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Combinatorial morphogenesis of dendritic spines and filopodia by SPAR and α -actinin-2

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Abstract

Rap small GTPases regulate excitatory synaptic strength and morphological plasticity of dendritic spines. Changes in spine structure are mediated by the F-actin cytoskeleton, but the link between Rap activity and actin dynamics is unclear. Here we report a novel interaction between SPAR, a postsynaptic inhibitor of Rap, and α -actinin, a family of actin-crosslinking proteins. SPAR and α -actinin engage in bidirectional structural plasticity of dendritic spines: SPAR promotes spine head enlargement, whereas increased α -actinin2 expression favors dendritic spine elongation and thinning. Surprisingly, SPAR and α -actinin2 can function in an additive rather than antagonistic fashion at the same dendritic spine, generating combination spine/filopodia hybrids. These data identify a molecular pathway bridging the actin cytoskeleton and Rap at synapses, and suggest that formation of spines and filopodia are not necessarily opposing forms of structural plasticity.

Keywords

Rap small GTPase; actin cytoskeleton; hippocampal neurons; postsynaptic density; structural plasticity

INTRODUCTION

Dendritic spines are the primary sites of excitatory synaptic transmission in the CNS [1]. Spines are small, highly dynamic protrusions that are morphologically responsive to many types of environmental stimuli and patterns of synaptic activity, suggesting that changes in spine structure may underlie molecular encoding of information [2]. Spine morphology is developmentally regulated, progressing from thin filopodia-like structures in immature neurons to mushroom headed spines as neurons age [3], implicating filopodia as spine precursors. Functionally, spine head size is correlated with synaptic strength but inversely correlated with potential for further plasticity, and spines are dysmorphic in many dementias [4]. Thus, dendritic spine morphology may play critical roles in synaptic function and cognitive disorders.

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Actin is the primary cytoskeleton in spines, and alterations in spine morphogenesis are intimately associated with regulation of actin dynamics [5]. Accordingly, several actin binding proteins have been implicated in synaptic function and plasticity [6]. SPAR (Spine-Associated Rap GTPase-activating protein (GAP)) is an attractive candidate linking synaptic activity to actin remodeling. SPAR binds to the postsynaptic density scaffold protein PSD-95 in association with NMDA receptors (NMDARs) in brain [7]. In addition, SPAR is highly associated with and induces dramatic reorganization of F-actin filaments, and promotes massive spine head growth [7]. SPAR also contains enzymatic GAP activity that inhibits Rap1 and Rap2, small GTPases involved in synaptic depression, depotentiation and spine shrinkage [7-12]. Inhibition of Rap, or remodeling of F-actin, may contribute to the ability of SPAR to cause spine enlargement.

Here we report that SPAR interacts with the actin cross-linking protein α -actinin. Actinin comprises a family of proteins that play multiple roles at synapses [13], in association with glutamate receptors [14,15] and other excitatory synaptic proteins [16-19]. α -Actinin2 overexpression leads to elongation/thinning of dendritic protrusions and increased numbers of filopodia [20] at the expense of mature spines [21], in apparent opposition to the spine enlargement mediated by SPAR. We examined in detail the physical association of SPAR and α -actinin and determined their functional interaction in spine morphogenesis.

MATERIALS AND METHODS

Constructs

The following constructs have been described: PSD-95 PDZ1/2 in pGAD10 [22], α -actinin2 in pCDNA3 and NR1 C0-C1 tail in pBHA [14], myc-Act2 GW1 and myc-SPAR GW1 [7], and SPAR-Act2 in pBHA and SNK in pGAD10 [23]. Deletion constructs were generated by PCR and cloned into pGW1, pGAD10, or pBHA. Deletions for SPAR-Act2 domain were Δ N (aa1313-1505), Δ NC (aa1313-1415) and Δ C (aa1216-1415). All constructs were verified by DNA sequencing.

Yeast two hybrid

Two-hybrid screens and assays used the yeast strain L40 harboring β -gal and HIS3 reporters, as described [24]. 1×10^6 clones of a rat or human brain cDNA library in pGAD10 (Clontech) were screened against SPAR Act2 domain (aa1216-1505) in pBHA as bait.

