fact, Satoh et al. showed that GST-Spino(155–495) did not bind F-actin in overlay analysis. This negative result may represent a sensitivity of this new ABD to a native conformation within actin or spinophilin that is not maintained in the overlay assay. Both the failure of the overlay assay and the lack of similarity of Spino(164–282) to previously identified actin-binding sites may have excluded this region as a candidate for further study at the time. Our co-sedimentation experiments strengthen the evidence that an additional ABD exists within spinophilin.

The results of transient transfection experiments are consistent with the actin-binding activity already described for aa 1–154. The second ABD described in this paper, amino acids 164–282, also exhibited co-localization with F-actin. However, this was limited to a pool of actin in the cell periphery. It is not clear whether this pattern of actin is induced by the expression of this construct or is merely due to a unique affinity of this fragment for a sub-population of actin.

Further experiments demonstrate that aa 164–282 are not sufficient to co-localize with actin when expressed contiguously with aa 283–817. This result is not surprising given that several lines of evidence indicate that longer constructs of spinophilin or neurabin do not completely co-localize with actin. In vitro, Oliver et al. [12] observed reduced co-sedimentation of actin with aa 103–1095 of neurabin I, while our data (Fig. 1B) indicate that Nrb(100–372) has a similar avidity for actin as Spino(151–284). Together these results suggest that Nrb(100–372) may also be regulated by its more carboxyl regions. This possibility is consistent with the localization pattern of neurabin I (aa 145–1095) in COS7 cells [11] and the incomplete localization of spinophilin with actin seen in work using PC12 cells [22] that is contrasted by the complete overlap seen in the 1–230 construct (Fig. 3D).

The limited spine targeting seen with the second ABD is also informative as to the necessary requirements for the spine targeting of proteins since the spine targeting of inositol 1,4,5-triphosphate 3-kinase and the beta isoform of Ca^{2+}/calmodulin-dependent protein kinase II been shown to be dependent on actin binding [16,18]. However, our results with spinophilin would seem to indicate that while actin binding is required, other characteristics of the actin-binding domain (ABD) may determine the ability of a particular protein to target a subcellular domain since in fibroblasts, ABD2 is still able to associate with F-actin.

Alignment of amino acids 1–281 of rat spinophilin/neurabin II with amino acids 1–283 of rat neurabin I reveals several regions of high homology within the first ~ 220 amino acids (Fig. 1A). Segments of aa 1–230 shared between the two proteins are also found to be conserved in such genera as *Xenopus* and *Fugu* (unpublished observation). This conservation would imply a functional role for these regions in both proteins. It is clear that amino acids 1–154 of spinophilin/neurabin II, initially identified as the F-actin-binding domain, contain enough of the actin-binding domain to bind F-actin by overlay [15]. However, Nakanishi et al. [11] showed for neurabin I that while amino acids 1–144 bound F-actin by overlay, amino acids 1–210 appeared to bind F-actin more avidly. It remains to be determined whether these two F-actin-binding regions of spinophilin represent segments of a single binding site or two independent sites. If the latter is the case, then this would provide a model by which a single molecule of spinophilin could potentially bundle two actin filaments. This activity, in combination with the multimeric nature of spinophilin, quickly illustrates how spinophilin can serve as an actin bundling protein.

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