CaMKII-dependent Phosphorylation Regulates SAP97/NR2A Interaction*

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Synapse-associated protein 97 (SAP97), a member of membrane-associated guanylate kinase protein family, has been implicated in the processes of targeting ionotropic glutamate receptors at postsynaptic sites. Here we show that SAP97 is enriched at the postsynaptic density where it co-localizes with both ionotropic glutamate receptors and downstream signaling proteins such as Ca2+/calmodulin-dependent protein kinase II (CaMKII). SAP97 and αCaMKII display a high co-localization pattern in hippocampal neurons as well as in transfected COS-7 cells. Metabolic labeling of hippocampal cultures reveals that N-methyl-D-aspartic acid (NMDA) receptor activation induces CaMKII-dependent phosphorylation of SAP97: co-incubation with the CaMKII-specific inhibitor KN-93 reduces SAP97 phosphorylation to basal levels. Our results show that SAP97 directly interacts with the NR2A subunit of NMDA receptor both in an in vitro “pull-out” assay and in coinmunoprecipitation experiments from homogenates and synaptosomes purified from hippocampal rat tissue. Interestingly, in the postsynaptic density fraction, SAP97 fails to co-precipitate with NR2A. We show here that SAP97 is directly associated with NR2A through its PDZ1 domain, and CaMKII-dependent phosphorylation of SAP97-Ser-232 disrupts NR2A interaction both in an in vitro pull-out assay and in transfected COS-7 cells. Moreover, expression of SAP97(S232D) mutant has effects similar to those observed upon constitutively activating CaMKII. Our findings suggest that SAP97/NR2A interaction is regulated by CaMKII-dependent phosphorylation and provide a novel mechanism for the regulation of synaptic targeting of NMDA receptor subunits.

The correct delivery of synaptic proteins is a key process in the regulation of physiological neuronal response. This mechanism requires the coordination of a number of molecular components. In fact, it implies the formation of multimolecular complexes that bring transmembrane receptors together with molecular organizers and involves the action of signaling elements, i.e. specific kinases, that govern the regulatory processes responsible for a dynamic assembly/disassembly of these complexes.

Membrane-associated guanylate kinase (MAGUK) protein family has been addressed as organizing elements in excitatory neurons. MAGUKs are characterized by a common multimodular structure including three PDZ domains, an Src homology domain 3, and a guanylate kinase-like domain (1, 2). Members of this family act as molecular scaffolds for synapse-targeted components mainly by direct interaction with a SVX motif on the cytoplasmic termini of their binding proteins. MAGUK partners are ionotropic glutamate receptor subunits, as well as other signal transduction elements (1, 2).

Among MAGUKs, SAP97 (3) is the rat homologue of the Drosophila (Dlg) and human (hDlg) discs large tumor suppressor protein. Dlg is highly concentrated in postsynaptic membranes where it has been shown to interact with synaptic molecules such as Shaker potassium channels (4). In the mammalian central nervous system, SAP97 has been described to be enriched both at pre- and postsynaptic compartments (3, 5). Recently, SAP97 has been implicated in the early processing of the GluR1 subunit of the AMPA receptor (6), and a possible interaction with NR2-type NMDA subunits has been put forward. In particular, the NR2A subunit has been proposed to interact in vitro with SAP97 (7, 8) even if the physiological relevance and the mechanisms regulating these interactions are still unclear.

It is well known that the cytosolic domain of the NR2 subunits can serve as a docking site not only for members of MAGUK protein family (9) but also for signaling molecules such as CaMKII (10). CaMKII is a flower-shaped enzyme highly enriched in brain neurons and represents the main signaling protein of the postsynaptic density (PSD). It is well established that a fine modulation of its activity and autophosphorylation state is central to the regulation of glutamatergic synapse (11). Several data have demonstrated that CaMKII uses different molecular strategies to spatially and temporally control signal transduction in the hippocampal neuron, including phosphorylation of a variety of substrates. Nevertheless, although several CaMKII substrates have been identified in the last few years (12), little is known about the functional role of specific CaMKII phosphorylation processes that take place in vivo in the postsynaptic

