Neuroscience Letters 533 (2013) 44–49

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

# Ligand binding of PDZ domains has various roles in the synaptic clustering of SAP102 and PSD-95

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## HIGHLIGHTS

- Ligand binding-deficient PDZ domains enhance the synaptic clustering of SAP102.
- PDZ domains play distinct roles in the synaptic clustering of SAP102 and PSD-95.
- Binding of NMDAR with SAP102 is required for its efficient synaptic clustering.

## ARTICLE INFO

*Article history:*
Received 11 August 2012
Received in revised form 6 November 2012
Accepted 8 November 2012

*Keywords:*
SAP102
PSD-MAGUK
PDZ domain
NMDA receptor
Postsynaptic density
Synaptic clustering

## ABSTRACT

Synapse-associated protein 102 (SAP102) and postsynaptic density-95 (PSD-95) bind to NMDA receptors through PDZ domains and cluster at excitatory postsynaptic sites called postsynaptic densities (PSD). We previously reported that PSD-95 containing mutated PDZ domains incapable of ligand binding clustered at synaptic sites with reduced efficiency. Here, we compared the synaptic clustering of the same series of full-length SAP102 mutants in hippocampal neurons. Unexpectedly, ligand-binding deficient mutant SAP102 showed more efficient synaptic localization than wild-type SAP102. Further, when SAP102-PDZ mutants were co-expressed with either the GluN2A or GluN2B NMDA receptor subunit, both subunits showed decreased synaptic clustering, although the mutants were efficiently targeted to the synapses. This finding suggests that direct binding of NMDA receptors with SAP102 is involved in the efficient targeting of NMDA receptors to the synapses, whereas ligand binding of the PDZ domains is not essential for the synaptic clustering of SAP102.



## 1. Introduction

Excitatory synapses in the mammalian brain are characterized by a dense network of proteins known as postsynaptic densities (PSD), where the membrane-associated guanylate-kinase (MAGUK) family proteins PSD-95, synapse-associated protein 102 (SAP102), PSD-93, and SAP97 are abundantly expressed and form a protein complex with receptors, ion channels, cytoskeletal proteins, and signaling molecules to regulate signal transduction [11]. PSD-MAGUKs share a common domain structure comprising three PDZ domains in the N-terminus, followed by SH3 and GK domains [3]. They bind directly to the C-termini of the GluN2 subunits of NMDA receptors and the auxiliary subunit of AMPA receptors (TARP) via the PDZ domains. Knock-in mice expressing GluN2B lacking the intracellular C-terminal domain or TARPγ-8 lacking the C-terminal four residues exhibit perinatal lethality or reduced AMPAR-mediated basal transmission, respectively, suggesting the crucial role of the binding of PSD-MAGUKs with GluN2B or AMPAR in normal signal transduction [12,13]. Thus, PSD-MAGUKs are key molecules for regulating targeting, anchoring, and signaling of receptors and ion channels via protein–protein interactions.

PSD-95 is a dominant PSD-MAGUK protein. The SH3-GK domains and the N-terminal region, including palmitoylated Cys-3 and Cys-5, are essential for efficient targeting and clustering of PSD-95 at synaptic sites [3,16]. Similarly, the N-terminus of SAP102 with no cysteine palmitoylation site is suggested to play a role in synaptic clustering, and the SH3-GK domains of SAP102 are also essential for efficient synaptic targeting through their interaction with mPins, which directly bind the G protein inhibitory subunit and modulate SAP102 trafficking [3,10]. SAP102 is highly mobile in dendritic spines, however, compared to PSD-95, suggesting the

*Abbreviations:* AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; DIV, day *in vitro*; EGFP, enhanced green fluorescent protein; MAGUK, membrane-associated guanylate kinase; PBS, phosphate-buffered saline; PDZ, PSD-95/discs large/zona occludens-1; PSD, postsynaptic density; PSD-95, postsynaptic density protein-95; SAP102, synapse-associated protein 102; SCI, synaptic clustering index; VGLUT, vesicular glutamate transporter.

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http://dx.doi.org/10.1016/j.neulet.2012.11.019

versatility of MAGUK in their function [17]. The PSD-MAGUKs also have developmentally different roles in the trafficking of NMDA receptors and AMPA receptors to synaptic sites, but partially compensate for each other [2]. Thus, how the four PSD-MAGUKs with remarkable similarity play different roles, and the structural bases of their specialization are interesting topics of study.

We previously studied synaptic clustering of PSD-95 containing mutated PDZ domains that are incapable of ligand binding to determine the significance of tandem PDZ domains in synaptic clustering [8]. Our findings revealed that individual PDZ domains independently and additively contribute to the synaptic clustering of PSD-95. In the present study, we introduced the same sets of mutations into SAP102 to assess the effects of ligand binding of PDZ domains on the efficacy of the synaptic clustering of SAP102 in hippocampal neurons. Surprisingly, the clustering of mutated SAP102 was highly efficient, whereas the clustering of GluN2A and GluN2B subunits was impaired by co-expression of the mutated SAP102, suggesting that the mechanisms underlying synaptic clustering are distinct for SAP102 and PSD-95, and that SAP102 has a fundamental role in trafficking the NMDA receptor complex to the synaptic sites.

