Selective Binding of Synapse-associated Protein 97 to GluR-A α-Amino-5-hydroxy-3-methyl-4-isoxazole Propionate Receptor Subunit Is Determined by a Novel Sequence Motif*©

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A family of closely related PDZ domain-containing membrane-associated guanylate kinase homologues (MAGUKs) is involved in the regulation of the amount and functional state of ionotropic glutamate receptors in excitatory synapses. To understand the mechanisms that determine the specificity of these interactions, we examined the structural basis of the highly selective association between the ionotropic Glur subunit GluR-A and synapse-associated protein 97 (SAP97). The C terminus of GluR-A bound to the PDZ domains of SAP97, but not to those of three related MAGUKs, PSD-93, PSD-95, and SAP102. Experiments with single PDZ domains indicated that the strongest contribution was by the second PDZ domain. Unexpectedly, mutation analysis of the GluR-A C terminus revealed that a tripeptide sequence SSG at position −9 to −11 plays an essential role in this binding, in addition to a C-terminal type I PDZ binding motif (leucine at C terminus and threonine at the −2 position). Analysis of the in vitro MAGUK-binding properties of a GluR-D mutant with a one-residue deletion at the C terminus provides further support for the view that an SSG sequence located N-terminally from a type I PDZ binding motif can mediate selective binding to SAP97 and suggest the existence of a novel variation of the PDZ domain–peptide interaction.

Synapse-associated protein 97 (SAP97) and the closely related SAP90/PDS-95, SAP102, and PSD-93/psd-93 form a family of membrane-associated guanylate kinase homologues (MAGUKs), characterized by the presence of three PDZ domains, an SH3 domain, and a C-terminal guanylate kinase homologous domain (1–5). Interactions of the PDZ domains of MAGUK proteins with type I C-terminal binding motifs present in ionotropic glutamate receptor subunits have been implicated in the regulation of the organization and functional activity of glutamatergic synapses (for review, see Refs. 6 and 7). Direct physical association of these MAGUK proteins with the subunits of N-methyl-D-aspartate (NMDA) (4, 8–10), kainate (11), and α-amino-5-hydroxy-3-methyl-4-isoxazole propionate (AMPA)-selective glutamate receptors (12) has been demonstrated, but the regulation and detailed physiological functions of these interactions are still unclear.

Interaction between the AMPA receptor subunit GluR-A (GluR1) and SAP97 is particularly interesting (12). First, an interaction between the C terminus of GluR-A and a type I PDZ domain protein has been implicated in several models of activity-dependent regulation of synaptic strength (13). Second, it is to date the only PDZ domain interaction of AMPA receptors that does not involve the Glur-B subunit and, therefore, may be relevant for the synaptic organization of calcium-permeable AMPA receptors, which lack this subunit (14). Third, in contrast to the majority of synaptic MAGUKs, which generally share binding partners, GluR-A appears to bind only to SAP97. However, the true selectivity and its underlying mechanisms have yet to be defined. In the present study, we show that GluR-A binds to the second PDZ domain of SAP97 but not to the PDZ domains of PSD-93, PSD-95, or SAP102 and that the recognition of the GluR-A C terminus by SAP97 is critically dependent on an SSG sequence located outside the canonical PDZ binding motif.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotide primers were purchased from Sigma Genosys. Restriction and modifying enzymes were from New England Biolabs, Finnzymes, or Fermentas. Anti-FLAG M1 antibody was purchased from Sigma Chemical Co., anti-His antibody was from Roche Molecular Biochemicals, and anti-GFP antibody-agarose conjugate was obtained as kind gifts from Dr. C. Garner (University of Alabama, Birmingham, AL) and Dr. D. Bredt (University of California, San Francisco, CA), respectively.

