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Structure-Function Analysis Of SAP97, A Modular Scaffolding Protein That Drives Dendrite Growth

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Abstract

Activation of AMPA receptors assembled with the GluA1 subunit can promote dendrite growth in a manner that depends on its direct binding partner, SAP97. SAP97 is a modular scaffolding protein that has at least seven recognizable protein-protein interaction domains. Several complementary approaches were employed to show that the dendrite branching promoting action of full length SAP97 depends on ligand(s) that bind to the PDZ3 domain. Ligand(s) to PDZ1, PDZ2 and I3 domains also contribute to dendrite growth. The ability of PDZ3 ligand(s) to promote dendrite growth depends on localization at the plasma membrane along with GluA1 and SAP97. These results suggest that the assembly of a multi-protein complex at or near synapses is vital for the translation of AMPA-R activity into dendrite growth.

Keywords

PDZ domain; GluA1; spinal cord neuron

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Introduction

The interaction of a young animal with its environment evokes patterns of synaptic activity within the nervous system that influences the form and function of neurons (Hubel and Wiesel, 1970; Hubel et al., 1977; Wiesel, 1982). The molecular mechanisms by which synaptic activity operates have been well studied when activation of N-methyl-D-aspartate-type glutamate receptors (NMDA-Rs) is the initiating event. This has crystallized into a version of the “synaptotrophic hypothesis” in which transient pre- and post-synaptic contacts (mediated by adhesive molecules (Chen et al., 2010)) are stabilized if they lead to activation of NMDA-Rs (Constantine-Paton, 1990; Cline and Haas, 2008; Chia et al., 2014). Stabilized synapses lead to stabilized neurites while preliminary contacts (and the neurites that bear them) that fail to lead to active NMDA-Rs decay (Ruthazer et al., 2003; Hua and Smith, 2004; Niell et al., 2004; Haas et al., 2006). This form of activity-dependent development has been extensively studied in the sensory systems (most notably the visual system).

An additional form of activity-dependent development is initiated by activation of (2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)) propanoic acid-type glutamate receptors (AMPA-Rs) assembled with the GluA1 subunit. The developing locomotor system has provided a useful platform for mechanistic insight into this form of activity-dependent plasticity. Neonatal motor neurons display calcium-permeable AMPA receptors (Carriedo et al., 1996; Vandenberghe et al., 2000) – consistent with the observation that they express particularly high levels of GluA1 during this period (Jakowec et al., 1995a,b). Elimination of GluA1 leads to stunted motor neuron dendrite growth, deranged segmental spinal cord circuitry and sub-normal motor behavior (Zhang et al., 2008). Overexpression of GluA1 in mature motor neurons leads to remodeling of the dendritic tree with an increase in branch abundance (Inglis et al., 2002). These events occur in an NMDA-R independent manner (Inglis et al., 2002). These observations provide the first clues that GluA1 containing receptors can control activity-dependent plasticity in parallel with the better understood NMDA-R mediated pathway.

The capacity of AMPA-Rs assembled with GluA1 to control the various aspects of motor system development is entirely dependent on its ability to form a native complex with synapse associated protein of 97 kDa molecular weight (SAP97). This has been most convincingly shown by studies of mice with a knock-in allele of GluA1 that lack the C-terminal 7 amino acids (originally designated GluR1 7, now called GluA1 7) (Zhou et al., 2008). AMPA-Rs are processed normally in the GluA1 7 mice, show normal synaptic currents and normal synaptic plasticity (such as NMDA-R-dependent hippocampal plasticity) (Kim et al., 2005; Zhou et al., 2008). Yet motor neuron dendrite growth is aberrant both in vitro and in vivo. These affects can be ascribed to the inability of GluA1 to chaperone SAP97 to synapses because the GluA1 7 phenotype can be completely rescued by membrane targeting SAP97. As would be expected, conditional deletion of SAP97 from motor neurons phenocopies the loss of GluA1 (Zhou et al., 2008). These observations are most parsimoniously explained by a GluA1-mediated form of neuronal plasticity that is driven by a GluA1/SAP97 synaptic complex that does not involve activation of NMDA-Rs.

SAP97 is a scaffolding protein with numerous protein-protein interactions domains (Leonard et al., 1998; Lee et al., 2002). Here we systematically examine the contribution of each domain to the dendrite promoting action of SAP97 and the importance of membrane targeting.

Materials and Methods

Plasmids

The SAP97 related plasmids (Figure 1) employed in this study were the β -isoform (Zhou et al., 2008): FL-SAP97 (full length); SAP97N (from N-terminal to PDZ2 domain); myc-L27 (SAP97 L27 domain); myc-PDZ1 (SAP97 PDZ1 domain); myc-PDZ2 (SAP97 PDZ2 domain); myc-PDZ3 (SAP97 PDZ3 domain); myc-SH3 (SAP97 SH3 domain); myc-I3 (SAP97 I3 domain); myc-GUK (SAP97 GUK domain); FL PDZ3mut SAP97 (full length SAP97 with 2 point mutations in the PDZ3 domain rendering it ligand-binding incompetent); pal-myc-PDZ3 (palmitoylated SAP97 PDZ3 domain), FL-WT SAP97 that resists shRNA knockdown ("res-WT SAP97") and FL-PDZ3 mutant-SAP97 that resists shRNA knockdown ("res-PDZ3mut SAP97") and YFP-tagged SAP97. All the target genes were subcloned into pGW expression vector except YFP-SAP97, which employs a β -actin promoter.

Antibodies

The following primary antibodies were used in all the biochemical and histological studies except for the quantitative synapse studies: Mouse anti-SAP97 (#75-030; UC Davis/NIH NeuroMab Facility); Rabbit anti-SAP97 (PA1-741; ThermoFisher Scientific). Mouse anti-c-MYC (#M5546; Sigma); Rabbit anti-c-MYC (#C3956; Sigma); Rabbit anti-GluA1 (#PC246; Calbiochem and #AB1504; Millipore); Rabbit anti-actin (#A2066; Sigma). Fluorescent conjugated secondary antibodies used in this study include: Alexa Fluor 488-conjugated anti-mouse (#A11001) or anti-rabbit IgG (#A11008); Alexa Fluor 594-conjugated anti-mouse IgG (#A11005) or anti-rabbit IgG (#A11012); Alexa Fluor 680 goat anti-mouse IgG (#A21058) or anti-rabbit IgG (#A21109); IR DYE 800 CW goat anti-mouse (#926-32210) and IR DYE 800 CW goat anti-rabbit (#926-32211). All secondary antibodies are from Life Technologies with the exception of the IR DYE antibodies which come from Li-COR.

Mixed spinal cord neuron cultures

Mixed spinal cord neuron cultures were prepared as described previously (Zhang et al., 2008). Briefly, neocortical astrocyte monolayers were established from the forebrain of Sprague Dawley rats (postnatal day 2). When they were 80% confluent, dissociated embryonic day 15 (E15) spinal cord cells were added. AraC was added later to arrest astrocyte proliferation. The neurons and astrocytes were maintained in the glia-conditioned medium. Half of the medium was replaced twice per week. Growth media was supplemented with 10 ng/ml glial-derived neurotrophic factor, ciliary neurotrophic factor and cardiotropin-1 (all from Alomone labs, catalogue numbers # G-240, # C-245 and # C-200 respectively) as previously described (Jeong et al., 2006).

Transfection of spinal cord neurons and human embryonic kidney 293 (HEK 293) cells

Lipofectamine 2000 (#11668-019, Life Technologies) was used to transfect the constructs into mixed spinal cord neuron cultures according to the manufacturer's recommendations. The constructs were transfected into neurons that had been in culture for 5 days. The constructs were co-transfected with a GFP plasmid at a proportion of 3:1 (construct of interest : GFP) to ensure all GFP positive cells are also transfected with the interested constructs.

HEK 293 cells were grown in Delbecco's Modified Eagle Media (DMEM) with 10 % fetal bovine serum and 1% Penicillin/Streptomycin and transfected at 80% confluence with Lipofectamine 2000 + constructs according to the manufacturer's recommendations.

Membrane protein preparation

Cells were washed twice with cold PBS and harvested in lysis buffer: 10 mM KCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl, pH7.4; 1.5 ml/60mm plate. After incubation on ice for 10 minutes, cell lysis was finalized using a homogenizer (Dounce). The cell lysate was first centrifuged at 2000 g for 2 minutes to remove nuclei and unlysed cells. The collected supernatant was then centrifuged at 100, 000 g for 30 min at 4°C to pellet cell membranes.

Western Blot analysis

Cells were washed twice with cold PBS and harvested in lysis buffer: 50 mM Tris (pH8.0), 10 mM MgCl₂, 15 mM NaCl, 1% Triton X-100, 5% glycerol and 1% protease inhibitor cocktail (#P2714-1BTL; Sigma). Cell lysates were sonicated for 10 seconds and sat on ice for 20 min. Then, cell lysates were centrifuged at 12, 000 g for 20 min at 4°C, supernatants were collected, and the protein concentration determined using the Bradford Assay (BioRad).