## 2. Materials and methods

### 2.1. DNA constructs

The mutated PSD-95 cDNAs fused with enhanced green fluorescent protein (EGFP) were described previously [8]. The cDNA of SAP102 (GI:118131177) coding 849 amino acids was prepared from a mouse brain full-length cDNA library (Clontech) and introduced into the chicken β-actin promoter-based expression vectors, pCA-flag and pCA-EGFP, kindly provided by Drs. H. Niwa and M. Takeichi (RIKEN, Center for Developmental Biology). The ligand-binding deficient PDZ1 domain (1d) of SAP102 was prepared by introducing S162N and A164V mutations and deleting I167 to V172. Similarly, the mutant PDZ2 domain (2d) contained S257N and A259V mutations and a deletion of I262 to I267. The mutated PDZ3 domain (NS) of SAP102 contained an N417S mutation. The myc-tag (EQKLISEEDL) was inserted between N35 and I36 of the mouse GluN2A subunit and between A53 and H54 of the GluN2B subunit. These cDNAs were introduced into the pCA expression plasmid.

### 2.2. Immunoprecipitation

The full-length mutant SAP102 was co-expressed with myc-tagged GluN2B in COS cells and cell lysates, prepared in 1% Triton X-100, 50 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and protease inhibitors, were incubated with 5 μg myc antibody overnight at 4 °C. The mixtures were further incubated with 30 μl Protein G agarose for 1 h at 4 °C. The extracts from the Protein G agarose with sodium dodecyl sulfate sample buffer were analyzed by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent immunoblotting with the anti-myc antibody 9E10 (Roche Applied Science), and the anti-GFP antibody JL-8 (Clontech, Takara Bio Inc.).

### 2.3. Hippocampal neural cell cultures and transfection

Rat hippocampal dissociated neurons were prepared from embryonic day 18 Wister rats, as described previously [7] and transfected with cDNAs using a Rat Neuron Nucleofector kit (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's instructions. Transfected neurons were then plated directly onto poly-L-lysine (Sigma, St. Louis, MO)-coated culture dishes at a density of $3 \times 10^3$ cells/cm$^2$ and incubated in Neurobasal medium (Invitrogen, Grand Island, NY) supplemented with B27 (Invitrogen) and 1 mM L-glutamine. At ~15–17 days in vitro (DIV), cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde/4% sucrose. The fixed cells were quenched in 1 mM glycine/PBS, permeabilized with 0.2% Triton X-100/PBS for 10 min at room temperature, thoroughly washed with PBS, incubated in blocking solution (3% bovine serum albumin/PBS) for 30 min at room temperature, and then incubated overnight at 4 °C with primary antibodies diluted in the blocking buffer as described below, washed three times with PBS, and incubated with AlexaFluor-conjugated secondary antibodies (Molecular Probes) at a 1:500 dilution in blocking buffer. Antibodies were purchased and diluted as follows: mouse monoclonal anti-synaptophysin (Sigma, 1:1000), rabbit polyclonal anti-VGLUT1 (Synaptic Systems, Goettingen Germany; 1:1000), and AlexaFluor-conjugated anti-rabbit and anti-mouse antibodies (Invitrogen; 1:500). All animal experiments were performed according to the guidelines approved by the Animal Experimentation Committee of Kyoto University.

### 2.4. Quantitative microscopy and image analysis

All immunofluorescence images were acquired using a cooled charge-coupled device camera, the Cool SNAP HQ (Photometrics) controlled by Meta Morph software (Molecular Devices), mounted on a BX50 upright microscope (Olympus) with 100× (N.A.1.35) or 40× (N.A.0.85) objectives. For quantification, because the intensity values derived from fused GFP varied due to overexpression, neurons that expressed each mutant-GFP within the following average intensity values in the dendritic shafts were randomly picked; 102-WT, 102-1d2d, and 102-1d2dNS: 50–220; 95-WT, 95-1d2d, 95-1d2dNS: 100–300 in a 12-bit dynamic range, 0–4096, at a fixed gain setting. Most of the cells with a normal morphology fell within these values.

Calculation of the synaptic clustering index (SCI) shown in Fig. 2 was performed as described previously [8]. Briefly, synaptic PSD-95 or SAP102 clusters were defined as 0.3–1.0 μm diameter spots of increased GFP fluorescence, at least twice as bright as the dendritic shaft, which were juxtaposed to synaptophysin-immunopositive puncta. To calculate the SCI, after subtracting the background, the maximal intensity of each synaptic cluster was divided by the mean intensity of the proximal parent dendritic shaft. In each neuron, ~20–30 synaptic clusters that colocalized with synaptophysin was selected to calculate the SCI. A total of 6–10 cells was analyzed for each transfected construct. Three independent trials of transfection with a series of mutants were performed, and the same tendency shown in Fig. 2 was observed for each imaging and quantification. All data are expressed as the mean ± SEM. For statistical analysis, the SCI measurement was evaluated by one-way ANOVA with Dunnett's multiple comparison post hoc test.

For quantification of images of neurons co-expressing SAP102 with GluN2A or GluN2B in Fig. 3, we calculated the mean SCI (mSCI) using the following averaging method with Meta Morph software, because calculation of the SCIs for both of SAP102 and GluN2A or GluN2B for individual synapses of each mutant construct was time- and labor-intensive. Basically, the mSCI was calculated as the mean synaptic cluster intensity at the sites juxtaposed to the presynaptic markers divided by the mean dendritic shaft intensity. The synaptic clusters were defined as the areas between 10 and 300 pixels with an intensity greater than 10% of the maximum in a neuron that were colocalized with a presynaptic marker, VGLUT1. Mean synaptic intensity was calculated by dividing the total synaptic intensity by the corresponding total synaptic area. Mean dendritic shaft intensity was calculated by dividing the total dendritic shaft intensity by the total dendritic shaft area. The mSCI was defined as the ratio of the mean spine intensity to the mean dendritic shaft intensity. A total of 6–10 cells was analyzed