Ion Channel Clustering by Membrane-associated Guanylate Kinases

DIFFERENTIAL REGULATION BY N-TERMINAL LIPID AND METAL BINDING MOTIFS*

Received for publication, December 9, 1999, and in revised form, April 20, 2000
Published, JBC Papers in Press, April 21, 2000, DOI 10.1074/jbc.M909919199

Alaa E. El-Husseini‡, J. Rick Topinka‡, Joshua E. Lehrer-Graiwer‡, Bonnie L. Firestein‡, Sarah E. Craven‡, Chiye Aoki§, and David S. Bredt¶

From the ‡Departments of Physiology and University of California, San Francisco, California 94143 and §Center for Neural Science, New York University, New York, New York 10013

Neurotransmission requires appropriate assembly of signal transduction machinery at synaptic sites. Although mechanisms for organization of these synaptic signaling complexes are unclear, recent studies suggest a general role for PDZ domain-containing membrane-associated guanylate kinases (MAGUK)1 in receptor clustering at pre- and postsynaptic sites (1–4).

Molecular cloning has identified four neuronal MAGUK proteins in mammals: PSD-95 (SAP-90), PSD-93 (Chapssyn-110), SAP-97 (hDLG), and SAP-102 (5–10). Immunohistochemical studies indicate that PSD-95 and PSD-93 occur primarily in a somatodendritic distribution and are specifically enriched at the PSD (11). Consistent with this postsynaptic localization, targeted disruption of PSD-95 in mouse disrupts N-methyl-D-aspartate receptor-dependent synaptic plasticity (12). Recent studies show that SAP-102 can be found at postsynaptic sites (7, 13). On the other hand, SAP-97 occurs in presynaptic terminals in forebrain neurons (8). Although SAP-97 knockout mice have not yet been reported, SAP-97 is the mammalian homologue of Drosophila discs large (DLG), and flies lacking DLG show prominent presynaptic defects (14).

Mechanisms for differential localization and function of MAGUK proteins remain uncertain. MAGUK proteins share extensive sequence homology in their PDZ, SH3, and guanylate kinase domains. Significant differences between these proteins occur primarily at their N termini, suggesting roles for these regions in differential functions of these proteins. Importantly, PSD-95 contains a pair of cysteine residues that are sites for protein palmitoylation (15), a post-translational modification that is essential for postsynaptic sorting (16). In contrast, SAP-97 lacks N-terminal cysteines, and it is not palmitoylated. PSD-95 is alternatively spliced at the N terminus to yield two major isoforms: the first, which we now term PSD-93α, has cysteines at positions 5, 7, and 12; and the second, which we term PSD-93β, has cysteines at positions 3 and 5, like PSD-95 (9). Finally SAP-102 has cysteines at positions 7, 8, 10, and 13 (7). It is uncertain whether PSD-93 or SAP-102 are palmitoylated and whether this regulates cellular trafficking and ion channel clustering by these proteins.

Here, we report that both PSD-95 and PSD-93 occur prominently at postsynaptic sites in hippocampal neurons, whereas SAP-102 localizes to both dendrites and axons in these cells. Similarly, we find that PSD-95 and both isoforms of PSD-93 mediate surface ion channel clustering, although SAP-97 and SAP-102 do not. Metabolic labeling studies show that PSD-95 and both isoforms of PSD-93 are robustly palmitoylated and that SAP-97 and SAP-102 are not. Mutants of PSD-95 and PSD-93 that disrupt palmitoylation also block surface receptor clustering, indicating a critical role for this lipid modification in ion channel aggregation at the plasma membrane. Rather than being palmitoylated, we find that N-terminal cysteines in SAP-102 tightly bind to zinc and that this unique N terminus of SAP-102 confers axonal targeting in addition to postsynaptic sites within dendrites in neurons. These data suggest that lipid modifications and heavy metal associations with the N termini of MAGUKs mediate differential functions and subcellular localizations of these synaptic scaffolds.