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The abbreviations used are: MAGUK, membrane-associated guanylate kinase; SAP97, synapse-associated protein 97; PSD, postsynaptic density; CaMKII, Ca2+/calmodulin-dependent protein kinase II; NMDA, N-methyl-D-aspartic acid; PBS, phosphate-buffered saline; GST, glutathione S-transferase; TIF, Triton-insoluble fraction; GFP, green fluorescent protein; WB, Western blot; HRSS, Hanks’ balanced saline solution; AMPA, 1-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid.
COMPARTMENT. Recently, different members of MAGUK as possible new targets for CaMKII have been suggested (12, 13), raising the possibility of a new mechanism regulating MAGUK function in the postsynaptic neuron. Here we show that, in hippocampal neurons, SAP97 interacts with NR2A subunits through its PDZ1 domain and that Ser-232 within this domain is phosphorylated by CaMKII in vitro and in vivo in metabolically labeled transfected COS-7 cells and in cultured hippocampal neurons. CaMKII-dependent phosphorylation of this site disrupts SAP97 interaction with NR2A suggesting that CaMKII activation in the postsynaptic compartment can serve as a molecular mechanism influencing NR2A–SAP97 complex thereby regulating synaptic targeting of this NMDA receptor subunit.

EXPERIMENTAL PROCEDURES

COS-7 Cell Culture and Transfection—COS-7 cells at 20–50% confluency (24 h after plating on glass coverslips in 12-well plates) were transfected by Superfect® Transfection Reagent (Qiagen, Valencia, CA) with cDNA expression constructs (1–1.5 μg of DNA/well) for 2–3 h at 5% CO2, 37 °C. Cells were washed twice with PBS, fed with Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, and 1% penicillin/streptomycin, and grown for 24–48 h before fixation for immunocytochemistry or before metabolic labeling experiments. Mutated products were obtained by using the QuikChange™ Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). Where indicated in the text, Ile-Glu-Arg-Val-Ile-Phe-Gln-Ser-Amn SAP97 (34–44) or Leu-Glu-Ary-Gly-Amn-Ser-Glu-Leu-Gly-Phe-Ser SAP97 (227–237) peptides were transfected in COS-7 by means of the Pro-Jet Protein Transfection Reagent (Pierce) 4 h before immunolabeling.

Neuronal Cultures and Immunofluorescence Labeling—Low density hippocampal neuronal cultures were prepared from E18 to E19 rat hippocampi as described (14, 15). Hippocampal neurons or transfected COS-7 cells were fixed (−20 °C for 15 min), Primary and secondary antibodies were applied in GDB buffer (30 mM phosphate buffer, pH 7.4 containing 0.2% gelatin, 0.5% Triton X-100, and 0.8 mM NaCl). Fluorescence images were acquired using Bio-Rad Radiance 2100 confocal microscope.

Image Acquisition and Quantification—Confocal images were obtained using a Nikon 60× objective with sequential acquisition setting at 1024 × 1024 pixel resolution. Each image was a z series projection of −8 to 12 images taken at 0.5–μm depth intervals. Labeled transfected COS-7 cells were chosen randomly for quantification from two to five coverslips from three to five independent experiments for each construct. Co-localization analysis was performed using Laserpix software (Bio-Rad). Quantification of Western blot analysis and autoradiography were performed by means of computer-assisted imaging (Quantity-One® System; Bio-Rad), and statistical evaluations were performed according to one-way analysis of variance followed by Bonferroni as post hoc comparison test.

Cloning, Expression, and Purification of GST Fusion Protein—SAP97 fragments were subcloned downstream of glutathione S-transferase (GST) in the BamHI and HindIII site of the expression plasmid pGEX-KG by PCR using the Pfu polymerase (Promega, Madison, WI). The inserts were fully sequenced with ABI Prism 310 Genetic Analyzer (ABI Prisma). In particular, SAP97-PDZ2 and SAP97-PDZ3 GST fusion proteins were expressed in Escherichia coli, purified on glutathione-agarose beads, and eluted as described previously (16).

Fusion Protein Phosphorylation—GST–SAP97-PDZ1 and GST–SAP97-PDZ3 purified fusion proteins were incubated with αCaMKII (1–325) (New England Biolabs) at 37 °C, in the presence of 20 nm HEPES, pH 7.4, 10 mM MgCl2, 1 mM diethiothreitol, 2.4 μM calmodulin, 2 mM CaCl2, with 100 μM ATP (γ32P)ATP 2 μCi/μl; 3000 Ci/mmol; Amersham Biosciences). The reaction was initiated by the addition of kinase solution to the reaction mixture. The reaction was carried out for 5 min and stopped by the addition of SDS sample buffer.