DNA Constructs—General molecular biological procedures were performed according to standard procedures (15). For the expression of GST fusion proteins, pGEX-3T vector (Amersham Biosciences) or its
In E. coli strain BL21 (GST fusions), or BL21(DE3)/pLysS (His-tagged proteins) according to standard procedures. Briefly, overnight cultures from single colonies grown in 10 ml of LB medium containing 100 μg/ml ampicillin were diluted 1:10 with LB medium, and then growth was continued under continuous shaking at 37 °C for 2–4 h until the culture reached an A 600 of about 0.5. Then, isopropyl-β-D-thiogalactopyranoside was added to the final concentration of 0.1–0.2 mg/ml, and the culture was transferred to 30 °C and incubated on a shaker platform for another 2 h. The cells were harvested by centrifugation, suspended in 10 ml of PBS or TBS containing 1 μg/mGL 100 μg/ml aprotinin, and 10 μg/ml leupeptin, and sonicated on ice with a tip sonicator by applying three bursts for 10 s on a low intensity setting. Insoluble material was removed by centrifugation (20,000 g, 15 min, 4 °C). For purification of the recombinant proteins, the supernatants were incubated with glutathione-Sepharose 4B (1 ml per 10 ml of culture) at 4 °C for 1–2 h, then the resin was washed five times with PBS or TBS. Finally, GST fusion proteins were eluted in 15 mM glutathione (2 × 1 ml), and His-tagged proteins were eluted in 0.2 M imidazole (2 × 1 ml).

**Results**

**Interaction of GluRA with SAP97**—First, we used immunoprecipitations and GST pull-down assays to demonstrate the specific interaction between GluRA and SAP97, originally reported by Leonard and co-workers (12). GluRA was present as a 100-kDa species in an immunoprecipitate prepared from rat cerebellar extract by using an antisera raised against the N-terminal domain (residues 2–100) of SAP97 but not with the corresponding preimmune serum. Likewise, an antisera raised against the C-terminal domain of GluRA subunit specifically precipitated immunoreactive SAP97, appearing as a doublet in the 120-kDa range (Fig. 1A). GST pull-down assays were used to study the specificity of the interaction between SAP97 and GluRA. Instead of C-terminal 10- to 11-mer peptides used previously (12), we used the entire C-terminal domains (CTDs) of AMPA receptor subunits. Myc-tagged SAP97, solubilized from transfected HEK293 cells, bound to GST-fused microtubule-binding protein CRIP and to the residues 1326–1464 of the NMDA receptor subunit NR2A, both of which were used as positive controls, but did not bind to GST alone (Fig. 1B). The CTD of GluRA but not of GluR-B, GluR-C, or GluR-D subunits bound SAP97 (Fig. 1B). An identical binding pattern was observed when the purified, bacterially expressed, His-

d-tagged PDZ1–3 segment of SAP97 was used instead of the myc-tagged full-length molecule, indicating that the binding is mediated by the PDZ domains in all cases (see below).

**MAGUK: Selectivity of GluRA PDZ Interaction**—We examined the selectivity of the GluRA interaction with respect to the MAGUK protein by using GST pull-down assays. First, we tested the binding of histidine-tagged PDZ1–3 domains of SAP97, PDG55, PDG93, and SAP102 to GST-fused CTDs of GluRA, GluR-D, and GluR-6, the C-terminal segment of NR2A, and the microtubule-binding protein CRIP. All four PDZ1–3 proteins bound to CRIP and to the C-terminal domains of GluR6 and NR2A, but only SAP97-PDZ1–3 bound to GluRA CTD (Fig. 2A). The specificity of the observed interactions is suggested by the finding that none of the PDZ1–3 proteins bound to the CTD of the GluR-D subunit (Fig. 2A) or...
to GST alone (results not shown). We used a reverse approach to determine the binding of GluR-A, solubilized from rat cerebellum to GST-fused PDZ1–3 domains of PSD-93, PSD-95, SAP97, and SAP102. Immunoreactive GluR-A was observed in the glutathione eluate from SAP97PDZ1–3 but not in the eluates from PSD-95_PDZ1–3, PSD-93_PDZ1–3, and SAP102_PDZ1–3 (Fig. 2B). These findings demonstrate the highly selective nature of the interaction both with respect to the AMPA receptor subunit and the MAGUK protein.

GluR-A Binds to the Second PDZ Domain of SAP97—We next analyzed the relative contributions of individual SAP97 domains to the interaction with GluR-A. Binding of AMPA receptors solubilized from rat cerebellum to separate PDZ domains of SAP97 was determined in a GST pull-down assay. Immunoblotting showed association of GluR-A-containing receptor subunits GluR-A and GluR-D. CRIPT and a C-terminal segment (residues 1326–1464) of the NMDA receptor subunit NR2A were used as positive controls. The bound SAP97 was eluted and detected by Western blotting using anti-myc antibody. The lower panel shows the corresponding protein stained eluates.