MATERIALS AND METHODS

Antibodies and Immunoblotting—The following primary antibodies were used: rabbit polyclonal antibodies to Kv1.4 (17), PSD-95 (9),

* This work was supported by a pre-doctoral research grant from the National Science Foundation and an Achievement Reward for College Scientists Foundation scholarship (to S. E. C.), a grant from the American Heart Association (to J. L.-G.), and postdoctoral grants from the Medical Research Council of Canada (to A. E. H.) and the National Institutes of Health (NIH) (NICHD) and Spinal Cord Research Foundation (to B. L. F.). This research was also supported by NIH Grant R01-NS36017 (to D. S. B.) and grants from the National Science Foundation and an Achievement Reward for College Scientists Foundation scholarship (to S. E. C.), a grant from the American Heart Association (to J. L.-G.), and postdoctoral grants from the National Institutes of Health (NIH) (NICHD) and Spinal Cord Research Foundation (to B. L. F.). This research was also supported by NIH Grant R01-NS36017 (to D. S. B.) and grants from the National Science Foundation, the National Association for Research on Schizophrenia and Depression, the EJLB, and the Culpeper and Beckman Foundations. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: University of California, San Francisco, School of Medicine, 513 Parnassus Ave., San Francisco, CA 94143-0444. Tel.: 415-476-6310; Fax: 415-476-4929; E-mail: bredt@itsa.ucsf.edu.

¶ The abbreviations used are: MAGUK, membrane-associated guanylate kinase; PSD, postsynaptic density; dg, discs large; GFP, green fluorescent protein; PDZ, postsynaptic density-95, discs large, zonula occludens; SAP, synapse-associated protein; GST, glutathione S-transferase; MAP-2, microtubule-associated protein-2; PAGE, polyacrylamide gel electrophoresis.
MAGUK Clustering Regulated by Lipid/Metal Binding Motifs

Fig. 1. Differential cellular distributions of neuronal MAGUKs in hippocampal cultures detected by immunofluorescence. PSD-95 (A, green) and PSD-93 (B, green) are found only at clusters that occur along MAP-2 (red)-positive dendritic processes. The small panels on the right show enlargements of the boxed regions on the left (yellow indicates regions in which the red and green overlap). SAP-102 (C, green) is also clustered along MAP-2 (red)-positive dendrites. D, these SAP-102 (green)-positive clusters are synaptic as they co-localize with synaptophysin (red). E, SAP-102 (green) is also present in a dense network of axonal processes that stain positively for neurofilament-H (NH, red). Scale bar = 10 μm.

PSD-95 (9), SAP-97 (15), SAP-102 (18) and monoclonal antibodies to PSD-95 (#046; Affinity Bioreagents) and GFP (Quantum, CLONTECH). GAD-65 antibody was a gift from Dr. Steinunn Baekkeskov (Dept. of Medicine, UCSF). All antisera were affinity-purified on columns containing the immunizing antigen linked to Affi-Gel-10 resin. For immunoblotting, protein extracts were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Primary antibodies were diluted in block solution containing 3% bovine serum albumin, 0.1% Tween 20 in Tris-buffered saline and incubated with membranes overnight at 4°C. Labeled bands were visualized using ECL (Amersham Pharmacia Biotech).

cDNA Cloning and Mutagenesis—Subcloning of wild-type, mutant, and chimeric forms of PSD-93, SAP-97, and SAP-102 as N-terminal fusions with GFP in pGW1 were analogous to those of PSD-95 previously described (15, 16). Sequences of all polymerase chain reaction primers are available upon request. Proper introduction of all mutations was verified by DNA sequencing.

Cell Transfection, Metabolic Labeling, and Immunoprecipitation—COS7 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin, and streptomycin. Cells were transfected using LipofectAMINE reagent according to the manufacturer’s protocol (Life Technologies, Inc.). For studies of palmitoylation, transfected COS7 cells were labeled in media containing 1 μCi/ml [3H]palmitic acid (50 Ci/mmol, NEN Life Science Products). Cells were washed with ice-cold phosphate-buffered saline and resuspended in 0.4 ml of lysis buffer containing TEE (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA), 150 mM NaCl, and 0.2% SDS. After extracting for 20 min at 4°C, Triton X-100 was added to 1% to neutralize the SDS, and insoluble material was removed by centrifugation at 10,000 × g for 10 min. For immunoprecipitation experiments, the samples were then incubated with GFP antibodies (1:150 dilution, CLONTECH) for 1 h at 4°C. After the addition of 20 μl of protein A-Sepharose beads (Amerham Pharmacia Biotech), samples were incubated for 1 h at 4°C. Immunoprecipitates were washed three times with buffer containing TEE, 150 mM NaCl, and 1% Triton X-100, boiled in SDS-PAGE sample buffer with 1 μl dithiothreitol for 2 min, and analyzed by SDS-PAGE.

For fluorography, protein samples were separated by SDS-PAGE and stained with Coomassie Blue. Gels were treated with Amplify (Amerham Pharmacia Biotech) for 30 min, dried under vacuum, and exposed to Hyperfilm-MP (Amerham Pharmacia Biotech) at −80°C for 3 to 5 days.