Antibodies

The following antibodies have been described: rabbit-anti-SPAR [7]; rabbit Shank [25]. The following antibodies were purchased from commercial sources: myc 9E10, myc agarose conjugate, and goat anti-SPAR V20 (Santa Cruz Biotechnology); GFP monoclonal 3E6 (Quantum Biotechnologies); α -tubulin B-5-1-2 and α -actinin EA-53 monoclonals, and goat, mouse and rabbit IgG (Sigma).

COS-7 transfections, immunostaining, and immunoprecipitation

COS-7 cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer's protocol, and immunostained as described [22]. For immunoprecipitation, cells were harvested in RIPA buffer and lysates centrifuged at 16,000g for 15 minutes. Antibodies, or non-immune rabbit and mouse IgG/protein A or protein G sepharose conjugates were mixed with supernatants for 2 hr at 4°C. After washing 5x in RIPA buffer, immunoprecipitates were analyzed by immunoblotting using standard methods.

Brain immunoprecipitation

Immunoprecipitations from rat brain homogenates were performed as described [26] except that whole brain lysates were extracted by 1% sodium deoxycholate for 1 hr at 4°C. For each immunoprecipitation, clarified extract (200 µg protein) was incubated with 10 µg of desired antibodies (or nonimmune IgG) for 2 hr at 4°C. Precipitates were washed in 50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Triton X-100.

Neuronal culture, transfection and immunocytochemistry

Hippocampal primary neuronal cultures prepared from embryonic day (E) 18-19 rat embryos were plated at medium density (~150 cells mm⁻²) as described [27]. Rats were treated in accordance with NIH and Georgetown guidelines on animal care and use. Neurons were transfected at ~14 days in vitro (DIV) using a modified calcium phosphate procedure [28]. Immunostaining was performed essentially as described [27] at 19 DIV. Secondary antibodies used were donkey-anti-rabbit AlexaFluor 555 (1:300) and donkey-anti-mouse AlexaFluor 488 (1:100) (Invitrogen).

Quantitation and image analysis

Images were acquired using a Fluoview confocal microscope (Olympus) or Axiovert 200M (Zeiss) using consistent laser intensity or camera exposure levels for each fluorophore in each experiment. Confocal z-series image stacks encompassing dendrite segments were analyzed using MetaMorph software (Universal Imaging Corporation). For morphological classification, filopodia were defined as dendritic protrusions >2 µm in length and < 0.6 µm in width. All other protrusions < 5 µm in length were classified as spines.

Statistical analyses

All data are expressed as mean ± standard error of the mean. Data were analyzed for multiple groups using one-way ANOVA with Tukey's post hoc test or using Student's t-test for two group comparisons. Significance was determined at p<0.05.

RESULTS AND DISCUSSION

To elucidate the mechanism by which SPAR remodels actin and spine morphology, yeast two-hybrid (Y2H) interaction screens were conducted with the SPAR Act2 domain as bait (Fig. 1A). Act2 was chosen because this region is sufficient for SPAR association with F-actin and is required for spine enlargement [7]. Two separate screens were performed, against either a rat or a human brain cDNA library. Two α -actinin family members were identified as extremely strong interactors in these screens, clone HB15 representing rat α -actinin-1 and clone HB20 encoding human α -actinin-2. The primary structure of α -actinin proteins consists of an N-terminal actin binding domain, a series of central spectrin repeats, an EF hand motif in the carboxy-terminal region, and a PDZ-domain binding ligand at the very C-terminal tail [29]. DNA sequencing revealed that HB15 and HB20 encompassed strikingly similar coding regions, spanning from spectrin repeat 2 to the C terminus (Fig. 1A). Further Y2H deletion analysis showed that the α -actinin domains required for interaction with SPAR could be narrowed down to spectrin repeats 2-4 (Fig. 1A), a region that also binds to the C0-C1 exons of the NMDAR subunit NR1 C-terminal tail [14].

Conversely, deletion mapping of SPAR showed that clones HB15 and HB20 both bound preferentially to the C-terminal part of the SPAR Act2 domain (Act2 Δ N) (Fig. 1B). In contrast, the activity-inducible polo kinase SNK/Plk2, which targets SPAR for degradation via phosphorylation-dependent ubiquitination [23,30,31] and also interacts with the Act2 domain [23], bound preferentially to the N-terminal portion (Act2 Δ C). Such adjacent Act2 binding

sites suggest that SNK and α -actinin could bind SPAR simultaneously and independently (Fig. 1B; see Fig. 1C for schematic network of proposed interactions).