Cell lysates were boiled for 3 min in 6X sample buffer: 300 mM Tris-HCl (pH6.8), 12% SDS, 0.6% bromophenol blue, 30% glycerol. Equal amounts of proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane. The membrane was blocked in PBST buffer (0.1% tween 20 in PBS) with 5% non-fat dried milk for one hour at room temperature. Then, the membranes were incubated with different primary antibodies overnight at 4°C. After washing with PBST, the blots were incubated with the secondary antibodies: either Alexa Fluor 680 goat anti-rabbit /anti-mouse IgG or Alexa fluor 800 goat anti-rabbit/anti-mouse IgG. Finally the immunoblots were imaged with Odyssey imaging system from Li-Cor Biosciences.

Immunoprecipitation

Dynabeads Protein G (#10004D; Life Technologies) were pre-cleared and diluted in 200μl PBS. Primary antibody was added and incubated at room temperature for 30 minutes. Dynabeads and primary antibody were isolated (with the magnet), the supernatant was removed, cell lysate was added and incubated for one hour at room temperature. Dynabeads were then washed with PBS and sample buffer was added. After boiling, the precipitated proteins were subjected to SDS-PAGE electrophoresis and immunoblotting.

Knockdown and rescue constructs

The pSUPER RNA interference system was used as previously described (Zhang et al., 2008). The targeted SAP97 sequence is 5'-AAACCCAAATCCATGGAAAATATC-3'. To generate a version of SAP97 that would not be recognized by the pSUPER-based knockdown construct, six silent nucleotide exchanges were introduced in the SAP97 cDNAs (5'-AAACCCAAATCCATGGAAAATATC-3' → 5'-AAGCCGAAGTCGATGGAGAACATC-3'). QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) was employed for mutagenesis. The resulting constructs were called resistant SAP97 ("res-SAP97").

Immunocytochemistry

Transfected cells used for camera lucida drawings were prepared as follows. Cells were fixed with 4% paraformaldehyde and washed with PBS buffer. 5% Goat serum in 0.1% Triton X-100 in PBS was used to permeate and block cells for one hour at room temperature. Then the cells were incubated with primary antibody mouse anti-myc (1:1000) overnight at 4°C. After washing with PBS, the cells were reacted with secondary antibody Alexa Fluor 594 goat anti mouse (1:500) for 2 hours at room temperature. After further washing with PBS, the coverslips were mounted on a slide and imaged.

For GluA1 cell surface staining and analysis, we followed the Malenka Laboratory protocol (Soler-Llavina et al., 2013). Briefly, mixed spinal cord neuron cultures were co-transfected at a 1:3 ratio with GFP expression vector: plasmids expressing individual domains: PDZ1, PDZ2, PDZ3, SH3, I3, GUK and no domain (ensuring that virtually every GFP expressing cells also expressed the individual domain). Five days later, the cells were fixed with 4% paraformaldehyde and blocked with 5% goat serum in PBS for 1 hour. Triton X-100 was not added so as to retain cell membrane integrity and allow staining of endogenous, cell-surface GluA1 immunostaining. After blocking with goat serum, the cells were incubated with primary antibody directed to an extracellular epitope on GluA1 (Calbiochem rabbit anti-GluA1,) overnight at 4°C, and washed then incubated with secondary antibody Alexa Fluor 594 for one hour. After washing, coverslips were mounted with Fluorogel (#17985-10; Electron Microscopy Sciences) and viewed with fluorescent microscopy. Using this technique, bright cell surface staining was present using the Calbiochem rabbit antibody directed against an extracellular epitope (amino acids 271-285 of rat GluA1, numbered from the signal peptide) but not with the Millipore antibody directed against an intracellular epitope (amino acids 895-907 of rat GluA1, numbered from the signal peptide). The images (20 – 25 cells per experimental group) were acquired using an Olympus Fluoview FV300 laser confocal microscope with an oil immersion 40x objective (1.0 numerical aperture) and were acquired at the same non-saturating laser power settings and photomultiplier voltage, gain and offset. Images were analyzed offline using ImagePro software (Media Cybernetics) for quantification of GluA1 and GFP signals. When we examined the range of GFP values for each experimental group we found no statistically significant group differences. We then determined the ratio of GluA1/GFP fluorescence for each individual cell and group comparisons were made using ANOVA.

Imaging and neuron tracing

Cell images were taken using Olympus Fluoview system. Cells were chosen according to the following criteria: the maximum diameter of the cell should be over 20 μm ; the cell must have an axon; and the cells must have at least three primary dendrites. Only processes 4 microns were considered to be dendritic branches; shorter processes that could be confused for filopodia were thus excluded from this analysis. Drawings were made blind to the experimental group. Cells were traced using the Neurolucida program (Jeong et al., 2006). Neuron data were obtained using the Neuroexplorer program. Soma area, number of primary dendrites, number of branches, total dendrite length, the average dendrite length, and the longest dendrite length of neurons were collected as previously described (Zhang et al., 2008).

Statistics

We ran the normality test on all data generated and selected the appropriate statistical tool based on these results. If the data were normally distributed and there were two experimental groups, we ran Student's t-test. If the data were normally distributed and there were 3 experimental groups, the data was analyzed by ANOVA (with the p values reported). Post-hoc analysis employed the Student-Newman-Keuls test and the threshold for significance set at $p < 0.05$. If the data were found to be non-normally distributed and there were two experiment groups, we employed the non-parametric statistical tool, Mann-Whitney Rank Sum tests. If the data were found to be non-normally distributed and there were 3 or more experiment groups, we employed the non-parametric statistical tool Kruskal-Wallis One Way ANOVA on Ranks (with the p values reported). Post-hoc analysis was performed using Dunn's Method and the threshold for significance set at $p < 0.05$. Data are expressed as mean $\pm$ standard error of the mean in all figures.

Results

Development and characterization of the primary screening tools

SAP97 (and its human homologue discs large, hDLG) contains several recognizable protein-protein interaction domains: NH₂ – L27, PDZ1, PDZ2, PDZ3, SH3, 4.1 binding sequence (known as “hook” domain), I3, guanylate kinase (GUK) -like domain – COOH. Our initial goal was to test the hypothesis that specific domains of SAP97 play a critical role in its ability to translate activity of AMPA-R assembled with GluA1 into dendrite growth. A number of approaches to achieve this goal could be imagined and each has limitations owing to *a priori* assumptions. For example, prior work utilized an extensive series of deletion mutants of SAP97 to define domains important for targeting lin-2/CASK to the lateral membrane of epithelial cells (Lee et al., 2002) and synaptogenesis (Regalado et al., 2006). We obtained this collection of deletion mutants, cloned them into a suitable neuronal expression vector and studied the effects on dendritogenesis *in vitro*. When co-transfected with a reporter construct (that expresses green fluorescence protein, GFP), we unexpectedly found that either the SAP97 domain deletion mutants were toxic to neurons, or that the transgene failed to leave the cell body (data not shown). Thus at least in neurons, overexpression of deletion mutants of SAP97 would not be an effective tool to achieve our goal.

We reasoned that heterologous overexpression of individual domains of SAP97 might act as a decoy and prevent the endogenous ligand(s) from associating with the GluA1/SAP97 complex at synapses. For this approach to be effective, there must be: 1) a favorable stoichiometry between the ligand and the endogenous SAP97 domain, 2) the affinity/stability of this endogenous interaction must be amendable to displacement by the exogenously provided decoy domain, 3) the subcellular compartment where endogenous binding partner(s) interact with SAP97 must be accessible to the heterologously expressed decoy domain, 4) to make comparisons valid, each decoy domain would need to be expressed at similar levels, and 5) the domain would need to be widely distributed throughout the dendritic tree of the neuron. A series of experiments were undertaken to determine the viability of this approach as a screening tool with the expectation that follow up experiments would be required to provide validation.

A cartoon depicting the domain structure of SAP97 is provided in Figure 1, panel A. In western blot analysis we found that the abundance of each individual myc-tagged domain was generally similar and comparable to the expression level of full length SAP97 (FL-SAP97) (Figure 1, panel B). To determine the distribution of heterologously expressed domains, neurons in culture were co-transfected with the myc-tagged domain expression plasmid + a GFP reporter and immunostained for the myc-tagged domain. As an example, we found that myc-PDZ3 was widely distributed through the neuron, even into spine-like structures in distal dendrites and this was true for all of the constructs (Figure 1, panel C). We tested each construct used in these experiments and every one showed the same neuron-wide distribution pattern (not shown). We considered the possibility that expression of individual domains might influence the neuronal surface expression of GluA1. Quantitative imaging of cell surface GluA1 staining of neurons expressing domains showed group differences by ANOVA ($p = 0.0026$) (Figure 1, panel D). The post-hoc analysis revealed that there were no differences between cell surface GluA1 expression in the “no domain” neurons versus neurons expressing any SAP97 domain. The only group differences found were between neurons expressing the SH3 domain versus neurons expressing PDZ2 or I3 domains. Here the SH3 domain expressing neurons had significantly more surface GluA1 in comparison with the other two groups. In sum these experiments indicate that individual SAP97 domains are expressed at similar levels in cells and distribute widely in neurons; thus are suitable for determining which portions of SAP97 are involved in promoting dendrite growth. The effects of expressing individual SAP97 domains on dendrite architecture and synapses are unlikely to be linked to changes in cell surface expression of GluA1.