Transfection of Primary Neuronal Cultures and Immunofluorescent Labeling—Hippocampal cultures were transfected as described previously (16). Briefly, acutely dissociated hippocampal neurons from E18 rats were transfected in suspension by lipid-mediated gene transfer. Cells were then plated at a density of 600/mm² on glass coverslips (Fisher) and maintained in Neurobasal media (Life Technologies, Inc.) supplemented with B27. After washing with phosphate-buffered saline containing 0.3% Triton X-100 3 times for 5 min each, the cells were incubated in phosphate-buffered saline/Tween containing 3% normal goat serum for 1 h at room temperature to block nonspecific antibody interactions. Primary antibodies were added in block solution for 1 h at room temperature, followed by donkey anti-mouse or goat anti-rabbit secondary antibodies conjugated to Cy2 or Cy3 fluorophores (diluted 1:200 in block solution) for 1 h at room temperature. Coverslips were then mounted on slides (Frost Plus slides; Fisher) with Fluoromount-G (Southern Biotechnology Associates, Inc.), and images were taken under fluorescence microscopy with a 100× oil immersion objective (numerical aperture = 1.4) affixed to a Leica upright microscope.

Quantitative Measurement of GFP Expression—Quantification of polarized protein expression in dendrites versus axons was performed on 10–15 neurons from 2–3 independent transfections. Images of neurons were acquired with a CCD camera and quantitated using Metamorph imaging software (Universal Imaging). The exposure time of the camera was adjusted to limit photobleaching and so that maximum pixel intensity was approximately one-half to three-fourths saturating for cells with low to moderate expression levels, as determined by total pixel

Fig. 2. Localization of SAP-102 at presynaptic, postsynaptic, and non-synaptic sites in cerebral cortex. Adult cortical sections were immunolabeled using horseradish peroxidase-3,3′-diaminobenzidine tetrahydrochloride as the label. Four synapses are shown in this panel, with arrows placed across synaptic clefts in the pre-to-post-synaptic direction. Synapses 1 through 3 are all axo-spinous and asymmetric, indicating that they are glutamatergic but differ in terms of SAP-102 distribution. SAP-102 occurs only postsynaptically at synapse 1, neither pre- nor postsynaptically at synapse 2, and only pre-synaptically at synapse 3. Synapse 4 is symmetric, axo-dendritic, and probably GABAergic. Neither side of this synapse is immunolabeled. In addition, the field shows two more labeled axonal varicosities (ia) and an unlabeled axon varicosity (ia). Calibration bar = 500 nm.
counts. The average background fluorescence of untransfected cells was subtracted. The degree of polarized expression was determined by calculating the average pixel intensity in the axon versus that in the dendrites. The average pixel intensity was calculated through three dendrites and three sections of the axon (each 20 μm in length). These averages were then converted into a ratio of axonal versus dendritic expression. The data were analyzed by paired r test.

Electron Microscopic Immunocytochemistry—Adult Harlan Sprague-Dawley rats were fixed by transcardial perfusion with an aldehyde mixture consisting of 4% paraformaldehyde and 3% acrolein, buffered with 0.1 M phosphate buffer, pH 7.4. Sodium borohydride was used to terminate tissue fixation. Brain sections (40 μm) containing primary visual cortex were prepared using a Vibratome. Sections were blocked in saline buffered with 0.01 M phosphate (phosphate-buffered saline) containing 1% bovine serum albumin. After an overnight incubation with affinity-purified anti-SAP-102 antibody (1 μg/ml), sections were processed for immunolabeling with the avidin-biotin-horseradish peroxidase complex using the ABC Elite kit (Vector) and 3,3'-diaminobenzidine (Aldrich) and hydrogen peroxide as substrates (19). These sections were post-fixed with 1% glutaraldehyde for 10 min, then with 1% osmium tetroxide for 1 h, and stained as a whole with 4% uranyl acetate. Sections were dehydrated for infiltration with the EMBED 812 resin (EM Sciences) for ultrathin sectioning. Sections (80 nm) were examined without lead citrate counterstaining.

UV Absorbance—A GST fusion protein corresponding to amino acids 1–118 of SAP-102 was expressed and purified from Escherichia coli and dialyzed against 50 mM Tris-HCl, pH 7.6. The SAP-102 N-terminal peptide, corresponding to amino acids 1–20, was synthesized by AnaSpec. The fully reduced peptide was prepared by solubilizing in 0.1% trifluoroacetic acid, reducing with 50 mM dithiothreitol at 90 °C for 30 min, precipitating with 1% TCA at 0 °C for 15 min, centrifuging at 14,000 g for 5 min, and washing twice in ether. This reduced peptide was desiccated and stored at −70 °C until use. Immediately before use, the peptide was resuspended (100 μM) in 50 mM Tris-HCl, pH 7.6, containing various concentrations of ZnCl2 and spectra were recorded on a Cary 1E UV-visible spectrophotometer. Difference spectra were calculated by subtracting absorbance for peptide alone from absorbance for peptide with ZnCl2.