Metabolic Labeling and Immunoprecipitation—Hippocampal cultures were precultured for 2 h in phosphate-free minimal essential medium and then labeled with [32P]orthophosphate (1 μCi/ml) in phosphate-free essential medium at 37 °C for 2 h at 37 °C. The cultures were washed and incubated for an additional 15 min at 37 °C with either Hanks’ buffer (HBSS; for control cultures) or HBSS containing NMDA (50 μM) or NMDA (50 μM) + KN-93 (10−5 M). Following two quick washes with cold HBSS, the cells were harvested into 0.5 ml of ice-cold solubilization buffer containing (unless otherwise indicated) 50 mM HEPES, pH 7.4, 0.5 M NaCl, 0.5% Triton X-100, 0.1% deoxycholate, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF in the presence of a complete set of proteases inhibitors (Complete™, Roche Diagnostics). Each solubilized sample was then incubated for 2 h at 4 °C with 10 mg of protein A-Sepharose beads and anti-SAP97 polyclonal antibody. Following this incubation, the supernatants were removed, and the beads were washed five times with solubilization buffer. After the final wash, the beads were resuspended in 100 μl of SDS sample buffer, incubated with agitation for 5 min, and briefly centrifuged; the supernatants were loaded on 9% SDS-PAGE gels as described. Incorporation of 32P was quantified by exposing dried gels to film for varying lengths of time, and then densitometric analysis was performed by means of computer-assisted imaging (Quantity-One® System; Bio-Rad).

Cells were solubilized in 50 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and Complete™ where indicated. The homogenized cells were centrifuged at 40,000 rpm for 30 min. The resulting pellet (P1) was resuspended in an equal volume of 1% Triton X-100 and 150 mM KCl and centrifuged at 40,000 rpm for 30 min to obtain a Triton-insoluble fraction (TIP).

COS-7 cells were transfected with empty vectors, GFP–SAP97wt + αCaMKII-T286D or GFP–SAP97wt(S222D) + αCaMKII-T286D. 48 h after transfection, cells were preincubated for 1 h in phosphate-free minimal essential medium. The medium was aspirated and replaced with fresh phosphate-free minimal essential medium containing [32P]orthophosphate (500 μCi/ml). After 1 h of incubation, 0.2 μM αCaMKII was added to the medium, and cells were incubated for an additional hour. Cells were harvested into 0.5 ml of ice-cold solubilization buffer containing a complete set of protease inhibitors (Complete™, Roche Diagnostics). Each solubilized sample was then incubated overnight at 4 °C with anti-GFP polyclonal antibody. Protein A-Sepharose beads washed in the same buffer were added, and incubation was continued for 2 h. Western blots were performed on all immunoprecipitated samples to verify that equal amounts of SAP97 protein were being precipitated under all experimental conditions (data not shown).

PSD Preparation—To isolate PSD from rat hippocampus, a modification of the method by Carlin et al. (17) was used, as described previously (18).

“Pull-out” Assay—Aliquots containing 5 μg of hippocampal PSD were diluted with PBS, 0.1% or 1% SDS to a final volume of 1 ml and incubated (1 h, 37 °C) with glutathione-agarose beads saturated with GST fusion proteins or GST alone. After the incubation period, the beads were extensively washed with PBS, 0.1% Triton X-100. Bound proteins were resolved by SDS-PAGE and subjected to immunoblot analysis with polyclonal anti-NR2A and anti-GluR1 antibodies.

Antibodies—The following antibodies were used: mouse αCaMKII antibody, polyclonal GluR1, NR1, and NR2A antibodies were purchased from Chemicon International, Inc. (Temecula, CA); polyclonal SAP97 antibody was purchased from Affinity BioReagents Inc. (Golden, CO); polyclonal anti-GFP and AlexaFluor 488, 568, and 633 secondary antibodies were purchased from Molecular Probes (Eugene, OR).