The second PDZ domain of SAP97 represented an opportunity to test the validity of our proposed screening approach. The ability of endogenous GluA1 to promote dendrite growth is specifically dependent on the interaction of the C-terminal 7 amino acids of GluA1 with PDZ2 of SAP97 (Zhou et al., 2008). This was shown *in vitro* (in studies utilizing constructs: WT GluA1, GluA1 lacking its C-terminal 7 amino acids (GluA1⁻⁷), WT SAP97 and SAP97 mutated in PDZ2 so that it was ligand-binding defective) and *in vivo* (using GluA1⁻⁷ knock-in mice and mice with conditional ablation of SAP97 in motor neurons). If heterologous expression of PDZ2 acts as a decoy domain to block the GluA1/SAP97 interaction, this should be demonstrable biochemically and ought to prevent the pro-dendrite

growth effects of SAP97 overexpression. To test these propositions, we expressed GluA1, SAP97 and either of three putative decoy domains: L27, PDZ2 or PDZ3 in HEK 293 cells and half of the lysate was immunoprecipitated (IP) using anti-GluA1 and immunoblotted (IB) for SAP97. Expression of the PDZ2 domain inhibited the association of GluA1 with SAP97 in comparison with cells co-expressing L27 or PDZ3 (Figure 2 panel A upper). The input levels of all proteins and domains were equivalent. Consistent with prior work indicating that the C-terminus of GluA1 associates with PDZ2 of SAP97 (Leonard et al., 1998), our results suggest that expressing the PDZ2 domain acts as a decoy to reduce the physical association of GluA1 with SAP97.

Single-particle electron microscopy experiments demonstrate that SAP97 can exist in either a compact or an extended, rod-like conformation (Nakagawa et al., 2004). These conformations exist in a dynamic equilibrium that can be biased by the association of SAP97 with a binding partner, such as CASK (Lin et al., 2013). The compact conformation is driven by the intramolecular association of the N-terminus of SAP97 with its SH3 and GUK domains and heterologous expression of specific SAP97 domains can disrupt these intramolecular interactions (Wu et al., 2000). These observations raise the possibility that in our experiments expression of a decoy domain (i.e., PDZ2, PDZ3 or L27) binds to compact SAP97. In doing so, it alters SAP97 conformation and leads to the exposure of multiple protein-protein interaction domains. To examine this issue, we took the second half of the lysate from cells co-transfected SAP97 + GluA1 + individual decoy domains, immunoprecipitated SAP97 and immunoblotted for SAP97 or the myc-tagged domain. While SAP97 is present in the immunoprecipitate, myc-tagged domains are absent (Figure 2 panel A lower). In sum, we find no evidence that the three individual myc-tagged domains we studied here bind to SAP97. This suggests that these domains do not act by disrupting SAP97 intramolecular interactions.

Next we asked whether expression of PDZ2 decoy domain influenced dendrite growth in vitro. We transfected neurons with a GFP expression plasmid to delineate the dendritic tree along with the experimental transgenes (Figure 2 panels B and C). Total DNA content was the same in all transfections (using a LacZ expression plasmid, as needed, for normalization). In previous work we limited our quantitative dendrite analysis to four pre-specified criteria as described in the Methods section. However, we note that the distinctions between hairy appendages (long thin protrusions), filopodia and a growing branch may blur, particularly when studying neurons in vitro (Figure 2 D,1 – D,4). Branches, but not filopodia should have a growth cone, although transitional forms are likely to exist. While the extent to which a specific length criteria can distinguish between these emanations is uncertain, we restrict our analysis in camera lucida drawings to branches ≥ 4 microns and that do not qualitatively appear to be hairy appendages. Nonetheless we wondered if restricting our analysis to dendritic branches that were ≥ 10 microns, as opposed to ≥ 4 microns would lead to different conclusions. To this end, we made camera lucida drawings of four experimental groups: 1) GFP, 2) SAP97, 3) PDZ2, and 4) SAP97 + PDZ2 (Figure 2 panels E). We then undertook a statistical analysis of the data including all branches that were at least 4 microns in length (the “branches ≥ 4 microns” group – Figure 2 panel F) and compared this with a statistical analysis of the data including all branches that were at least 10 microns in length (the “branches ≥ 10 microns” group – Figure 2 panel G). Using the “branches ≥ 4 microns”

criteria, compared to GFP alone, SAP97 led to an ~20% increase in dendritic branches and a ~30% increase in total dendritic length. Using the “branches 10 microns” criteria, compared to GFP alone, SAP97 led to an ~40% increase in dendritic branches and a ~20% increase in total dendritic length. Using the “branches 4 microns” criteria, compared to GFP alone, PDZ2 led to an ~35% percent reduction in dendritic branches and a ~25% percent decrease in total dendritic length. Using the “branches 10 microns” criteria, compared to GFP alone, PDZ2 led to an ~25% percent reduction in dendritic branches and a ~25% percent decrease in total dendritic length. All of these observations were statistically significant in *post hoc* analysis (Figure 2 panels F and G). Co-expression of PDZ2 with SAP97 completely occluded the pro-dendrite growth activity of SAP97; there were no differences between the dendrites of neurons expressing PDZ2 versus SAP97 + PDZ2 (using either the “branches 4 microns” or “branches 10 microns” criteria). Several conclusions can be drawn from these results. First, the same basic observations are evident whether we use the “branches 4 microns” or “branches 10 microns” criteria in our data analysis. Since all prior work from this lab employed the “branches 4 microns” criteria (Jeong et al., 2006; Zhou et al., 2008; Zhang et al., 2008), we will employ it here for the sake of consistency. Second, SAP97 promotes dendrite arborization and this effect is unlikely to be accounted for by elaboration of primary dendrites or filopodia, *per se*. None of the data in Figure 2 includes filopodia (c.f. Figure 2 panels D,1 – D,4) and there were no group differences in the primary dendrite measurements. Third, these results suggest that reducing the physical association of GluA1 with SAP97 (by expression of the PDZ2 decoy domain) abrogates the dendrite pro-growth activity evoked by SAP97 overexpression. These findings are entirely consistent with prior work (Zhou et al., 2008) and provide impetus for the experimental approach: expression of individual SAP97 domains can be used to identify critical portions of full length SAP97 that mediate its ability to promote dendrite growth.

In our initial screen we simultaneously overexpressed SAP97 + individual SAP97 domains or only overexpressed SAP97 domains. Neurons overexpressing SAP97 have a substantially larger and more branched dendritic tree (Zhou et al., 2008) than control neurons; potentially making experimental group differences more robust. This advantage must be weighed against the fact that this initial screen is a “gain-of-function” approach. Simply expressing particular SAP97 domain(s) might be expected to reduce dendrite size and branching, but it might be difficult to distinguish a specific effect from a non-specific toxicity. We decided to begin with the gain-of-function approach and used a replacement strategy in the secondary analysis.

We commenced our screen by generating myc-tagged portions (domains alone or coupled to adjoining domains) of SAP97 (graphically displayed with nomenclature in Figure 1, panel A). To determine whether the protein-protein interactions domains in the amino terminus of the protein (up to and including PDZ2, denoted “SAP97N-PDZ2”) play a role in dendrite growth, we compared the following four groups: 1) GFP, 2) SAP97, 3) SAP97N-PDZ2 and 4) SAP97 + SAP97N-PDZ2 (Table 1). Compared with GFP alone, SAP97 led to an ~40% increase in dendrite branches ($p = 0.001$) and no other statistically significant changes in dendrite architecture. SAP97N-PDZ2 had no effect on dendrite structure when expressed on its own, but when co-expressed with SAP97 it completely eliminated the dendrite branch

promoting action of overexpressed SAP97. There were no statistically significant differences in dendritic architecture between the GFP versus SAP97N-PDZ2 + SAP97 groups. These observations suggest that protein(s) that bind to SAP97 between the amino terminus and PDZ2 is/are important for promoting dendrite branching upon overexpression of SAP97.

To explore this idea further, we investigated the relevance of two protein-protein interaction domains in the N-terminus of SAP97: PDZ1 and L27. In the first experiment we compared the following 4 groups: 1) GFP, 2) SAP97, 3) PDZ1, and 4) SAP97 + PDZ1 (Table 2). Compared with GFP alone, FL-SAP97 led to an ~ 40% increase in dendrite branches ($p = 0.001$), and an ~ 30% increase in overall arbor size ($p = 0.012$). Neurons expressing PDZ1 displayed no statistically significant morphological differences from the GFP alone group. When PDZ1 was co-expressed with SAP97 there was complete elimination of the pro-dendrite growth effects of SAP97. There were no statistically significant differences in any dendrite measurement between neurons expressing GFP alone versus PDZ1 + SAP97. In the second experiment we compared the following 4 groups: 1) GFP, 2) SAP97, 3) L27, and 4) SAP97 + L27 (Table 3). Compared with GFP alone, SAP97 led to an ~ 40% increase in dendrite branches ($p < 0.001$), and ~ 30% increase in overall arbor size ($p = 0.002$). Expression of L27 had no demonstrable effect on the neuronal dendritic tree when expressed alone. In addition, there were no statistically significant differences in any dendrite measurement between neurons expressing SAP97 alone versus SAP97 + L27. Together these results suggest that a binding partner(s) of PDZ1, but not L27, contributes to the dendrite growth promoting action of SAP97 overexpression.