Circular Dichroism—SAP102 1–20 peptide was prepared as above. CD spectra were recorded on a Jasco J710 spectropolarimeter, and each recording of peptide with ZnCl2 was corrected for the spectrum of the corresponding concentration of ZnCl2 without peptide.

FIG. 3. Differential localization of SAP-102 and PSD-95 in transfected hippocampal neurons mediated by N-terminal motifs. A, appending the N-terminal region of SAP-102 to PSD-95 redistributes the chimera to axons and dendrites. Hippocampal neurons were transfected with cDNAs encoding fusions of GFP tagged to PSD-95, SAP-102, or a chimera of PSD-95 containing the N terminus of SAP-102 (102/95). PSD-95 occurs primarily in dendrites, whereas both SAP-102 and 102/95 are present in axons (arrows) and dendrites. B, a bar graph summarizes expression data for PSD-95, SAP-102, and 102/95 transfected into hippocampal neurons. GFP signals were quantitated in axons and dendrites as described under "Materials and Methods." Significant fluorescence from SAP-102 and 102/95 is detected in axons (~65% that in dendrites). In contrast, minimal PSD-95 fluorescence is detected in axons (14%). Statistical analysis shows that the axon/dendrite fluorescence densities for SAP-102 (p < 0.027) and 102/95 (p < 0.014) are significantly different from PSD-95 but not from each other (p < 0.95). C, SAP-102 localizes to presynaptic sites. A high power fluorescence image of a hippocampal neuron transfected with GFP-SAP-102 shows the detailed localization of SAP-102 in the axon (arrow) and dendrites. Arrowheads point at puncta in the axon enriched for SAP-102. Bottom panels show that SAP-102 (green)-positive axonal puncta co-localize with the presynaptic marker GAD-65 (red) middle. The overlay of SAP-102 and GAD-65 is shown at bottom right. Scale bar = 10 μm.

FIG. 4. Differential palmitoylation of neuronal MAGUKs. A, sequence alignment of the N termini of PSD-95, PSD-93α, PSD-93β, PSD-97, and SAP-102 (cysteine residues are in bold) COS cells were transiently transfected with PSD-95, PSD-93β (PSD-93C5, 7), PSD-93α (PSD-93C3, 5), SAP-97, or SAP-102 fused to GFP and were metabolically labeled with [3H]palmitate. Cells were lysed in radioimmune precipitation buffer, and the solubilized material was immunoprecipitated with an antibody to GFP. Immunoprecipitates were loaded onto duplicate gels that were analyzed for [3H]palmitate by fluorography (upper gel) or were immunoblotted for GFP (lower gel). PSD-95 and both isoforms of PSD-93 are palmitoylated, whereas SAP-97 and SAP-102 are not. Mutating cysteine 5 of PSD-93 (PSD-93 C5S) to serine abolishes palmitoylation, and mutation of cysteine 7 (PSD-93 C7S) dramatically reduces palmitoylation.

The data were analyzed by paired t test.
RESULTS

Differential Localization of PSD-95/93 and SAP-102 in Neurons—To characterize differential functions for the neuronal MAGUK proteins, we compared their cellular distribution in primary hippocampal neuronal cultures. Low density neuronal cultures were maintained in neurobasal medium, and mature neurons at 2–3 weeks in culture were analyzed by immunofluorescence. As previously reported (11), PSD-95 and PSD-93 localize exclusively to small puncta that correspond to synaptic sites (Fig. 1), as they co-localize with synaptophysin (Ref. 11 and data not shown). These synaptic puncta occur along dendritic processes (Fig. 1) that are positive for the dendritic marker microtubule-associated protein-2 (MAP-2).

By contrast, SAP-102 shows a more diffuse localization in the hippocampal neurons; it is present in the dendritic cytoplasm and is modestly enriched at synapses (Fig. 1). Unlike PSD-95 and PSD-93, SAP-102 also occurs in processes that are labeled with the axonal marker neurofilament-H (Fig. 1). Axonal staining for SAP-102 is likely specific as it is blocked by preadsorbing the antibody with the immunizing antigen (data not shown). We find that exogenous PSD-95 is enriched in dendrites, whereas SAP-102 is present in both dendrites and axons (Fig. 3, A, B, and C). In the axons, SAP-102 shows diffuse staining but is also enriched at presynaptic sites, where it co-localizes in GABAergic neurons with the presynaptic marker GAD-65 (Fig. 3C; lower panels). Replacing the palmitoylation motif of PSD-95 with the unique N terminus of SAP-102 mediates localization of the chimera to both axons and dendrites (Fig. 3, A and B) in a pattern similar to SAP-102. These results indicate that the N terminus of SAP-102 may use a different mechanism than that of PSD-95 to localize to postsynaptic sites and to localize presynaptically.

