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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 four 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)[1] and the closely related SAP90/PSD-95, SAP102, and PSD-93/chapsyn-110 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 from Santa Cruz Biotechnology. Antibodies against the C-terminal domain (residues 827–907) of GluR-A and the N-terminal segment (residues 2–100) of SAP97 were prepared by immunizing rabbits with the corresponding purified GST fusion proteins, expressed and isolated from *Escherichia coli* cultures. In immunoblotting, SAP97 antiserum, but not the corresponding preimmune serum, recognized a broad 120-kDa species in rat cerebellar lysate and a 150-kDa band in human embryonic kidney 293 cells (HEK293) transfected with GFP-SAP97 but not in cells transfected with GFP-PSD-95. GluR-A antiserum, but not the corresponding preimmune serum, recognized a 105-kDa species in rat cerebellar lysate and in transfected HEK293 cells expressing GluR-A but not GluR-B, GluR-C, or GluR-D. Expression vectors encoding GFP-SAP97 and GFP-PSD-95 fusion proteins were 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, pGEX3-T4 vector (Amersham Biosciences) or its

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[1] The abbreviations used are: SAP97, synapse-associated protein 97; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; CTD, C-terminal domain; CRIPT, cysteine-rich interactor of PDZ three; GluR, glutamate receptor; GluR-A through GluR-D, equivalent to GluR1 through GluR4 by alternative nomenclature; GST, glutathione *S*-transferase; HEK, human embryonic kidney; MAGUK, membrane-associated guanylate kinase homologue; NMDA, *N*-methyl-D-aspartic acid; NR2A, NMDA receptor subunit 2A; PBS, phosphate-buffered saline; PSD-93, postsynaptic density-93; PSD-95, postsynaptic density-95; PDZ, PSD-95/Dlg/ZO-1 homology; SAP102, synapse-associated protein 102; TBS, Tris-buffered saline; GFP, green fluorescent protein; PMSF, phenylmethylsulfonyl fluoride.

derivative in which the *Bam*HI-*Not*I linker was replaced by a *Bam*HI-*Nco*I-*Hin*dIII-*Not*I polylinker (pGEX-BNH) were used. Coding sequences of the PDZ domains of SAP97 (Swiss-Prot/TrEMBL access code: Q62696; residues 213–549), PSD-95 (P31016; residues 97–434), PSD-93 (Q63622; residues 87–500), and SAP102 (Q62936; residues 138–455) were amplified by PCR from plasmid template (SAP97) or from rat brain cDNA (PSD-95, PSD-93, and SAP102) using appropriate primers and subcloned into *Nco*I-*Hin*dIII-treated pGEX-BNH. Coding sequences of the C termini of GluR subunits (GluR-A, residues 827–907; GluR-B, residues 834–883; GluR-C, residues 839–888; GluR-D, residues 835–902; GluR6, residues 834–909; NR2A, residues 1326–1465) were PCR-amplified from corresponding plasmid templates and cloned into *Bam*HI-*Hin*dIII sites of pGEX3-T4. The entire coding region for the rat microtubule-binding protein CRIPT (O70333 (16)) was amplified from rat brain cDNA and cloned into *Bam*HI/*Hin*dIII-treated pGEX3-T4. GST fusion proteins of 11-mer peptides representing residues 897–907 of GluR-A and 891–901 of GluR-D were generated by PCR using appropriately designed primers and cloned into the *Bam*HI/*Hin*dIII-treated pGEX-BNH. For the expression of His-tagged proteins, a T7 promoter-containing vector pTFT74HIPMC (obtained from Dr. A. Plückthun, University of Zürich, Switzerland) was modified so that a unique *Xba*I site was placed before a C-terminal His_6 tag, thereafter the PDZ1–3 segments of SAP97, PSD-95, PSD-93, and SAP102, single PDZ domains (PDZ1, 213–318; PDZ2, 311–409; PDZ3, 457–549), and the N-terminal segment (residues 2–100) of SAP97 were cloned into the *Nco*I-*Xba*I-treated vector. For mammalian cell expression, the N-terminally FLAG-tagged GluR-A construct was placed into pcDNA3.1(−) vector (Invitrogen). Fragments encoding C-terminally His-tagged GluR-A and the L907A point mutation were prepared by PCR and used to replace the corresponding fragments in the wild-type vector. All constructs were verified by restriction digestion, and all PCR-generated fragments were sequenced.