Previous work suggested that protein-protein interaction domains C-terminal to PDZ2 could also influence dendrite growth (Zhou et al., 2008). To explore this idea further, we investigated the relevance of four protein-protein interaction domains in the C-terminus of SAP97: PDZ3, SH3, I3 and GUK. In the first experiment we compared the following 4 groups: 1) GFP, 2) SAP97, 3) PDZ3 and 4) SAP97 + PDZ3 (Table 4). Compared with GFP alone, SAP97 led to an ~ 35% increase in dendrite branches ($p < 0.001$), and ~ 10% increase in overall arbor size ($p < 0.001$). Expression of PDZ3 alone had no effects, but it completely blocked the growth promoting action of SAP97 overexpression. There were no statistically significant differences in any dendrite measurement between neurons expressing GFP versus PDZ3 + SAP97.

In the second experiment we compared the following 4 groups: 1) GFP, 2) SAP97, 3) SH3 and 4) SAP97 + SH3 (Table 5). Compared with GFP alone, SAP97 led to an ~ 40% increase in dendrite branches ($p = 0.002$), and no increase in overall arbor size. Expression of SH3 alone had no effect on branches but did cause an ~20% decrease in overall arbor size compared with the SAP97 (but not GFP alone) group ($p = 0.002$). In co-expression studies, SH3 did not block the ability of overexpressed SAP97 to promote dendrite branching. There were no statistically significant differences in any dendrite measurement between neurons expressing SAP97 versus SH3 + SAP97.

In the third experiment we compared the following 4 groups: 1) GFP, 2) SAP97, 3) I3 and 4) SAP97 + I3 (Table 6). Compared with GFP alone, SAP97 led to an ~ 50% increase in

dendrite branches ($p < 0.001$) and an ~25% increase in overall arbor size ($p < 0.001$). Expression of I3 alone led to an ~35% decrease in branches compared with GFP alone expressing neurons ($p < 0.001$). When co-expressed with SAP97, I3 suppressed all of the dendrite growth promoting actions of overexpressed SAP97. There were no statistically significant differences in any dendrite measurement between neurons expressing GFP versus I3 + SAP97.

Finally in the fourth experiment we compared the following 4 groups: 1) GFP, 2) SAP97, 3) GUK and 4) SAP97 + GUK (Table 7). Compared with GFP alone, SAP97 led to an ~50% increase in dendrite branches ($p < 0.001$) and no change in overall arbor size ($p = 0.40$). GUK had no effects on dendrite morphology when expressed alone and in co-expression studies did not block the dendrite growth promoting actions of SAP97 overexpression. There were no statistically significant differences in any dendrite measurement between neurons expressing SAP97 versus GUK + SAP97.

Overall conclusions from this screen are: 1) Overexpression of SAP97 consistently leads to an ~40% increase in branches and a modestly less consistent ~30% increase in overall arbor size. In general, no other measures of dendrite architecture are affected in this *in vitro* model and these observations are entirely consistent with prior *in vivo* experiments (Zhou et al., 2008). The less-than-completely penetrant effect of SAP97 on overall arbor size may be due to technical factors (see Discussion) and 2) Four domains appear to be important for the dendrite-growth promoting actions of overexpressed SAP97; they are PDZ1, PDZ2, PDZ3 and I3. Regarding the possible mechanism by which these domains act, we found no consistent relationship between the ability of a SAP97 domain to occlude the dendrite growth promoting action of SAP97 overexpression and: 1) the expression level of the domain (Figure 1, panel B), or 2) the expression level of neuronal cell surface GluA1 levels (Figure 1, panel D). For the case of PDZ3, we find no evidence for direct binding to SAP97 (Figure 2, western blots). While the mechanism of PDZ3 action seems unlikely to be linked “prying open” a compact conformation of the protein, this has not been conclusively excluded. The remainder of the work reported herein focuses on PDZ3 of SAP97.

Secondary screening; ligand-binding mutation of PDZ3

To consolidate these findings we asked if a version of FL-SAP97 with point mutations in PDZ3 (to render it ligand-binding incompetent) was as effective as wild type FL-SAP97 in promoting dendrite growth. In previous work we found that introduction of K323A, K326A mutations in PDZ2 prevents binding of SAP97 to the extreme C-terminus of GluA1 (Zhou et al., 2008). Here we introduced two mutations into PDZ3 (H469A, R470A) which structural studies indicate will disrupt both the charge-charge interaction with the carboxyl group of the PDZ ligand and the “glycine-leucine-glycine-phenylalanine, GLGF” loop required for formation of the ligand binding pocket (Doyle et al., 1996; Zhang and Wang, 2003), personal communication, Mingjie Zhang) and called the construct PDZ3mut SAP97. Both constructs express at comparable levels (Figure 1 panel E). We compared the following 3 groups: 1) GFP, 2) SAP97, and 3) PDZ3mut SAP97 (Table 8). Compared with GFP alone, SAP97 led to an ~20% increase in dendrite branches ($p = 0.036$), an ~35% increase in overall arbor size ($p < 0.001$), an ~25% increase in the average dendrite length ($p < 0.001$),

and an ~ 30% increase in the longest dendrite ($p < 0.001$). Interestingly expression of PDZ3mut SAP97 had no effect on branching (indistinguishable from GFP expressing neurons). On the other hand, neurons expressing PDZ3mut SAP97 had an ~ 25% increase in total dendrites in comparison with GFP expressing neurons ($p = 0.001$). Average dendrite length (~40%) and longest dendrite (~35%) were also statistically significantly greater in the PDZ3mut SAP97 in comparison with GFP expressing cells. These results reinforce the idea that *ligand(s)* of the third PDZ3 domain of SAP97 are important for dendrite branch promotion.

Secondary screening; replacement strategy

All of the screening studies undertaken so far involve SAP97 over expression. We selected this approach because neurons overexpressing SAP97 have a substantially larger and more branched dendritic tree than control neurons and thus more robustly reveal group differences. A secondary screen strategy was devised involving the knockdown of endogenous SAP97 using RNA interference (RNAi) and re-expression of an RNAi resistant version of SAP97 (res-SAP97) that is either wild type or mutant in PDZ3. We generated shRNA to SAP97 and res-SAP97, expressed them in HEK 293 cells and by western blot analysis find that the abundance of WT SAP97 (the non-RNAi resistant form) is reduced by the shRNA to SAP97 (but not a scrambled “scr” shRNA control) (Figure 3, panel Ai). The RNAi resistant version of WT SAP97 is expressed at an equivalent level of the non-RNAi resistant version of SAP97 and there was no knock down of res-SAP97 by shRNA to SAP97. The same results were obtained with the PDZ3 mutant version of SAP97. That is, the shRNA to SAP97 only knock down expression of the non-resistant version of the construct (Figure 3, panel Aii). When active or control RNAi constructs were engineered into recombinant HSV and expressed in spinal cord neuron cultures, we found specific knockdown of SAP97 (Figure 3, panel Aiii). These results show that we can biochemically replace cellular SAP97 with a “designer” SAP97 and this can be accomplished in our neuronal culture system.

In our first cellular test of this replacement strategy we compared 4 experimental groups: 1) GFP, 2) pSUPER SAP97, 3) res-WT SAP97, and 4) pSUPER SAP97 + res-WT SAP97 (Figure 3, panel B). Compared with the GFP control, knock down of SAP97 with pSUPER SAP97 led to an ~ 20% reduction in primary dendrites ($p = 0.017$), ~ 30% reduction in branches ($p = 0.002$) and an ~20% reduction in overall arbor size ($p < 0.001$). Compared with the GFP control, over expression of SAP97 (res-WT SAP97) led to an ~ 20% increases in branches and overall arbor size (both statistically significant by *post hoc* analysis). Remarkably, when the GFP only expressing neurons were compared to pSUPER SAP97 + res-WT SAP97 expressing neurons, there were no statistically significant differences in any quantitative measure of the dendritic tree. These results indicate that it is possible to knockdown endogenous SAP97 and replace it with an exogenously provided SAP97 construct that restores the native activity but does not lead to a gain-of-function dendrite phenotype (i.e. stimulation of dendrite growth over baseline). This is strong evidence that the replacement strategy can be a viable approach for determining the dendrite growth activity of specific domains of endogenous SAP97.