Differential Palmitoylation of PSD-95/93 versus SAP-97/102—Recent studies demonstrate that N-terminal palmitoylation of PSD-95 is necessary for postsynaptic targeting (16). This raises the possibility that differential palmitoylation of the MAGUK proteins accounts for the differential subcellular localization of these proteins. To address this, cells transfected with neuronal MAGUK proteins were metabolically labeled with [3H]palmitate, and following immunoprecipitation and SDS/PAGE, protein palmitoylation was detected by autoradiography. As previously reported (15), we find that PSD-95 is robustly palmitoylated (Fig. 4). We also find that both isoforms of PSD-93, the α isoform, which has cysteines at positions 5 and 7, and the β isoform, which has cysteines at positions 3 and 5, are robustly palmitoylated (Fig. 4). On the other hand SAP-97 and SAP-102 are not detectably palmitoylated (Fig. 4). Previous studies have shown that mutations of cysteines 3 or 5 from

Fig. 5. PSD-95 and PSD-93 but not SAP-97 or SAP-102 can mediate cell surface ion channel clustering. COS cells were co-transfected with Kv1.4 and each of the neuronal MAGUKs. Cells were fixed 48 h post-transfection and double-labeled with antibodies to the appropriate MAGUK (green) and Kv1.4 (red). PSD-95 and both isoforms of PSD-93 co-cluster with Kv1.4, as evidenced by formation of large irregular patches on the cell surface. By contrast, SAP-97 and SAP-102 do not induce cell surface ion channel clustering but rather form intracellular clusters that accumulate on the nuclear membrane and in intracellular perinuclear structures. Scale bar = 10 μm.
PSD-95 disrupt palmitoylation (15). Similarly, we find that mutating cysteine 5 or cysteine 7 of PSD-93α to serine abolishes protein palmitoylation (Fig. 5 and data not shown).

Palmitoylation Determines Differential Cell Surface Ion Channel Clustering Activity of PSD-93/95 and SAP-97/102—At postsynaptic sites, certain MAGUK proteins cluster ion channels with downstream protein networks (2, 4, 20). Some aspects of this clustering activity can be reproduced in heterologous cell co-transfections. In this assay co-transfection of PSD-95 with an interacting ion channel results in co-localization of both proteins to surface patches on the plasma membrane (17). To determine systematically which MAGUK proteins have ion channel clustering activity in this assay we individually co-transfected each of the neuronal MAGUKs with K+ channel Kv1.4. When expressed alone, the neuronal MAGUKs or the K+ channel occur diffusely throughout the COS cells (Fig. 5). As previously reported (17), co-transfection of PSD-95 with Kv1.4 results in surface clustering of both molecules (Fig. 5), whereas co-transfection of SAP-97 with Kv1.4 results in prominent perinuclear intracellular structures (21). Here we find that both isoforms of PSD-93 (α and β) form membrane clusters with Kv1.4. On the other hand, co-transfection of SAP-102 with the K+ channel results in prominent co-localization on the nuclear membrane, and both proteins also accumulate in large round structures in the cytoplasm in a pattern similar to SAP-97 (Fig. 5). These data demonstrate that postsynaptic MAGUKs, PSD-95, and PSD-93 can mediate plasma membrane clustering of interacting ion channels, but the more diffusely localized MAGUKs, SAP-97, and SAP-102 cannot. These results also show that only palmitoylated MAGUKs are capable of clustering ion channels on the plasma membrane.