That the second PDZ domain of SAP97 provides the major contribution to the interaction with GluR-A.

Type I PDZ Binding Motif at the C Terminus of GluR-A—The experiments described above indicate that GluR-A interacts primarily with the PDZ2 domain of SAP97 and can discriminate against PSD-93, PSD-95, and SAP102. The PDZ2 domains of the other three synaptic MAGUK proteins are 79–88% identical to that of SAP97, and the residues that interact with the peptide ligand (as predicted on the basis of structural information) are fully conserved. Therefore, the observed selectivity of the in vitro interaction is surprising. Hence, it was of considerable interest to see if an analysis of the GluR-A C terminus would reveal any unusual features responsible for the binding to SAP97. In the majority of analyzed PDZ-peptide interactions, the C-terminal 4–5 residues play the major role in determining the binding specificity. Thus, we first analyzed the effects of individual alanine or glycine (at position 904 with a native alanine) substitutions at residues 903–907 of GluR-A upon SAP97_PDZ1–3 binding (Fig. 4A). The C-terminal amino acid sequence of GluR-A (-ATGL) conforms to the consensus motif for type I PDZ domain interactions, -(T/S)X(A/I), where Φ stands for a residue with a hydrophobic side chain (16, 17). As expected, SAP97_PDZ1–3 did not show any binding to GluR-A when positions for the CTD mutants L907A and T905A, whereas mutations at the positions -1, -3, and -4 relative to the C terminus (G903A, A904G, and G905A) did not cause any changes in the binding as judged by the intensities of the immunoreactive SAP97_PDZ1–3 bands in the corresponding eluates (Fig. 4A). Adding an extra alanine after Leu-907 (XA908, Fig. 4A) abolished the binding totally, demonstrating that the Leu-907 and Thr-905 residues have to be at 0 and -2 positions for the binding to occur (Fig. 4A).

Leucine as the C-terminal residue is somewhat less commonly observed than either valine or isoleucine in type I PDZ domain interactions, and most identified PDZ binding partners of synaptic MAGUKs have either an isoleucine or a valine as the C-terminal residue. Therefore, we decided to study the role of the C-terminal residue further. However, substituting an isoleucine or valine residue for Leu-907 did not, however, affect...
the binding to SAP97PDZ1–3 (Fig. 4B). Thus, the mutation analysis did not reveal any significant contributions to the binding other than those of the C-terminal residue and the threonine at the −2 position.

One-residue Deletion Reveals a Cryptic SAP97-selective PDZ Binding Motif in GluR-D—The absence of any major effects on PDZ domain binding by side chain substitutions at positions −1, −3, and −4 in the GluR-A C terminus suggested a possible involvement of structures beyond the archetypal PDZ binding motif in the interaction. Sequence comparison revealed that a single-residue deletion (P902) at the extreme C terminus of the AMPA receptor subunit GluR-D would expose a type I PDZ binding motif with a C-terminal leucine and a serine at the −2 position (Fig. 5A). This “cryptic” PDZ binding motif prompted us to study if the GluR-D mutant would bind to SAP97. Indeed, in a pull-down assay, GST-fused GluR-D CTD ΔP902 bound avidly to SAP97PDZ1–3, whereas the wild-type CTD did not show any binding under the same conditions (Fig. 5B). Surprisingly, in further analyses, the mutated GluR-D CTD displayed the same SAP97 selectivity as GluR-A CTD, having no interaction with PSD-95 PDZ1–3, PSD-93 PDZ1–3, or SAP102 PDZ1–3 (Fig. 5B). Therefore, the homologous CTDs of GluR-A and GluR-D were further compared, which enabled us to identify any “upstream” motifs involved in SAP97 binding.