In the next set of experiments we compared the following 4 groups: 1) GFP, 2) pSUPER SAP97, 3) pSUPER + res-WT SAP97 and 4) pSUPER + res-PDZ3mut SAP97 (Figure 3, panel C). Consistent with the prior experiment, compared with GFP, knockdown of SAP97 led to an ~ 30% reduction in primary dendrites ($p < 0.001$), ~ 35% reduction in branches ($p < 0.001$) and an ~ 15% reduction in overall arbor size ($p = 0.008$). Combining res-WT SAP97 with pSUPER SAP97 knockdown, completely reverted this phenotype back to control levels. However, replacement with the version of SAP97 that is mutated in PDZ3 leads to a different outcome. In comparison to GFP, neurons expressing pSUPER + res-PDZ3mut SAP97 have an ~ 25% reduction in primary dendrites and ~35% reduction in branches (both statistically significant by *post hoc* analysis). There are no other differences between these two groups in any other measure of dendritic architecture. The limited defects in dendritic architecture engendered by replacing WT SAP97 with the version of SAP97 with inactivating point mutations in PDZ3 lead to the following conclusions: 1) the PDZ3 mutant version of SAP97 is unlikely to be toxic to neurons since this protein has very specific effects on dendritic architecture, 2) results garnered by the over expression paradigm can be informative about the native situation when with appropriate following up experiments are performed, and 3) both the overexpression and replacement experiment approaches lead to the same basic result that the ligand(s) of PDZ3 appear to play a specific role in promoting dendrite branching.

Cellular mechanism of PDZ3 action to promote dendrite growth is linked to plasma membrane localization and relation to other SAP97 domains

In previous work we found that overexpression of GluA1 with SAP97 led to a synergistic enhancement of dendrite branching and overall growth (Zhou et al., 2008). The synergism was blocked by disrupting the physical association of GluA1 with SAP97 (either by elimination of the C-terminal 7 amino acids of GluA1 or mutating PDZ2 of SAP97 (K323A, K326A)). Targeting SAP97 to the plasma membrane by addition of a palmitoylation sequence rescued the synergistic growth phenotype. These results suggest that the synergism of GluA1 and SAP97 on dendrite growth does not strictly require a physical interaction – it is manifest as long as SAP97 is co-localized with GluA1 at the plasma membrane. In light of our observations with PDZ3mut SAP97, we wondered if co-expression of palmitoylated PDZ3 would rescue the dendrite growth effects of PDZ3mut SAP97. We began by adding a 9 amino acid motif from paralemnin that directs palmitoylation onto the N-terminus of PDZ3 (palPDZ3). HEK293 cells were transfected with palPDZ3 or PDZ3, lysed in hypotonic buffer and membrane and cytosolic fractions immunoblotted for the myc tag. Construct expression levels in the whole lysate were comparable; palPDZ3 partitioned with the membrane fraction and PDZ3 preferentially was found in the cytosolic fraction (Figure 4 panel A). Thus the chimeric protein behaved as designed.

We used these tools in the following 3 groups: 1) GFP, 2) PDZ3mut SAP97 and 3) PDZ3mut SAP97 + palPDZ3 (Figure 4 panels B and C). While PDZ3mut SAP97 had no effect on dendrite branching, co expression with palPDZ3 led to an ~20% increase in branches ($p = 0.027$). As seen above (Table 8), PDZ3mut SAP97 led to an ~20% increase in overall dendrite tree size (in comparison with GFP alone), and co-expression with palPDZ3 increased overall dendrite tree size an additional ~10% ($p < 0.001$). Also, PDZ3mut SAP97

led to an ~33% increase in average dendrite length (in comparison with GFP alone), and co-expression of palPDZ3 had minimal additional effects ($p < 0.001$). Overall these observations are consistent with the notion that a protein or proteins that bind PDZ3 of SAP97 play a specific role in dendrite branch elaboration and that the PDZ3 ligand(s) need only be co-localized with SAP97 and GluA1 at the plasma membrane to trigger the branching phenotype.

This last set of observations led us to wonder if the dendrite branching promoting actions of membrane targeted PDZ3 were dependent on the remainder of SAP97. That is, could palPDZ3 promote dendrite branching if SAP97 was eliminated from cells, or did palPDZ3 require the presence of SAP97 at the membrane? To address this issue we used an shRNA construct that specifically knocked down SAP97 ("pSUPER-SAP97"). As shown in Figure 3, this construct, but not an shRNA with a scrambled sequence ("pSUPER-scr"), specifically reduced the abundance of SAP97 in our cultures (Zhou et al., 2008). In addition we note that reducing SAP97 with pSUPER-SAP97 does not alter the expression level of its direct binding partner, GluA1 (Figure 5, panel A). We used these tools to compare the following four groups: 1) GFP + pSUPER-scr, 2) pSUPER-SAP97, 3) pSUPER scr + palPDZ3 and 4) pSUPER-SAP97 + palPDZ3 (Figure 5, panel B). As previously found, knockdown of SAP97 leads to an ~ 30% reduction in branches and here we show that expression of palPDZ3 completely rescues this phenotype ($p = 0.006$; e.g. there was no statistically significant difference in branch number between pSUPER SAP97 + palPDZ3 versus pSUPER scr). Interestingly, overexpression of palPDZ3 did not stimulate dendrite branching (over baseline) in neurons with reduced levels of SAP97 (e.g., there was no statistically significant difference in branch number between pSUPER-scr + palPDZ3 versus pSUPER-SAP97 + palPDZ3). These observations indicate that simply targeting PDZ3 of SAP97 to the membrane will rescue the dendrite branching defect seen in neurons with reduced levels of SAP97. Additional dendrite branch promoting activity of membrane targeted PDZ3 (over baseline) is restrained by other factors that are supplied when SAP97 is overexpressed (cf., Figure 3, panel C.).

GluA1 is required for the dendrite growth promoting actions of SAP97

Previously we showed that GluA1 chaperones SAP97 into synapses and if SAP97 does not reach the synapse (or at least the plasma membrane), overexpressed GluA1 loses all of its ability to stimulate dendrite growth (Zhou et al., 2008). Is the dendrite growth promoting action of SAP97 simply tied to the ability of GluA1 to chaperone this protein to the cell surface, or is some other property of GluA1 needed for the dendrite growth promoting action of membrane-targeted SAP97? We tackled this issue in two sets of experiments. In both sets of experiments we used a pSUPER construct that we have previously shown leads to a specific knockdown of GluA1 expression in neurons (Zhang et al., 2008). We re-confirmed this in a heterologous expression system. pSUPER-GluA1 reduced the abundance of GluA1 but not GluA2 or SAP97, when expressed in HEK293 cells, while pSUPER-scr had no effect on expression of GluA1, GluA2 or SAP97 (Figure 6, panel A). Next, we compared the following four groups: 1) GFP + pSUPER-scr, 2) pSUPER-GluA1, 3) pSUPER-scr + SAP97 and pSUPER-GluA1 + SAP97 (Figure 6, panel B). In comparison with neurons expressing the inactive pSUPER-scr construct, knockdown of GluA1 led to an ~ 25%

reduction in dendritic branches ($p = 0.007$) and an $\sim 25\%$ reduction overall dendritic tree size ($p < 0.001$). There was also a 15% reduction in primary dendrite branches ($p < 0.001$). This is in accord with our previous observations on GluA1 knockdown on dendrite growth (Zhang et al., 2008). GluA1 knockdown inhibited all of the dendrite growth promoting actions of SAP97 overexpression (there were no statistically significant differences between pSUPER-scr versus SAP97 + pSUPER-GluA1). These observations indicate that endogenous GluA1 is required for the dendrite growth promoting action of overexpressed SAP97.

Since SAP97 does not associate with membrane in the absence of GluA1, perturbation of subcellular localization of SAP97 may be the mechanism of epistasis. If so then membrane targeting of SAP97 in cells with GluA1 knockdown would be expected to promote dendrite growth. To test this idea compared three groups: 1) GFP, 2) pSUPER-GluA1 + SAP97 and 3) pSUPER-GluA1 + palSAP97 (Figure 6, panel C). In comparison with neurons expressing GFP, neurons with GluA1 knockdown and SAP97 overexpression, there was an $\sim 30\%$ reduction in branches ($p = 0.05$) and an $\sim 20\%$ reduction in overall tree size ($p = 0.03$). There was also an $\sim 25\%$ reduction in primary dendrite branches ($p = 0.001$). Interestingly there were no statistically significant differences in dendrite structure between groups of neurons with GluA1 knockdown that overexpress SAP97 or palSAP97. Thus even if SAP97 is targeted to the plasma membrane it loses its ability to stimulate dendrite growth in the absence of endogenous GluA1. This argues that in addition to bringing SAP97 to the plasma membrane, GluA1 has another biological activity that allows SAP97 to promote dendrite growth. Prior work strongly suggests that calcium permeation by AMPA-R assembled with GluA1 is the critical element (Jeong et al., 2006).