To evaluate a role for protein palmitoylation in the differential cell surface ion channel clustering activity of neuronal MAGUKs, we transfected COS cells with various mutant and chimeric MAGUKs together with Kv1.4. As previously reported (22), mutation of cysteines 3 and 5 of PSD-95 prevents ion channel clustering with Kv1.4. In co-transfections with this palmitoylation-deficient isoform of PSD-95, staining for both PSD-95 and Kv1.4 occurs along the nuclear membrane and in large perinuclear structures (data not shown). Similarly, co-transfections with palmitoylation-deficient forms of PSD-93α (C5,7S) also show no clustering and yield prominent staining of the nuclear membrane and of intracellular juxtanuclear spots (Fig. 5 and data not shown). This pattern is similar to that observed in co-transfections of Kv1.4 with wild-type SAP-97 or SAP-102. To further establish a role for palmitoylation in cell surface channel clustering, we evaluated chimeras in which the palmitoylated N termini of PSD-95 or PSD-93α replaced the N terminus of SAP-97 or the palmitoylated N terminus of PSD-93α replaced the N terminus of PSD-95. These constructs, which induce palmitoylation of the chimeras, also mediate cell surface clustering activity (Fig. 6). Kv1.4 does not co-cluster on the plasma membrane with a chimera in which a cysteine mutant form of the PSD-93α N terminus is fused PSD-95 (93:1–64(C5,7S)/PSD-95. Scale bar = 10 μm.

Zinc Binding Activity of the N Terminus of SAP-102—It is striking that SAP-102, which contains four N-terminal cysteines at positions 7, 8, 11, and 13 is not palmitoylated and does not mediate surface ion channel clustering. The presence of these cysteines in the absence of palmitoylation suggests other important functions for this unusual cluster of cysteines. Also conspicuous in the N terminus of SAP-102 are histidine residues at positions 2, 4, and 6. This concentrated cluster of histidine and cysteine residues at the N terminus suggests a possible role in heavy metal binding, as histidine and cysteine residues often participate in metal ligation (23, 24). In fact, the N-terminal sequence of SAP-102 bears considerable homology to RING fingers and LIM domains, which both bind to zinc (24).

To evaluate possible zinc binding by SAP-102, we purified a GST fusion protein containing the N-terminal 100 amino acids of SAP-102 from E. coli. Optical absorption spectroscopy provided evidence that this fusion protein binds zinc. The spectra obtained with reduced SAP-102 and 0–50 μM ZnCl2 show that the absorbency increases as Zn2+ is added (Fig. 7A). There is a maximum at ~240 nm in the difference spectra, indicating charge transfer transitions between the metals and sulfur ligands. Our Zn2+ spectra are quite similar to those found for other protein-metal complexes such as those of zinc finger or metallothionein proteins (23, 25). No metal binding to GST alone, as evidenced by a spectral shift with 1–10 μM ZnCl2, was detected (Fig. 7B).

To determine whether zinc binds to the unique cysteine-and histidine-rich region, we evaluated binding to a synthetic 20-
mer peptide corresponding to the extreme N terminus of SAP-102. Similar to what was found with the fusion protein, we found that zinc addition specifically induces a dramatic change in the absorbance spectrum with an absorbance maximum at 240 nm (Fig. 7C). Difference ultraviolet absorption spectroscopy with 0.5 to 4.0 m equivalents of ZnCl₂ shows that binding occurs at a stoichiometry of one Zn²⁺ per mole of SAP-102 peptide.

Circular dichroism studies demonstrate that zinc binding dramatically alters the secondary structure of the N-terminal SAP-102 peptide. The circular dichroism spectrum of the reduced apo-peptide displays a minimum near 200 nm (Fig. 7D), which is expected for a disordered polypeptide. The addition of ZnCl₂ produces marked changes in the spectrum, with an increase in the negative ellipticity, consistent with gain of secondary structure. Again, titration of the peptide showed a plateau at one molar equivalent of added ZnCl₂ (Fig. 7D).

**DISCUSSION**

This study demonstrates that the N termini of neuronal MAGUKs determine in large part the differential cellular distribution and neuronal functions for these proteins. PSD-93 and PSD-95 are robustly palmitoylated, and both are enriched at postsynaptic sites in neurons and mediate cell surface ion channel clustering in heterologous cells. By contrast SAP-97 and SAP-102 are not modified by this lipidation also occur in neuronal axons. The addition of the N terminus of SAP-102 to PSD-95 enhances its axonal localization and accumulation at both presynaptic and postsynaptic sites. The central role for palmitoylation in surface ion channel clustering by MAGUKs is demonstrated by analysis of chimeric constructs. Appending the N-terminal palmitoylation consensus of PSD-95 or PSD-93α onto SAP-97 induces ion channel clustering on the plasma membrane.

Considering that the palmitoylated N termini of PSD-95 and PSD-93 are critical for protein function, it is interesting to note that PSD-93 is alternatively spliced in this region (9). cDNA cloning from a rat brain library identified four distinct N-terminal forms of PSD-93. The two forms most commonly isolated, termed here PSD-93α and PSD-93β, contain N-terminal cysteines, whereas the other two minor forms of PSD-93 do not.