Immunoprecipitation—Cerebella from adult male Wistar rats were homogenized in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM sodium orthovanadate, 0.5 mM PMSF (1 ml per 200 mg of tissue) buffer; thereafter Triton X-100 was added to a final concentration of 1% (w/v), and the suspension was mixed at +4 °C for 2 h, followed by ultracentrifugation at 100,000 × *g* for 1 h. The supernatant was used for immunoprecipitation as described below. Lysates of transfected HEK293 cells (see below) were prepared by homogenizing the cells in 1% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 5.0 mM EDTA, 1.0 mM NaF, 1.0 mM Na_3VO_4, 1.0 mM PMSF, 10 μg/ml each of aprotinin and leupeptin (TNE buffer), followed by centrifugation at 20,000 × *g* for 15 min. One-milliliter aliquots of tissue and cell extracts were precleared by incubating with 20 μl of GammaBind G-Sepharose (Amersham Biosciences) for 2 h at 4 °C. For immunoprecipitation, lysates (0.5 ml) were incubated with an appropriate antibody (2 μg) or antisera (2 μl) overnight at 4 °C and then with GammaBind G-Sepharose for an additional 2 h. The precipitated beads were washed three times with TNE buffer and once with TNE buffer containing 0.5 M NaCl and finally once with PBS. The bound proteins were eluted by SDS sample buffer and were separated by SDS-PAGE. The gels were transferred to nitrocellulose membrane and immunoblotted with the appropriate antibodies. Blots were developed by using the ECL Plus (Amersham Biosciences).

Bacterial Expression—Recombinant proteins were produced 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-D-thio-β-galactopyranoside was added to a final concentration of 0.1–0.2 mM, 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 mM PMSF, 10 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 or Ni^{2+}-charged chelating Sepharose 4B (1 ml per 10 ml of supernatant) at 4 °C for 1–2 h, then the resins were extensively washed 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).

GST Pull-down Assay—Bacterial lysates, extracts prepared from rat cerebella or HEK293 cells, were incubated in a total volume of 1 ml with 10 μg of GST fusion protein attached to glutathione-Sepharose beads overnight at 4 °C. The gel was spun down at 1000 × *g* for 2 min and washed five times with 1% (w/v) Triton X-100 in TBS then twice with PBS. The bound proteins were eluted in SDS sample buffer containing 2 mM dithiothreitol, separated by SDS-PAGE, and transferred to nitrocellulose or polyvinylidene difluoride filters. Blots were stained with Ponceau S to verify the presence of all GST fusion proteins at a similar level and finally used for immunoblotting with anti-His, anti-myc, or anti-GluR-D antibodies as indicated in the figure legends. Blots were developed either by colorimetric reaction using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium or by using ECL Plus (Amersham Biosciences).

Expression in HEK293 Cells—HEK293 cells were grown in culture dishes or on poly-D-lysine-coated glass coverslips in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% (v/v) fetal calf serum, penicillin (50 units/ml), and streptomycin (50 units/ml) at 37 °C under 5% CO_2. HEK293 cells were transfected using calcium phosphate coprecipitation (10 μg of plasmid DNA per T-75, or 2 μg per 35-mm dish). Forty-eight hours after the transfection, the cells were either harvested in TNE buffer and used for preparation of cell extracts (see above) or processed for immunofluorescence microscopy as described below.

Immunofluorescence Microscopy—Transfected HEK293 growing on coverslips were processed for immunofluorescence microscopy 40–48 h post-transfection. Cells were fixed in 3% paraformaldehyde and preblocked with 3% goat serum. For total staining (as opposed to surface staining), the cells were additionally incubated in 0.05% Triton X-100 in PBS prior to blocking step. Cells were labeled with monoclonal anti-FLAG IgG (M1, Sigma, 5 μg/ml) followed by Cy3-conjugated anti-mouse IgG secondary antibody (Jackson Laboratories, 7 μg/ml). Cells were examined using an Olympus Provis AX70 epifluorescence microscope, and images were collected by a Photometrics SenSys air-cooled charge-coupled device camera using Image ProPlus software.