Discussion

The translation of synaptic activity into changes in dendrite architecture is a fundamental property of the developing, as well as mature, nervous system. These alterations in dendrite structure impact information processing in neural circuits and are reflected in organismal behavior (Stuart, 1999). Here we have begun the inquiry into the mechanism by which the GluA1/SAP97 complex operates in spinal cord neurons. We identify four domains (PDZ1, PDZ2, PDZ3 and I3) that are important for the ability of SAP97 to stimulate dendrite growth. We provide evidence that the ligand(s) for PDZ3, in particular, plays a special role in dendrite branching and this does not appear to be due to an intramolecular interaction. Our data suggest that SAP97 PDZ3 brings to the plasma membrane a key ligand(s) upon which the remainder of SAP97/GluA1 complex can act to foster dendrite growth. Together these findings indicate that a multi-component molecular machine, nucleated by the GluA1/SAP97 complex drives activity-dependent neuronal growth.

To understand the role of SAP97 in shaping neuronal architecture, we manipulated its expression level or replacing the wild version with a ligand binding incompetent version (here and in (Zhou et al., 2008)). The changes in SAP97 expression levels we induced may exceed what occurs under native conditions and thus may not perfectly report on the physiological operation of SAP97. At present there is no quantitative information on how much of the total pool of SAP97 occupies the critical niche at synapses/plasma membrane.

Nonetheless a recent fluorescence recovery after photobleaching (FRAP) study with GFP-tagged α - and β -isoform SAP97 showed that β SAP97 has a highly dynamic relationship with the postsynaptic density (Waites et al., 2009). One possibility is that synaptic activity increases the association of the kinetically mobile pool of SAP97 with GluA1. This would serve to localize the dendrite growth promoting machinery at the trigger site – active AMPA receptors assembled with the GluA1 subunit.

The values reported in Figures 2, 3, 4, 5 and 6 and Tables 1 – 8 show the natural variation that occurs when experiments are performed over a nine-year period. Although all the cultures were prepared by J. M.-P. and all of the drawings were made by L. Z., there are numerous sources of variability including: 1) batches of serum employed in tissue culture, 2) minor differences in the quality of each preparation of cells and their plating density, and 3) biological differences inherent in dams, pups and embryos. While these considerations must be kept in mind when comparing between experiments, the within-experiment data are less variable. This can be attributed to the fact that all comparison groups within an individual experiment were generated at the same time.

Reviewing the results of the decoy domain experiments raised a question: why does the expression of only some decoy domains *alone* appear to restrain dendrite growth (e.g., I3 or PDZ2 on branching and SH3 on longest dendrite)? Or put another way, why don't all of the decoy domains, when expressed in isolation, act in a “dominant-negative” manner? In a simplified schema, we assume that each decoy domain acts to specifically interfere (by competition) with the exclusive binding a single protein (“the ligand”) with the corresponding domain of endogenous SAP97. In a first scenario consider that the endogenous ligand is present at about the same abundance as endogenous SAP97. In this circumstance, the decoy domain overexpression could effectively deplete the ligand from interacting with endogenous SAP97. In a second scenario, consider that the endogenous ligand is present in excess of endogenous SAP97. In this circumstance, the decoy domain overexpression could not effectively deplete the ligand from endogenous SAP97 and thus overexpression of the decoy domain would not influence on dendrite growth (i.e. act in a dominant negative manner). In a third scenario consider that both SAP97 and the decoy domain are overexpressed and the endogenous ligand is present at about the same abundance as the summed number of binding sites. In this circumstance one would expect the decoy domain to inhibit the dendrite growth promoting action of overexpressed SAP97. Although we have no information about the stoichiometry of all the GluA1/SAP97 complex binding partners, we believe that differences in the abundance of the various relevant proteins could account our data. Other factors such as binding affinity, allostery, and subcellular ligand distribution are likely also to be in play and complicate the picture.

If we knew the specific binding partner of PDZ3 of SAP97 (for example), we would be in a better position to tease apart the above raised issues. This is, however, a daunting task for several reasons. First, there are at least 26 known SAP97 binding partners of which at least 19 are known to be expressed in the developing spinal cord (search - www.brain-map.org and www.stjudebgem.org). To screen these molecules in overexpression and knockdown paradigms for effects on dendrite growth will be time and effort consuming. Second, the GluA1/SAP97 may nucleate a supramolecular complex composed of many molecules and

without knowing the identity of all the members, the functional importance of a single protein may be unknowable. Third, a protein may have actions as part of the GluA1/SAP97 supramolecular complex as well as actions independent of GluA1/SAP97. Even if these actions on dendrite growth were synergistic, determining the relative contribution of a single protein to each pathway may be arcane. Fourth, overexpression or general membrane targeting (for example by introducing a palmitoylation sequence) may have non-physiological actions. While understanding the molecular mechanism by which the GluA1/SAP97 complex drives dendrite growth will depend, in part, on identifying all the binding partners, the next steps forward are not trivial.

PDZ-domain proteins of the post-synaptic density (MAGUKs) have a key role in clustering and trafficking of glutamate receptors and remodeling of spine cytoskeleton (Feng and Zhang, 2009). Knock out mouse and shRNA knockdown experiments have shown they have a remarkable ability to compensate for each other (Migaud et al., 1998; Elias et al., 2006; Schluter et al., 2006; Howard et al., 2010). In this regard it is interesting that although spinal cord neurons express other MAGUK proteins (PSD-93, PSD-95 and SAP102), none of them can compensate for the dendrite morphogenesis effects of the loss of SAP97 (Zhou et al., 2008). Thus SAP97 has a unique ability to translate the activity of AMPA receptors containing GluA1 into dendrite growth. Further inquiry into this property is merited.

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Highlights

- Study domains of SAP97 that promote dendrite growth.
- Identify PDZ3 domain of SAP97 as branch promoting.
- Show GluA1 is required for SAP97's activity.

Figure 1. Domain Structure of SAP97 constructs, expression levels, cellular distribution and effects on neuronal cell surface GluA1 abundance

Panel A. A cartoon depiction of recognizable protein-protein interaction domains of full length SAP97 (FL-SAP97) and the various truncation and point mutants employed in this study. Panel B. Western blots of various constructs expressed in HEK 293 cells. There are minor variations in the expression levels of individual constructs but no correlation between expression level and biological effects on dendrite growth. Panel C. Immunocytochemical localization of myc tagged PDZ3 shows that the protein is widely distributed throughout neuronal processes. GFP was used to delineate neuronal architecture and the myc-PDZ3 was imaged in the red channel. The merge image shows perfect co-localization. Calibration bar =

25 μ m. Panel D. Quantification of cell surface GluA1 immunoreactivity in neurons expressing individual domains. The reported values are a ratio of GluA1 fluorescence to GFP (the co-transfection marker). In comparison with no domain controls, there were few differences in surface GluA1 abundance. Neurons expressing the SH3 domain had significantly more surface GluA1 in comparison with neurons expressing the PDZ2 or the I3 domain. It is noteworthy that expression of the SH3 domain was associated with more GluA1 expression but had no effect on dendrite growth (see table 5). Panel E. Expression levels of FL WT SAP97 and FL PDZ3 mutant SAP97 are very similar as are actin levels.

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Figure 2. PDZ2 reduces the association of GluA1 with SAP97 although it (and the L27 and PDZ3 domains) does not associate directly with SAP97; the PDZ2 domain reduces dendrite growth

Panel A. After co-transfection of HEK 293 cells with GluA1 + SAP97 + either L27 or PDZ3 or PDZ2, cell lysates were prepared; half of the material was immunoprecipitated (IP'ed) with anti GluA1 and half was IP'ed with anti-SAP97. Aliquots from the initial lysate were reserved for input detection. In the upper panel, expression of PDZ2, but not PDZ3 or L27, reduces the abundance of the GluA1/SAP97 complex (IP GluA1, immunoblot (IB) SAP97). In the lower panel, SAP97 is found in the SAP97 immunoprecipitate (IB SAP97) but none of the domains are found in this material. Input blots show that the initial amount of all the

proteins used in these experiments were virtually identical. Panel B. A single GFP labeled neuron, typical of cells studied in this work. All the labeled processes are dendrites with the exception of axon emanating from the cell body at the 4 o'clock orientation and denoted by the “^”. Calibration bar = 25 microns. Panel C. A camera lucida drawing of the neuron in Panel A. Panel D,1. Enlargement of the distal portion of dendrite outlined in a rectangle in Panel B. (the boxed region has been rotated ~ 20° clockwise). In addition to two branch points, several thin hairy appendages are visible. Calibration bar = 6 microns. Panel D,2. An outline of the dendrite in panel D,1 super imposed on the GFP labeled dendrite. Panel D,3. The outline in Panel D,2 has been moved onto a white background to better visualize the dendrite, two branch points, a dendritic branch of approximately 20 microns length and hairy appendages. Panel D,4. The camera lucida rendering of the dendrite outlined in Panels D,2 and D,3. Only two branch points are drawn; hairy appendages are not included in the final stick figure used in the quantitative dendrite analysis Panel E. Representative camera lucida drawings of neurons expressing GFP alone or with various other constructs as labeled. SAP97 overexpression promotes dendrite growth and branching and this is blocked by co-expression of PDZ2. Calibration bar = 25 μ m. Panel F. Quantitative dendrite analysis using the “branches 4 micron criteria” indicates that PDZ2 blocks endogenous dendrite growth as well as the pro-dendrite growth action of SAP97. The “n” values in the first row indicate the number of individual neurons subjected to camera lucida drawing. Panel G. Quantitative dendrite analysis using the “branches 10 micron criteria” – the absolute values differ between the data in Panels F and G, the underlying observations match well.