![Fig. 7. Zinc binding to the N terminus of SAP-102. A and B, ultraviolet absorption spectra of SAP-102-GST or GST (160 μg/ml) with 0–10 μM ZnCl₂. C, ultraviolet absorption difference spectra of reduced SAP-102 N-terminal peptide (100 μM) with 50–400 μs added ZnCl₂. D, circular dichroism of SAP-102 N-terminal peptide (100 μM) with 0–150 μM ZnCl₂.](image-url)
contain these cysteines (9). Furthermore, alternative splicing of the PSD-93 N-terminal domains is tightly controlled in a tissue-specific fashion (9). Because alternative N-terminal splicing can alter ion channel clustering activity of MAGUK proteins, this may provide an important mechanism for modulating synaptic assembly and plasticity.

Why might PSD-95 and PSD-93, two major components of the postsynaptic density, be palmitoylated? As previous work has shown that this modification is essential for postsynaptic targeting (16), it is possible that palmitoylation functions primarily as a signal for postsynaptic sorting. However, many postsynaptic proteins are not palmitoylated, which suggests other functions for palmitoylation of MAGUKs. Unlike other lipid modifications, palmitoylation is readily reversible, and dynamic modulation of this lipidation may afford plasticity (26). In non-neuronal cells, many palmitoylated proteins occur together in caveolae, cholesterol-rich cave-like invaginations of the plasma membrane (27). Whereas neurons lack anatomical caveolae and lack the scaffolding protein caveolin, cholesterol-rich low density membrane fractions have been isolated from brain (28). Palmitoylated PSD-95 and PSD-93 may be analogous to caveolin and function to assemble signal transduction cascades at specialized lipid domains. Indeed several intracellular enzymes that associate with the PSD-95/N-methyl-d-aspartate receptor complex activity, including Fyn (29) and neuronal nitric-oxide synthase (30), are found in low density lipid compartments (31, 32).

Whereas PSD-95 and PSD-93 play critical roles in assembling signaling machinery at the PSD, we find that SAP-102 also occurs in axons. Insight into roles for MAGUK proteins in axons are suggested by the phenotype of Drosophila dlg, a MAGUK of invertebrates (33). Discs large mutant flies show structural defects at the postsynaptic neuromuscular junction (14) but also have presynaptic defects, which include increased neurotransmitter release (14). Both the pre- and postsynaptic defects are rescued by presynaptic but not postsynaptic expression of DLG (14). Furthermore, presynaptic expression of either SAP-97 or SAP-102 can rescue the phenotype of dlg mutants (34).

Because SAP-102 contains N-terminal cysteines, it was surprising to find that SAP-102 is not palmitoylated and does not mediate axon channel clustering on the plasma membrane. Instead, we find that the N terminus of SAP-102 contains a unique cysteine- and histidine-rich motif that binds to zinc. This zinc binding is high affinity and saturable, such that 1 mol of zinc binds/mol of SAP-102. Zinc binding to the N terminus of SAP-102 is reminiscent of several other presynaptic proteins that contain conserved zinc binding motifs including, RIM (35), rabphilin, bassoon (36), and piccolo (37). These proteins, many of which contain PDZ domains together with N-terminal zinc fingers, appear to regulate synaptic vesicle trafficking and fusion. It will now be important to determine how the N-terminal zinc binding motif of SAP-102 regulates both targeting and function at pre- and postsynaptic sites.