An SSG Sequence Located 9–11 Residues Upstream of the C Terminus Is Necessary for SAP97 Binding—CTDs of GluR-A and GluR-D differ at positions −1, −2, and −3, although both carry an alanine at position −4. Considered together with the finding that replacement of the −4 alanine by glycine in GluR-A did not visibly affect the interaction (Fig. 4A), our results strongly suggest that upstream elements shared by the
two subunits play a role in SAP97 binding. Further inscription of the C-terminal sequences of GluR-A and GluR-D reveals several regions of sequence identity (Fig. 5A). However, because the previous study by Leonard and co-workers (12) clearly demonstrated that the GST fusion protein of the last 11 residues of GluR-A was sufficient for binding to SAP97 and SAP97 PDZ domains, we focused on this region. The only striking similarity in this segment is the tripeptide SSG at position −8 to −10 of GluR-A. To test the potential importance of this sequence element for SAP97 interaction, we studied the binding properties of wild-type and mutated C-terminal 11-mer peptides of GluR-A. As shown in Fig. 6A, SAP97PDZ1–3 bound to the last 11 residues (897–907) of GluR-A (A-11SSG) but not to a mutant peptide in which the tripeptide was replaced by a triaryl sequence (A-11AAA). To confirm the importance of the SSG sequence for SAP97 binding, the effects of three additional mutations at the position of the middle serine were tested. No binding was observed to the mutant 11-mer peptides SS89R, SS89E, and SS89I. These results indicate that the SSG sequence located 9 to 11 residues N-terminally from the C terminus have a major role in SAP97 binding. Consistent with this, the replacement of the SSG sequence in the C-terminal domain of GluR-DAP902 by three alanines also abolished the binding of SAP97 PDZ domains (Fig. 6B).

**Localization of GluR-A in Transfected HEK293 Cells Is Not Affected by SAP97 Interaction**—Finally, to complement the in vitro binding assays performed by using GST pull-downs, we examined the interaction in transfected HEK293 cells co-expressing GFP-SAP97 together with N-terminally FLAG-tagged GluR-A, GluR-AΔL907A, or GluR-AHis. The importance of the C-terminal PDZ binding motif for the interaction was confirmed by immunoprecipitation experiments. GluR-A with a wild-type C terminus but not with either one of the two mutated C termini co-immunoprecipitated with GFP-SAP97 (Fig. 7A, upper panel). A direct anti-FLAG immunoblot of the cell homogenates showed that all constructs were expressed at similar levels (Fig. 7A, lower panel). We then studied if the cellular distribution of GluR-A is affected by the interaction with GFP-tagged SAP97. In immunofluorescence microscopy, an intense and highly similar anti-FLAG staining was observed for all three GluR-A constructs in Triton X-100-permeabilized cells (Fig. 7B and data not shown). The surface staining, which was analyzed in fixed nonpermeabilized cells, was substantially weaker, and there were no clear differences among the three constructs (Fig. 7B and data not shown). In conclusion, association with recombinant SAP97 does not appear to have any striking effects on the cellular distribution of GluR-A in transiently transfected cells.

**DISCUSSION**

Our initial in vitro binding experiments showed that the C terminus of GluR-A binds to the PDZ domains of SAP97 but not to the closely related ones of PSD-95, PSD-93, and SAP102. Under the same conditions, the C termini of the NR2A and GluR6 subunits and the microtubule-binding protein CRIP1 bound to all four proteins. These findings extend the original discovery of this interaction (12) by providing a clear demonstration of its highly selective nature. Intrigued by this unusual selectivity, we analyzed the interactions of GluR-A with single PDZ domains and the effects of C-terminal mutations on
the binding in an attempt to identify the structural features responsible for the specificity of the molecular association. First, experiments with single domains indicated that the second PDZ domain of SAP97 provides the major contribution to binding. No binding of PDZ1 or PDZ3 domains to GluR-A CTD was observed under conditions in which binding to GluR-6 CTD (PDZ1) or CRIPT (PDZ3) was clearly detectable. A recent report (19), based on immunoprecipitations from transfected HEK293 cells, suggested that the first PDZ domain of SAP97 is responsible for GluR-A binding. The reasons for this apparent discrepancy are unclear at the moment, but it should be noted that all three PDZ domains of PSD-95 have been found to bind the same peptide substrate albeit with widely different affinities (16, 20).