RESULTS

SAP97 Co-localization and Co-precipitation with NR2A and CaMKII—To determine SAP97 enrichment into different subcellular compartments of hippocampal neurons, we used a biochemical fractionation approach to isolate purified postsynaptic densities (PSD) from hippocampal rat tissue (Fig. 1). The expression of SAP97 as well as pre- and postsynaptic marker proteins in various subcellular compartments was investigated. SAP97 displays a high co-localization pattern with αCaMKII and ionotropic glutamate receptor subunits being enriched in the PSD fraction and present with a similar distribution in the total homogenate (H), crude synaptosomal membrane (Syn), and the low speed supernatant (S1) fractions. SAP97 was only barely detectable in the nuclei-associated membranes (P1). By using the same biochemical fractions, we also examined the subcellular distribution of synaptophysin, PSD-95, GluR1 subunit of AMPA receptor, and NR2A subunit of NMDA receptor. As expected, synaptophysin was present in all subcellular compartments analyzed but not in the PSD-purified fraction, whereas all the other proteins have a similar distribution pattern being enriched in synaptosomes and PSD fractions (Fig. 1A).
Recent data documented that SAP97 can interact with different subsets of proteins in vitro (5, 7). We performed immunoprecipitation experiments in homogenate, synaptosomes, and PSD fractions to evaluate the capability of SAP97 to bind putative interacting proteins. Anti-SAP97 immunoprecipitates were challenged in WB with NR1, NR2A, GluR1, and αCaMKII antibodies. Fig. 1B shows that in all tested compartments, SAP97 co-precipitates with GluR1 and αCaMKII; co-precipitation assay shows an association of SAP97 with NR2A subunit of NMDA receptor in homogenate and synaptosomal fraction but not in the PSD fraction. The absence of any NR1 signal in SAP97 co-immunoprecipitates tends to exclude that in our experimental conditions the co-precipitation of NR2A reflects an unspecific immunoprecipitation of insoluble proteins. Furthermore, anti-NR2A and anti-αCaMKII immunoprecipitates from synaptosomes challenged for SAP97 confirmed the co-precipitation of these proteins (Fig. 1C).

Co-clustering of SAP97 and αCaMKII in Transfected COS-7 Cells—It is well known that αCaMKII Thr-286 autophosphorylation is crucial for its persistent activation, keeping the enzyme in a catalytically active conformation independent of the presence of Ca^{2+}/calmodulin (11). Single amino acid substitution of Thr-286 with Asp (αCaMKII-T286D) mimics autophosphorylation, rendering the enzyme constitutively active. Clustering assay in heterologous cells was used to study possible interactions of both wild type and active-T286D αCaMKII with SAP97 in intact cells. COS-7 cells were first singly transfected with αCaMKII, αCaMKII-T286D, SAP97, or NR1. In single transfections both αCaMKII and αCaMKII-T286D showed a diffuse distribution, whereas both SAP97 and NR1 displayed a perinuclear accumulation with characteristic clusters (Fig. 2, A–D). Co-transfection of SAP97 with αCaMKII or αCaMKII-T286D caused a redistribution of SAP97 staining throughout the cell (Fig. 2, E–J). Of relevance, quantification of SAP97 co-clustering with T286D-mutated enzyme shows a higher and almost complete co-localization value (97.1 ± 2.3%) when compared with the wild type αCaMKII (75.4 ± 2.9%) suggesting a higher affinity of the activated form of the enzyme for SAP97. No effect on NR1 distribution was observed when it was co-transfected with either αCaMKII (E–G) or with αCaMKII T286D (H–J). No alteration of characteristic NR1 perinuclear staining is observed in cells double-transfected with NR1 and wild-type (K–M) or T286D αCaMKII (N–P). Scale bar, 10 μm.