REFERENCES
1. Kennedy, M. B. (1998) Brain Res. Rev. 26, 243–257
2. Sheng, M., and Wyszynski, M. (1997) Bioessays 19, 847–853
3. O'Brien, R. J., Lau, L. F., and Huganir, R. L. (1998) Curr. Opin. Neurobiol. 8, 364–369
4. Craven, S. E., and Bredt, D. S. (1998) Cell 93, 495–498
5. Cho, K. O., Hunt, C. A., and Kennedy, M. B. (1992) Neuron 9, 929–942
6. Kistner, U., Wenzel, B. M., Veh, R. W., Cases-Langhoff, C., Garner, A. M., Appeltauer, U., Voss, B., Gundelfinger, E. D., and Garner, C. C. (1993) J. Biol. Chem. 268, 4580–4583
7. Muller, B. M., Kistner, U., Kindler, S., Chung, W. K., Kuhlendahl, S., Fenster, S. D., Lau, L. F., Veh, R. W., Huganir, R. L., Gundelfinger, E. D., and Garner, C. C. (1996) Neuron 15, 255–265
8. Muller, B. M., Kistner, U., Veh, R. W., Cases-Langhoff, C., Becker, B., Gundelfinger, E. D., and Garner, C. C. (1996) J. Neurosci. 16, 1217–1229
9. Ray, A., Kim, E., Sheng, M., and Craig, A. M. (1998) J. Neurosci. 18, 1217–1229
10. Migaud, M., Charlesworth, P., Dempster, M., Webster, L. C., Watabe, A. M., Nakihara, M., He, Y., Ray, M. F., Morris, R. G., Morrison, J. H., O'Dell, T. J., and Grant, S. G. (1998) Nature 396, 433–439
11. Sans, N., Petralia, R. S., Wang, Y. X., Blaho, J. Jr., Heil, W. J., and Wenthold, R. J. (2000) J. Neurosci. 20, 1260–1271
12. Budnik, V., Koh, Y. H., Guan, B., Hartmann, B., Hough, C., Woods, D., and Gorczyca, M. (1996) Neuron 17, 627–640
13. Topinka, J. R., and Bredt, D. S. (1998) Neuron 20, 125–134
14. Cravens, S. E., El-Hussein, A. E., and Bredt, D. S. (1999) Neuron 22, 497–509
15. Kim, E., Niethammer, M., Sheng, M., and Su, M. (1999) Nature 378, 85–88
16. Firestein, B. L., Brennan, J. E., Aoki, C., Sanchez-Perez, A. M., El-Hussein, A. E., and Bredt, D. S. (1999) Neuron 24, 659–672
17. Hau, S. M., Raine, L., and Fanger, H. (1991) J. Histochem. Cytochem. 29, 577–580
18. Kornaz, H.-C., Seeburg, P. H., and Kennedy, M. B. (1997) Curr. Opin. Neurobiol. 7, 368–373
19. Kim, E., and Sheng, M. (1996) Neuropharmacology 35, 993–1000
20. Huseh, Y. P., Kim, E., and Sheng, M. (1997) Neuron 18, 803–814
21. Berg, J. M. (1990) Annu. Rev. Biophys. Biophys. Chem. 19, 405–421
22. Schwabe, J. W., and Klug, A. (1994) Nat. Struct. Biol. 1, 345–349
23. Vallen, B. L., and Auld, D. S. (1995) EXS (Basel) 73, 259–277
24. Milligan, G., Parenti, M., and Magee, A. I. (1995) Trends Biochem. Sci. 20, 181–187
25. Lisanti, M. P., Tang, Z., Scherer, P. E., Kubler, E., Koleske, A. J., and Sargiacomo, M. (1995) Mol. Membr. Biol. 12, 121–124
26. Wu, C., Butz, S., Ying, Y., and Anderson, R. G. W. (1997) J. Biol. Chem. 272, 3554–3559
27. Tang, Z., Scherer, P. E., Koleske, A. J., Gorczyca, M. (1996) J. Neurosci. 16, 577–578
28. Wu, C., Butz, S., Ying, Y., and Anderson, R. G. W. (1997) J. Biol. Chem. 272, 3554–3559
29. Tanaka, K., Umemori, H., Akiyama, T., Nakashima, S., and Namamoto, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 435–440
30. Brennan, J. E., Chen, D. S., Ge, S. H., McGee, A. W., Cravens, S. E., Santilliano, D. R., Huang, F., Xia, H., Peters, M. F., Frohnhauer, S. C., and Bredt, D. S. (1996) Cell 84, 757–767
31. Harder, T., Scheiffele, P., Verkade, P., and Simons, K. (1998) J. Cell Biol. 141, 929–942
32. Keshet, G. I., Ovarda, H., Taraboulos, A., and Gabizon, B. (1999) J. Neurochem. 72, 1224–1231
33. Woods, D. F., and Bryant, P. J. (1991) Cell 66, 451–464
34. Thomas, U., Plankinauven, B., Muller, B., Garner, C. C., and Gundelfinger, E. D. (1997) Mech. Dev. 62, 161–174
35. Wang, Y., Okamoto, M., Schmitt, F., Hofmann, K., and Sudhof, T. C. (1997) Nature 388, 593–598
36. tom Dieck, S., Sanmarti-Vila, L., Langnaese, K., Richter, K., Kindler, S., Sattel, A., Wex, H., Smalls, K. H., Kumpf, U., Franz, J. T., Stumm, M., Garner, C. C., and Gundelfinger, E. D. (1998) J. Cell Biol. 142, 499–509
37. Cases-Langhoff, C., Voss, B., Appeltauer, U., Takei, K., Kindler, S., Veh, R. W., De Camilli, P., Gundelfinger, E. D., and Garner, C. C. (1996) Eur. J. Cell Biol. 69, 214–23