Figure 3. RNA interference knockdown of WT but not resistant (“res”) versions of WT and PDZ3 mutant versions of full length SAP97; effects on dendrite growth

Panel Ai. Expression plasmids for WT or res full length SAP97 was co-transfected in HEK 293 cells with pSUPER construct that targeted SAP97 (pSUPER-SAP97) or a scrambled control (pSUPER scr). By western blot, pSUPER-SAP97 knocked down WT SAP97 but not res-SAP97. None of these constructs were toxic as evidenced by equivalent levels of actin. Panel Aii. Expression plasmids for WT or res full length PDZ3 mutant SAP97 was co-transfected in HEK 293 cells with pSUPER construct that targeted SAP97 (pSUPER-SAP97) or a scrambled control (pSUPER scr). By western blot, pSUPER-SAP97 knocked down WT PDZ3 mutant SAP97 but not res-PDZ3 mutant SAP97. None of these constructs were toxic as evidenced by equivalent levels of actin. Panel Aiii. HSV was engineered to express a control (“scr” for scrambled) or active (“SAP97 RNAi”) constructs and applied to

spinal cord cultures. By western blot, the active RNAi construct reduced the abundance of SAP97 in comparison with the control. Neither construct was toxic as evidenced by equivalent levels of actin. Panel B. Quantitative dendrite analysis indicate that knockdown of SAP97 stunts dendrite growth and there is rescue to normal growth (that is, equivalent to expression of GFP alone) when knockdown is accompanied by expression of RNAi resistant WT SAP97. Panel C. Quantitative dendrite analysis indicate that rescue of dendrite growth abnormalities evoked by knockdown of SAP97 occurs with expression of RNAi resistant WT SAP97 but not PDZ3mut SAP97.

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Figure 4. PDZ3 binding mutant SAP97 (PDZ3mut SAP97) does not stimulate dendrite branching; rescue by co-expression of palmitoylated PDZ3 (palPDZ3)

Panel A shows immunoblots for myc tagged palmitoylated and non-palmitoylated PDZ3 (palPDZ3 and PDZ3, respectively). Membranes were isolated from transfected cells by hypotonic lysis and centrifugation. Palmitoylated PDZ3 preferentially associates with membranes while non-palmitoylated PDZ3 is enriched in cytosolic fractions. Panel B shows representative camera lucida images of neurons expressing the noted constructs. Overexpression of PDZ3mut SAP97 has no effect on dendrite branching (in comparison with GFP only expressing neurons). Co-expression of PDZ3mut SAP97 with palPDZ3 rescues the dendrite growth promoting action of SAP97. Calibration bar = 25 μm . Panel C. There is a statistically significant increase in dendritic tree size and branching in neurons overexpressing PDZ3mut SAP97 + palPDZ3.

Figure 5. Simply localizing PDZ3 to the plasma membrane rescues the loss of SAP97 phenotype

Panel A. Western blots showing that an active pSUPER RNAi construct knocks down SAP97 but not GluA1. Panel B. Quantitative dendrite analysis indicates that endogenous SAP97 provides a limiting amount of substrate for palPDZ3 to operate upon.

A.

B.

Cell Parameter	pSUPER-scr (n=23)	pSUPER-GluA1 (n=23)	pSUPER-scr + SAP97 (n=23)	pSUPER-GluA1 + SAP97 (n=23)	p
Soma area (μm^2)	275 $\pm$ 12 ^a	271 $\pm$ 16	274 $\pm$ 10	255 $\pm$ 12	0.529
1 st dendrites, #	6.6 $\pm$ 0.3	5.2 $\pm$ 0.3*	6.6 $\pm$ 0.3 [†]	5.3 $\pm$ 0.2 ^{††}	< 0.001
Branches, #	12.9 $\pm$ 1.1	9.7 $\pm$ 1.0*	14.3 $\pm$ 1.0 ^{*†}	10.1 $\pm$ 0.8 ^{*†}	0.007
Σ length (μm)	1021 $\pm$ 38	774 $\pm$ 45*	1211 $\pm$ 72 ^{*†}	798 $\pm$ 31 ^{*†}	< 0.001
Average dendrites (μm)	164 $\pm$ 11	158 $\pm$ 12	195 $\pm$ 16	159 $\pm$ 10	0.381
Longest dendrite (μm)	170 $\pm$ 10	168 $\pm$ 9.8	198 $\pm$ 12	168 $\pm$ 9	0.168

^aData shown as mean $\pm$ SEM

*Differs from pSUPER-scr group, *p<0.05

[†]Differs from pSUPER-GluA1 group, [†]p<0.05^{††}Differs from pSUPER-scr+ SAP97 group, ^{††}p<0.05

C.

Cell Parameter	GFP (n = 25)	pSUPER-GluA1 + SAP97 (n = 25)	pSUPER-GluA1 + palSAP97 (n = 25)	p
Soma area (μm^2)	262 $\pm$ 8 ^a	271 $\pm$ 10	277 $\pm$ 11	0.572
1 st dendrites, #	7.1 $\pm$ 0.3	5.6 $\pm$ 0.3*	6.1 $\pm$ 0.3	0.001
Branches, #	14.2 $\pm$ 1.2	11.1 $\pm$ 0.6	12.4 $\pm$ 1.0	0.130
Σ length (μm)	1057 $\pm$ 62	869 $\pm$ 42*	896 $\pm$ 30*	0.032
Average dendrites (μm)	155 $\pm$ 12	161 $\pm$ 10	153 $\pm$ 8	0.598
Longest dendrite (μm)	182 $\pm$ 9	169 $\pm$ 9	162 $\pm$ 9	0.104

^aData shown as mean $\pm$ MEM

*Differs from GFP group, *p<0.05

Figure 6. GluA1 is required for the dendrite growth promoting effects of SAP97

Panel A. Western blots showing that an active pSUPER RNAi construct knocks down GluA1 but not SAP97 or GluA2. Panel B. Quantitative dendrite analysis indicates that knockdown of GluA1 reduces dendritic branching and overall tree size compared to controls and prevents dendrite growth evoked by overexpression of SAP97. Panel C. Quantitative dendrite analysis indicates that GluA1 is required for SAP97-dependent dendrite growth both because it localizes SAP97 to synapses and because it provides agency. The nature of this second activity is likely to be linked to calcium permeability (Jeong et al., 2006).

Table 1

Blockade of the pro-dendrite growth action of SAP97 by N-PDZ2 decoy domain.

Cell Parameter	GFP (n=21)	SAP97 (n=22)	SAP97N-PDZ2 (n=20)	SAP97N-PDZ2 + SAP97 (n=21)	<i>p</i>
Soma area (μm^2)	254 $\pm$ 12	233 $\pm$ 13	233 $\pm$ 13	246 $\pm$ 9.0	0.273
1 ⁰ dendrites, #	6.4 $\pm$ 0.2	6.5 $\pm$ 0.3	6.3 $\pm$ 0.3	6.2 $\pm$ 0.2	0.935
Branches, #	13.6 $\pm$ 1.3	18.7 $\pm$ 1.1 [*]	13.7 $\pm$ 0.7 [†]	14.1 $\pm$ 0.9 [†]	0.001
length (μm)	1144 $\pm$ 82	1271 $\pm$ 66	1069 $\pm$ 67	1210 $\pm$ 58	0.033
Average dendrites (μm)	185 $\pm$ 16	196 $\pm$ 8	177 $\pm$ 15	200 $\pm$ 12	0.112
Longest dendrite (μm)	209 $\pm$ 17	222 $\pm$ 21	193 $\pm$ 13	196 $\pm$ 10	0.850

Data shown as mean $\pm$ SEM^{*} Differs from GFP group, $p < 0.05$ [†] Differs from SAP97 group, $p < 0.05$

Table 2

Blockade of the pro-dendrite growth action of SAP97 by PDZ1 decoy domain.