Coimmunoprecipitation and colocalization of actinin and SPAR

To confirm the Y2H results, lysates from adult rat whole brain were detergent extracted using 1% deoxycholate and subjected to immunoprecipitation. Native SPAR and α -actinin (detected using pan- α -actinin antiserum that is nonselective for α -actinin family members) were robustly co-immunoprecipitated from brain lysates using a goat SPAR antibody, but not by nonimmune goat IgG (Fig. 2A). As another specificity control, immunoblots were stripped and reprobed for tubulin, but no association with SPAR was observed (Fig. 2A). Thus, the co-immunoprecipitation of α -actinin and SPAR was unlikely to be due to nonspecific cytoskeletal aggregation. We also performed the reciprocal coimmunoprecipitation, using monoclonal α -actinin antibodies. These antibodies efficiently precipitated brain α -actinin as well as a substantial amount of SPAR (Fig. 2B). Furthermore, COS-7 cells were cotransfected with myc epitope-tagged SPAR and α -actinin-2. The full-length recombinant proteins could be readily co-immunoprecipitated in the absence of neuron-specific proteins (Fig. 2C).

Next, we performed immunocytochemistry of heterologous cells. As reported previously, recombinant SPAR overexpressed in COS-7 cells formed self-aggregating clusters (Fig. 2D) that reorganize and recruit F-actin [7]. Endogenous α -actinin also became recruited to these SPAR clusters (Fig. 2D, arrows). When the minimal α -actinin binding Act2AN portion of SPAR was cotransfected with α -actinin2, the exogenous proteins colocalized even more robustly (Fig. 2E).

We then determined the degree of colocalization of native proteins using immunocytochemistry of primary cultured hippocampal neurons. Extensive colocalization of α -actinin with SPAR was observed in a punctate pattern and at the tips of dendritic spines (Fig. 3A-C and G, quantified in I, J). However, many SPAR puncta did not overlap with α -actinin (Fig. 3C, arrowhead), and α -actinin puncta often did not colocalize with SPAR (Fig. 3C, arrow). A similar partial colocalization existed between α -actinin and the excitatory synaptic marker Shank (Fig. 3D-F and H, quantified in I, J). Taken together, these data have identified a novel protein complex between SPAR and α -actinin that was readily observed in a variety of biochemical, genetic, and immunocytochemical assays, consistent with a robust, high affinity interaction.

Combinatorial regulation of spine morphology

Previously, both α -actinin2 and SPAR were implicated in shaping dendritic spine morphology, albeit in different ways. α -Actinin promotes thinning and elongation of spines [21]. In contrast, SPAR causes spine head enlargement, with increased abundance of glutamate receptors and other synaptic markers [7]. We initially confirmed both of these findings (Fig. 4B-C, quantified in F-G). These properties implied that SPAR and α -actinin2 may drive opposing morphological changes, the former toward more stable and larger spines and synapses, and the latter toward more dynamic and immature filopodia with smaller and weaker synapses. This potential antagonism was also suggested by the similarity between boosting α -actinin-2 levels and blocking SPAR with dominant negative constructs [7].

Thus, we cotransfected hippocampal neurons with both SPAR and α -actinin-2 to test whether their actions were mutually antagonistic, and predicted that their morphogenetic effects would cancel out, or that one would predominate. Instead, we found that the coexpression of α -actinin-2 and SPAR resulted in a mixed phenotype, with highly enlarged spines formed in addition to overly abundant filopodia (Fig. 4D, quantified in F-G). This result could represent a situation in which SPAR 'won' at certain synapses, yielding large spines, but 'lost' to actinin

at other synapses, resulting in filopodial transformation. However, it is not known what factors influence one outcome over the other.

Unexpectedly, we observed that many of the greatly enlarged dendritic spines sprouted multiple filopodia directly from the head itself (Fig. 4E), a 'hybrid' phenomenon that was rarely observed in control neurons (Fig. 4F). Thus, spine and filopodia morphogenetic pathways controlled by SPAR and α -actinin are not mutually exclusive, even at the same locus. It is presently unclear whether such hybrid spine/filopodia ('spinopodia') are physiological. These structures could be artifacts of misregulated overexpression of α -actinin and SPAR; perhaps mechanisms exist normally to prevent both proteins from being active simultaneously at the same synapse. On the other hand, filopodia atop spines are reminiscent of spinules [32,33], thin protrusions on spines observed in vivo that penetrate into presynaptic terminals and may play a role in trans-synaptic retrograde signaling [34]. Thus, the extraordinary spine plasticity we observed here could be exaggerated versions of physiologically relevant processes. Regardless, our data extend the traditional view of the relationship between spines and filopodia, and argue that filopodia cannot simply be precursors of spines or products of spine shrinkage.