Mutation analysis of the extreme C terminus of GluR-A confirmed, as expected, the importance of the type I PDZ binding motif for the interaction but provided no explanation for its unexpected MAGUK selectivity. The finding, that similar SAP97-selective binding is exhibited by a GluR-D subunit mutated to uncover a cryptic PDZ binding motif, led us to identify a tripeptide sequence SSG as an important determinant for SAP97 binding. The crucial role of this segment in the binding was indicated by the complete loss of binding resulting from replacement of the SSG sequence by three alanine residues, both in the whole CTDs and the C-terminal 11-mer peptides of GluR-A and GluR-DAP902. Moreover, three different substitutions of the “middle” S in the SSG sequence invariably led to loss of SAP97 interaction in 11-mer C-terminal peptide of GluR-A. Mechanistically, the critical role of the SSG sequence can be explained either by direct contacts between this sequence element and the PDZ domain or by an indirect conformational effect the SSG tripeptide sequence exerts on the extreme C terminus of GluR-A.

The structural mechanisms underlying the specificity of PDZ interactions are only partially understood (18, 21). PDZ domains bind preferentially to C-terminal peptide sequences of target proteins that contain a binding motif typical for each class of PDZ domain (22). The crystal structures of several PDZ domains have been determined with type I peptide ligands and show that the PDZ domain consists of a core of five or six antiparallel β-sheets (A–F) and two α-helices (A and B) (18, 22). The last four to five residues of the peptide ligand associate as an β-strand in an antiparallel orientation with one strand (B) of the PDZ domain. The C-terminal residue of the peptide ligand is accommodated in a pocket created by an α-helix (B), the B strand, and a conserved GLGF loop connecting the A and B strands, whereas the serine or threonine residue at the −2 position forms an essential hydrogen bond with a conserved histidine residue in helix B. These features explain the requirement for a PDZ type I binding motif, but the specificity of the interaction seems to be determined mostly by further interactions between the side chains of residues at positions −1, −3, and −4 of the peptide ligand and the PDZ domain (17, 18, 21–25). In the present study, we did not observe any clear effects of side-chain alteration at these positions in the GluR-A subunit. It should be noted, however, that the GST pull-down assay would not be able to identify small differences in binding affinity between the mutants. Therefore, it is possible that more detailed binding analyses, using solution binding assays, might well reveal a minor contribution by residues −1, −3, and −4. In contrast, mutations at positions −8 to −10 totally abolished the interaction between GluR-A C terminus and SAP97 PDZ domains, indicating the presence of a major contribution to binding. There are some previous reports of the importance of sequence elements, which are located upstream from the C-terminal 4–5 residues for PDZ domain binding. However, in most cases, these elements have not been clearly defined (17, 26) or their contribution to the binding has been relatively modest (27).

GluR-A CTD appeared to interact with the single PDZ2 domain or with the PDZ1–3 segment with similar strengths. Thus, it is probable that the SSG sequence interacts directly with the PDZ2 domain as the C-terminal motif does. In the absence of a three-dimensional structure, the mode of interaction can only be speculated for the moment. It seems, however, that the interaction between the C-terminal 4–5 residues of GluR-A with SAP97 is too weak, and a second interaction between the SSG sequence and the PDZ domain would be necessary to provide a stable complex. This second interaction may involve specific hydrogen bonds because two of the three residues are serines. An interesting possibility is that this second interaction site might serve a regulatory function, perhaps involving phosphorylation or further protein interactions. SAP97 has been reported to provide a link between protein kinases and phosphatases and GluR-A-containing AMPA receptors (28, 29). However, the two serines in the SSG sequence are not part of any known consensus motif for protein kinase substrates. Further studies to analyze the physiological importance of the SSG sequence element are clearly warranted.

In conclusion, our present findings demonstrate the highly
selective nature of the interaction between GluR-A and SAP97. Furthermore, they show that, in addition to a typical type I PDZ binding motif, an upstream SSG sequence, located 9 to 11 residues N-terminally, is essential for the interaction. Considered together with the relatively minor influence of residues at positions \(-1, -3, \text{and} -4\), our results suggest that the interaction between SAP97 and GluR-A represents a novel variation of PDZ domain-peptide interactions.

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