We then examined the distribution pattern of SAP97 and αCaMKII in 2–3-week-old cultured hippocampal neurons exposed or not exposed to NMDA (50 μM) for 15 min in order to allow Ca^{2+} influx and endogenous CaMKII activation (14). In untreated cultures, SAP97 labeling was diffused in the somatic cytoplasm of cultured neurons; the same diffuse staining was also observed within dendrites (Fig. 3). Both in the dendrites and in the soma of cultured neurons, it appears to have a moderate co-localization pattern with αCaMKII. Fifteen-minute treatment of hippocampal cultures with NMDA (50 μM),
known to maximally activate neuronal αCaMKII, leads to a punctate staining of αCaMKII in the dendrites. Interestingly, SAP97 staining in these experimental conditions changed from a diffuse to a punctate labeling in the dendritic compartment leading to a higher co-localization degree between the two proteins, thus confirming a higher co-clustering of SAP97 with activated CaMKII as shown in COS-7 cells. CaMKII-dependent Phosphorylation of SAP97 in Purified PSD and in Cultured Hippocampal Neurons—We then tested whether αCaMKII/SAP97 interaction may lead to SAP97 phosphorylation. PSDs from hippocampi were reacted under conditions optimal to maximally activate αCaMKII; immunoprecipitation studies were performed on 32P-phosphorylated PSD with a polyclonal SAP97 antibody. A phosphorylated band at about 130 kDa, corresponding to SAP97, is clearly visible (Fig. 4A, lane 3). In the same autoradiography, a phosphorylated band at 50 kDa is also present. This phospho-band corresponds to the autophosphorylated αCaMKII that co-precipitates with SAP97 as demonstrated by the WB analysis reported in the figure (lanes 1 and 2).

In a more direct approach, hippocampal neurons were metabolically labeled with [32P]orthophosphate and then exposed for 15 min to NMDA in the absence or presence of CaMKII protein bands induced by NMDA (50 μM) or NMDA (50 μM) + KN-93 (10 μM). Cultures were harvested, and neuronal lysates were immunoprecipitated with a polyclonal SAP97 antibody. WB was performed on all immunoprecipitated samples to verify that equal amounts of SAP97 protein were being precipitated under these experimental conditions (data not shown). Autoradiography of anti-SAP97 immunoprecipitates showed a major 32P band at about 130 kDa co-migrating with the SAP97 immunoreactive band. NMAD treatment significantly increased 32P incorporation into the 130-kDa SAP97 phospho-band (+81.2 ± 8.9%, NMAD versus control, p < 0.01).

In the same autoradiogram a phosphorylated band at 50 kDa corresponding to αCaMKII co-precipitation with SAP97 is also present, consistent with the above finding (Fig. 1B and Fig. 4A). The increase in phosphorylation of both SAP97 and αCaMKII protein bands induced by NMAD was completely blocked by co-incubation of the CaMKII inhibitor KN-93. Of relevance, NMAD and KN-93 treatment modulated both αCaMKII activation-inhibition and kinase association/dissociation from SAP97 (Fig. 4B, lower panel). In addition, Fig. 4C shows that SAP97 phosphorylation is restricted only in the Triton-insoluble fraction (TIF) and in a P1 crude membrane fraction where both SAP97 and αCaMKII are present (lower panel).

SAP97-Ser-232 Is a CaMKII Phosphorylation Site—Several CaMKII phosphorylation consensus sequences are distributed along SAP97 protein (19). In particular, two optimal CaMKII consensus sites are present at the beginning of the PDZ1 domain (RGN-Ser-232-GLG) (13) and inside the PDZ3 domain (HRG-Ser472-TLG), respectively. Fusion proteins of GST with SAP97-PDZ1-(217-318) or SAP97-PDZ3-(465-545) were prepared and incubated with purified CaMKII in presence of [γ-32P]ATP as phosphate donor. Fig. 5A shows a representative autoradiography corresponding to in vitro CaMKII-dependent phosphorylation of SAP97-PDZ1-(217-318) and SAP97-PDZ3-(465-545) GST fusion proteins; a radioactive band correspond-
ing to both fusion proteins is clearly visible confirming the presence of the CaMKII phosphosites into the two PDZ domains. The phosphorylation signal shown by SAP97 fragments is specific because GST alone did not show any phospho-band (rightmost lane).

We then tested whether the full-length SAP97 was phosphorylated in vitro by CaMKII. GFP, GFP-SAP97wt, or GFP-SAP97(S232D) was transiently expressed in COS-7 cells, immunoprecipitated with anti-GFP, and subsequently phosphorylated in the presence of recombinant αCaMKII. Representative autoradiography in Fig. 5B shows that SAP97wt is effectively phosphorylated by exogenous αCaMKII; abrogation of Ser-232 as phosphosite by S232D mutation resulted in a significant reduction (−54.3 ± 4.4%, SAP97 S232D versus SAP97wt, p < 0.01; values are mean ± S.E. obtained from three experiments, each including two independent samples) of 32P incorporation in GFP-SAP97(S232D) when compared with GFP-SAP97wt.