In addition to SPAR, α -actinins interact with several other synaptic proteins and likely play a wide variety of roles at synapses [16-19,35]. As an actin-crosslinking protein, α -actinin may directly link SPAR to actin to enable the Act2 domain to remodel F-actin cytoskeleton. α -Actinin-2 also binds to NR1 via the same region as it binds to SPAR (spectrin repeats 2-4) [14]. Because of the known role of α -actinin in calcium-dependent inactivation of NMDARs [36,37], it will be interesting to examine whether SPAR regulates this process by competitive interference with the α -actinin-NR1 interaction.

A final intriguing possibility is suggested by the observation that α -actinin is associated with numerous receptors that activate Rap in different contexts, including integrins [38,39], NMDARs [12,14,37], cadherins [40,41], insulin receptors [42,43], and mGluRs [44,45]. It is thus tempting to speculate that the α -actinin-SPAR complex may act as a widespread convergence point and general tethering mechanism between cell surface receptors, intracellular Rap signaling, and actin remodeling.

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Figure 1. Identification of α -actinin interaction with SPAR

(A) Y2H screens were performed using SPAR Act2 domain as bait in vector pBHA. Interaction strength was scored by β -gal activity (+++, 0-30 min; +, 30 min-2 hrs; -, >12 hrs). Interaction time for α -actinin-SPAR was consistently under 10 minutes. Residues encompassed by positive clones (HB15 and HB20) and α -actinin deletion constructs are indicated below α -actinin schematic diagram (interacting clones shown in black). SNK in pGAD10 vector was positive control for interaction with Act2-pBHA; NR1 C0-C1 exon-containing C-terminus in pBHA was positive control for the α -actinin interactions. PSD-95 PDZ1/2 in pGAD10 was negative control for all pBHA constructs.

(B) α -Actinins bind specifically to the C-terminal portion of Act2 (Act2 Δ N), whereas SNK binds primarily to the N-terminal part (Act2 Δ C).

(C) Schematic of SPAR domains and interactions. Act2, actin-associated domain 2. GKBD, guanylate kinase binding domain. ACTN, α -actinin. Double headed arrow indicates SPAR binding to α -actinin may displace NR1.

Figure 2. Biochemical and immunocytochemical association of SPAR and α -actinin

(A) Rat brain lysates were immunoprecipitated with SPAR V20 goat antibodies or with nonimmune goat IgG control, and precipitates were immunoblotted for SPAR, α -actinin and tubulin as indicated.

(B) Rat brain lysates were immunoprecipitated with mouse monoclonal antibodies to α -actinin or with nonimmune mouse IgG control, and precipitates were immunoblotted for SPAR and α -actinin. IN represents 10% of input used in each immunoprecipitation reaction in (A, B).

(C) COS-7 cells transfected with myc-tagged SPAR or α -actinin2 as indicated were immunoprecipitated with myc agarose and immunoblotted for α -actinin. 10% of input is shown as expression control. (D-E) COS-7 cells transfected with myc-tagged SPAR alone (D) or with mycAct2 Δ N domain of SPAR together with α -actinin2 (E). Cells were immunostained with myc and α -actinin antibodies, as indicated. Arrows show examples of colocalization. Scale, 5 μ m.

colocalized between Shank/ α -actinin and SPAR/ α -actinin. 'Marker only' represents puncta that contain only the marker indicated out of the pair examined.

Figure 4. SPAR and α -actinin2 regulation of dendritic spine morphogenesis

(A-E) Cultured hippocampal neurons (15 DIV) were transfected with DNA constructs as indicated and with pEGFP to visualize neuronal morphology. Insets show higher magnification views of representative dendritic protrusions. (E) Representative hybrid spine/filopodia from transfected neurons in (D), shown at same scale as higher magnification insets in (A-C). Scale bars: 5 μ m for dendrite images and 1 μ m for all higher magnification images. (F) Quantification of dendritic spine morphological classifications as indicated. Hybrid refers to combination spine/filopodia protrusions. (G) Dendritic protrusion length, width, and length-to-width ratio (L/W) for transfected neurons as indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to control.