Cell Parameter	GFP (n=23)	SAP97 (n=23)	PDZ1 (n=23)	PDZ1 + SAP97 (n=22)	<i>p</i>
Soma area (μm^2)	218 $\pm$ 8	240 $\pm$ 13	231 $\pm$ 11	247 $\pm$ 14	0.546
1 ⁰ dendrites, #	6.4 $\pm$ 0.3	6.8 $\pm$ 0.3	6.3 $\pm$ 0.2	7.1 $\pm$ 0.2	0.112
Branches, #	15.0 $\pm$ 0.7	20.8 $\pm$ 1.2 [*]	16.3 $\pm$ 1.0 [†]	16.6 $\pm$ 0.9 [†]	0.001
length (μm)	1193 $\pm$ 45	1565 $\pm$ 89 [*]	1277 $\pm$ 80 [†]	1287 $\pm$ 62 [†]	0.012
Average dendrites (μm)	195 $\pm$ 12	240 $\pm$ 18	210 $\pm$ 17	187 $\pm$ 13	0.207
Longest dendrite (μm)	210 $\pm$ 8	230 $\pm$ 12	219 $\pm$ 12	213 $\pm$ 15	0.640

Data shown as mean $\pm$ SEM^{*} Differs from GFP group, $p < 0.05$ [†] Differs from SAP97 group, $p < 0.05$

Table 3

No blockade of the pro-dendrite growth action of SAP97 by L27 decoy domain.

Cell Parameter	GFP (n=25)	SAP97 (n=24)	L27 (n=20)	L27 +SAP97 (n=20)	<i>p</i>
Soma area (μm^2)	220 $\pm$ 8	241 $\pm$ 12	240 $\pm$ 10	253 $\pm$ 14	0.261
1 ⁰ dendrites, #	6.5 $\pm$ 0.3	6.9 $\pm$ 0.3	7.0 $\pm$ 0.4	6.8 $\pm$ 0.3	0.803
Branches, #	15.3 $\pm$ 0.8	20.8 $\pm$ 1.2 [*]	14.6 $\pm$ 0.8 [†]	19.6 $\pm$ 1.3 ^{*†}	<0.001
length (μm)	1221 $\pm$ 50	1569 $\pm$ 86 [*]	1399 $\pm$ 88	1606 $\pm$ 95 [*]	0.002
Average dendrites (μm)	196 $\pm$ 11	239 $\pm$ 18	211 $\pm$ 16	250 $\pm$ 21	0.221
Longest dendrite (μm)	213 $\pm$ 9	229 $\pm$ 11	213 $\pm$ 10	238 $\pm$ 15	0.335

Data shown as mean $\pm$ SEM

* Differs from GFP group, $p < 0.05$

[†] Differs from SAP97 group, $p < 0.05$

[!] Differs from L27 group, $p < 0.05$

Table 4

Blockade of the pro-dendrite growth action of SAP97 by PDZ3 decoy domain.

Cell Parameter	GFP (n=34)	SAP97 (n=35)	PDZ3 (n=34)	PDZ3 + SAP97 (n=35)	<i>p</i>
Soma area (μm^2)	179 $\pm$ 8	183 $\pm$ 6	187 $\pm$ 6	175 $\pm$ 5	0.336
1 ⁰ dendrites, #	4.1 $\pm$ 0.2	4.4 $\pm$ 0.2	3.9 $\pm$ 0.1	4.1 $\pm$ 0.2	0.517
Branches, #	14.5 $\pm$ 0.8	19.7 $\pm$ 1.2 [*]	12.8 $\pm$ 1.0 [†]	13.8 $\pm$ 0.9 [†]	<0.0001
length (μm)	950 $\pm$ 60	1153 $\pm$ 52 [*]	938 $\pm$ 51 [†]	853 $\pm$ 42 [†]	<0.0001
Average dendrites (μm)	240 $\pm$ 15	281 $\pm$ 17	250 $\pm$ 16	217 $\pm$ 12	0.051
Longest dendrite (μm)	206 $\pm$ 10	202 $\pm$ 11	207 $\pm$ 11	187 $\pm$ 9	0.493

Data shown as mean $\pm$ SEM^{*} Differs from GFP group, $p < 0.05$ [†] Differs from SAP97 group, $p < 0.05$

Table 5

No blockade of the pro-dendrite growth action of SAP97 by SH3 decoy domain.

Cell Parameter	GFP (n=35)	SAP97 (n=35)	SH3 (n=34)	SH3 + SAP97 (n=35)	<i>p</i>
Soma area (μm^2)	212 $\pm$ 6	214 $\pm$ 6	211 $\pm$ 7	241 $\pm$ 10	0.089
1 ⁰ dendrites, #	4.3 $\pm$ 0.2	4.4 $\pm$ 0.2	4.3 $\pm$ 0.2	4.4 $\pm$ 0.2	0.992
Branches, #	13.1 $\pm$ 0.9	18.6 $\pm$ 1.2 [*]	14.6 $\pm$ 1.1 [†]	17 $\pm$ 1.2	0.002
length (μm)	978 $\pm$ 54	1078 $\pm$ 63	889 $\pm$ 48 [†]	1200 $\pm$ 71 ^{*,†}	0.002
Average dendrites (μm)	237 $\pm$ 14	250 $\pm$ 15	217 $\pm$ 11	289 $\pm$ 21 [†]	0.008
Longest dendrite (μm)	203 $\pm$ 9	173 $\pm$ 9	175 $\pm$ 9	199 $\pm$ 12	0.051

Data shown as mean $\pm$ SEM

^{*} Differs from GFP group, $p < 0.05$

[†] Differs from SAP97 group, $p < 0.05$

[†] Differs from SH3 group, $p < 0.05$

Table 6

Blockade of the pro-dendrite growth action of SAP97 by I3 decoy domain.

Cell Parameter	GFP (n = 31)	SAP97 (n = 33)	I3 (n = 32)	I3 + SAP97 (n = 34)	p
Soma area (μm^2)	178 $\pm$ 10	192 $\pm$ 8	200 $\pm$ 8	189 $\pm$ 6	0.054
1 ⁰ dendrites, #	4.0 $\pm$ 0.2	4.3 $\pm$ 0.2	3.8 $\pm$ 0.1	4.0 $\pm$ 0.1	0.229
Branches, #	13.1 $\pm$ 1.0	19.8 $\pm$ 1.4 *	9.6 $\pm$ 0.9 [†]	12.2 $\pm$ 1.0 [†]	< 0.001
length (μm)	1084 $\pm$ 75	1362 $\pm$ 80 *	909 $\pm$ 56 [†]	1055 $\pm$ 58 [†]	< 0.001
Average dendrites (μm)	229 $\pm$ 17	330 $\pm$ 21 *	243 $\pm$ 15 [†]	268 $\pm$ 13 [†]	< 0.001
Longest dendrite (μm)	217 $\pm$ 11	224 $\pm$ 13	210 $\pm$ 8	225 $\pm$ 11	0.721

Data shown as mean $\pm$ SEM

* Differs from GFP group, p<0.05

[†] Differs from SAP97 group, p<0.05

Table 7

No blockade of the pro-dendrite growth action of SAP97 by GUK decoy domain.

Cell Parameter	GFP (n = 35)	SAP97 (n = 32)	GUK (n = 33)	GUK + SAP97 (n = 32)	<i>p</i>
Soma area (μm^2)	184 $\pm$ 11	176 $\pm$ 7	204 $\pm$ 9	190 $\pm$ 7	0.031
1 ⁰ dendrites, #	4.2 $\pm$ 0.2	4.2 $\pm$ 0.2	4.1 $\pm$ 0.2	4.2 $\pm$ 0.2	0.876
Branches, #	12.3 $\pm$ 0.8	18.4 $\pm$ 0.9 [‡]	12.7 $\pm$ 1.0 [!]	17.2 $\pm$ 1.3 [‡]	<0.001
length (μm)	1149 $\pm$ 72	1230 $\pm$ 55	1308 $\pm$ 83	1311 $\pm$ 73	0.404
Average dendrites (μm)	225 $\pm$ 16	246 $\pm$ 15	264 $\pm$ 17	264 $\pm$ 18	0.261
Longest dendrite (μm)	241 $\pm$ 14	214 $\pm$ 11	267 $\pm$ 14	244 $\pm$ 13	0.052

Data shown as mean $\pm$ SEM

* Differs from GFP group, $p < 0.05$

[‡] Differs from SAP97 group, $p < 0.05$

[!] Differs from GUK group, $p < 0.05$

Table 8

Blockade of the pro-dendrite growth action of SAP97 by mutation of PDZ3.

Cell Parameter	GFP (n = 27)	SAP97 (n = 26)	PDZ3mutSAP97 (n = 27)	<i>p</i>
Soma area (μm^2)	262 $\pm$ 15	282 $\pm$ 24	290 $\pm$ 16	0.237
1 ^o dendrites, #	6.7 $\pm$ 0.2	7.2 $\pm$ 0.3	6.2 $\pm$ 0.3 [†]	0.035
Branches, #	16.3 $\pm$ 0.9	19.8 $\pm$ 1.3	16.4 $\pm$ 1.0	0.036
length (μm)	1086 $\pm$ 46	1457 $\pm$ 61 [*]	1372 $\pm$ 75 [*]	<0.001
Average dendrites (μm)	166 $\pm$ 9	211 $\pm$ 12 [*]	232 $\pm$ 15 [*]	<0.001
Longest dendrite (μm)	173 $\pm$ 9	231 $\pm$ 13 [*]	234 $\pm$ 10 [*]	<0.001

Data shown as mean $\pm$ SEM^{*} Differs from GFP group, $p < 0.05$ [†] Differs from SAP97 group, $p < 0.05$