To confirm that Ser-232 represented also in vivo a “major” phosphorylation site in SAP97, we performed metabolic labeling with [32P]orthophosphate in COS-7 cells transfected with either GFP-SAP97wt + αCaMKII-T286D or GFP-SAP97-(227–318) + αCaMKII-T286D (Fig. 5C). Cell lysates were immunoprecipitated with anti-GFP antibody. Autoradiography revealed a significant reduction of 32P incorporation in SAP97-(227–318)-transfected cells when compared with SAP97wt, indicating that Ser-232 is a major phosphorylation site in SAP97 also under these experimental conditions (−63.3 ± 5.1%, SAP97 S232D versus SAP97wt, p < 0.01; values are mean ± S.E. obtained from three experiments, each including two independent samples and normalized for SAP97wt and SAP97 S232D expression levels in total cell lysates). However, SAP97wt and SAP97 S232D expression levels were comparable in all experimental conditions as tested by Western blotting analysis using a polyclonal SAP97 antibody (data not shown).

CaMKII-dependent Phosphorylation of SAP97-Ser-232 Disrupts NR2A but Not GluR1 Interaction—By using the pull-out procedure, we tested whether the CaMKII-dependent phosphorylation of SAP97-PDZ1 region could influence the association of SAP97-interacting proteins (see Fig. 1B). GST-SAP97-PDZ1 fusion protein, phosphorylated in vitro by purified CaMKII in the presence or absence of ATP as phosphate donor, was incubated in a pull-out assay with solubilized hippocampal PSD. As expected, SAP97-PDZ1 domain was able to directly bind both the GluR1 subunit of the AMPA receptor and the NR2A subunit of the NMDA receptor (Fig. 6A). Phosphorylation of the CaMKII consensus site inside the SAP97-PDZ1 domain affects NR2A but not GluR1 binding (Fig. 6A); on this line, SAP97-(227–237) peptide containing SAP97-Ser-232 phosphosite competes with NR2A but not with GluR1 association in the in vitro pull-out assay (Fig. 6B).

CaMKII-dependent Phosphorylation Affects NR2A/SAP97 Co-clustering in Transfected COS-7 Cells—We then performed a clustering assay in transfected COS-7 cells to determine NR2A-SAP97 protein interaction in a mammalian cell context. As expected, double NR1 and SAP97 transfection confirmed the lack of interactions between the two proteins (Fig. 7, D–F). In contrast, cells double-transfected with NR2A and SAP97 formed plaque-like clusters in which SAP97 immunoreactivity co-localized precisely with NR2A (Fig. 7, G–I). In cells transfected with NR2A + SAP97 in presence of the competing SAP97-(227–237) peptide, however, SAP97 immunoreactivity remained diffuse and did not co-localize with NR2A clusters (Fig. 7, J–L). In addition, the SAP97(S232D) mutation mimick-
WB analysis was performed in total cell lysates (cell lysates were immunoprecipitated (i.p.) antibodies. Immunoprecipitated material with SAP97 and NR2A polyclonal

COS-7 cells were transfected with combinations of NR1, NR2A, SAP97, and NR2A/SAP97. CELLS were fixed 2 days after transfection and labeled with polyclonal SAP97, NR2A, and NR1 antibodies. A-C, single transfections with constructs encoding NR2A (A), SAP97 (B), or NR1 (C). SAP97 and NR1 when co-transfected do not show any co-localization pattern (D–F); co-transfection of SAP97 with NR2A leads to plaque-like clusters with a precise co-localization of the two proteins (G–J). Co-transfection with SAP97-(227–237) peptide (J–L) or mutation of SAP97 phosphosite S232D (M–O) disrupts NR2A/SAP97 co-localization leading to a diffuse SAP97 staining. No effect on NR2A/SAP97 clusters is observed by co-transfection with SAP97-(34–44) peptide (P–R). Scale bar, 10 µm. S, COS-7 cells were transfected with NR2A + GFP-SAP97 or with NR2A + GFP-SAP97(S232D), and cell lysates were immunoprecipitated (I.p.) with polyclonal anti-GFP. WB analysis was performed in total cell lysates (INPUT) and in the immunoprecipitated material with SAP97 and NR2A polyclonal antibodies.

CaMKII has been implicated in a variety of brain functions including activity-dependent regulation of synaptic plasticity by multiple mechanisms (11). Particularly relevant is its ability to transform a transient change in calcium levels into a long lasting change in kinase activity. CaMKII can phosphorylate a large number of proteins in vitro, and recently several substrates involved in synaptic plasticity have been identified (12, 19–21).

In this paper, we show that SAP97 directly interacts with the NR2A NMDA receptor subunit and that CaMKII-dependent phosphorylation of SAP97-Ser-232 modulates the dynamic association/dissociation of this complex. The co-localization of SAP97 and CaMKII in Western blot from different subcellular compartments, the in vitro phosphorylation of SAP97 fusion proteins, the metabolic labeling of cultured hippocampal neurons and transfected COS-7 cells, the co-immunoprecipitation of the two proteins, and the distribution pattern in transfected COS-7 cells, all these results clearly indicate that SAP97-Ser-232 is in vivo phosphorylated by CaMKII.

Basal phosphorylation of SAP97 in cultured resting hippocampal neurons is quite low, and phosphorylation is markedly enhanced by NMDA treatment. KN-93 treatment is able to reduce SAP97 phosphorylation to basal levels, indicating a central role for CaMKII in NMDA-mediated phosphorylation. This represents the first direct evidence of phosphorylation of a mammalian MAGUK protein by CaMKII, confirming recent in vitro data that identified MAGUK protein family as possible substrates in PSD for CaMKII (12, 21).

Metabolic labeling experiments in transfected COS-7 cells clearly address Ser-232 as a major CaMKII phosphorylation site in SAP97 protein. The SAP97-Ser-232 CaMKII consensus site here described corresponds to the Drosophila MAGUK Dlg-Ser-48 site indicated previously (13) to be implicated in the regulation of Dlg association with synaptic complex. On the other hand, inspection of SAP97 sequence reveals the presence of at least two other domains containing serine residues (Ser-39 in the N terminus and Ser-472 inside the PDZ3 domain) that might represent putative phosphate acceptor sites for CaMKII (19). Both sites are phosphorylated in vitro by the kinase even if with lower affinity when compared with PDZ21-Ser-232 (data not shown). Future experiments are needed to determine the possible role of these consensus sites that are located in two important strategic domains of SAP97 (22).

Recent studies (6–7, 23) have put forward the idea of a direct physical interaction between SAP97 and ionotropic glutamate receptor subunits, identifying GluR1 subunit of AMPA receptor and NR2A subunit of NMDA receptor as major binding targets for SAP97 in hippocampal neurons. In particular, the NR2A subunit has been demonstrated to display in vitro a strong interaction with SAP97; the C-terminal SDV motif of the NR2A
subunit has been proposed to be mandatory for efficient interaction with SAP97 (7). In addition, immunohistochemistry experiments comparing the distribution of NR2A and SAP97 demonstrated a high level of regional, cellular, and subcellular co-localization of the two proteins (3, 7).

Our results confirm and extend these findings. In fact, here we show that the SAP97-PDZ1 domain binds both NR2A and GluR1 and that CaMKII phosphorylation of SAP97-Ser-232 inside its PDZ1 consensus site is critical for SAP97 interaction with NR2A but not with GluR1. Significantly, we demonstrate that constitutive activation of CaMKII results in alteration of SAP97/NR2A clustering in triply transfected COS-7 cells. No effect is obtained when wild type enzyme is used, suggesting that constitutive activation of CaMKII results in dissociation of NR2A-SAP97 complex could take place in a Triton-soluble fraction mimicking the PSD fraction but not in a Triton-soluble fraction. This suggests that CaMKII-mediated dissociation of NR2A-SAP97 complex can take place in the PSD fraction or close to it where the kinase is highly enriched.

Together, our results demonstrate that the association of SAP97 with the NR2A subunit is regulated by CaMKII-dependent phosphorylation and provide a novel mechanism for the regulation of synaptic protein interactions during synaptic plasticity events.

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