RESULTS

Interaction of GluRA with SAP97—First, we used immunoprecipitations and GST pull-down assays to demonstrate the specific interaction between GluR-A and SAP97, originally reported by Leonard and co-workers (12). GluR-A was present as a 100-kDa species in an immunoprecipitate prepared from rat cerebellar extract by using an antiserum raised against the N-terminal domain (residues 2–100) of SAP97 but not with the corresponding preimmune serum. Likewise, an antiserum raised against the C-terminal domain of GluR-A subunit specifically precipitated immunoreactive SAP97, appearing as a doublet in the 120-kDa range (Fig. 1*A*). GST pull-down assays were used to study the specificity of the interaction between SAP97 and GluR-A. 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 CRIPT 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. 1*B*). The CTD of GluR-A but not of GluR-B, GluR-C, or GluR-D subunits bound SAP97 (Fig. 1*B*). An identical binding pattern was observed when the purified, bacterially expressed histidine-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 GluR-A PDZ Interaction—We examined the selectivity of the GluR-A 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, PSD-95, PSD-93, and SAP102 to GST-fused CTDs of GluR-A, GluR-D, and GluR-6, the C-terminal segment of NR2A, and the microtubule-binding protein CRIPT. All four PDZ1–3 proteins bound to CRIPT and to the C-terminal domains of GluR6 and NR2A, but only $SAP97_{PDZ1–3}$ bound to GluR-A CTD (Fig. 2*A*). 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. 2*A*) or

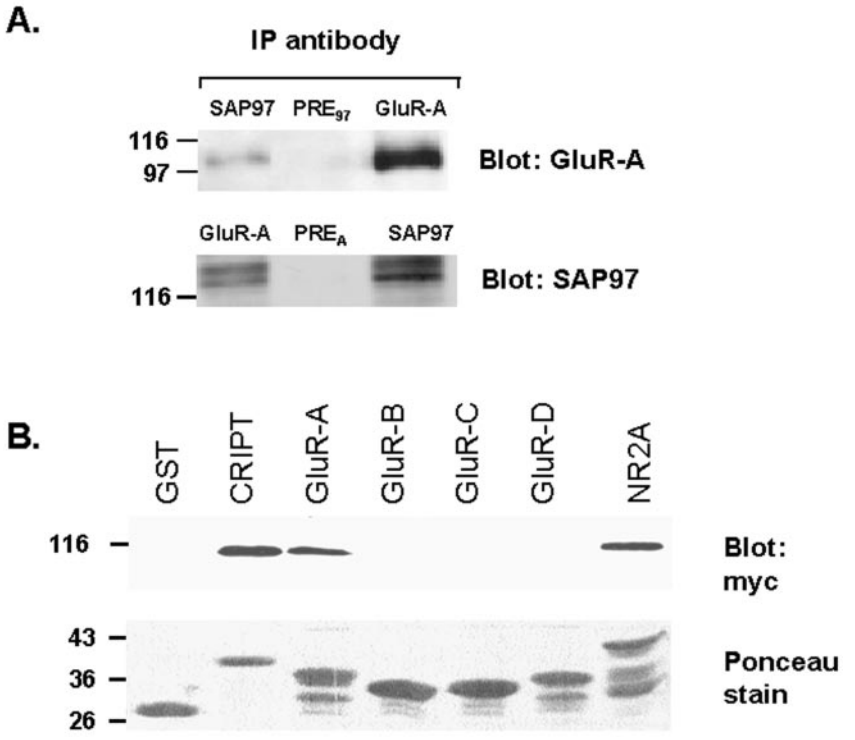

FIG. 1. **Association of GluR-A with SAP97.** *A*, coimmunoprecipitation of SAP97 and GluR-A. Rat cerebellar detergent extract was subjected to immunoprecipitation using the specific GluR-A and SAP97 antisera and the corresponding preimmune sera. The immunoprecipitates were analyzed by Western blotting for the presence of GluR-A and SAP97, as indicated. *B*, interaction of myc-SAP97 with CTDs of AMPA receptor subunits. Myc-SAP97 expressed in transfected HEK293 cells was bound to and eluted from immobilized GST fusion proteins of the C-terminal domains of AMPA receptor subunits GluR-A to 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.

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 $SAP97_{PDZ1–3}$ but not in the eluates from $PSD\text{-}95_{PDZ1–3}$, $PSD\text{-}93_{PDZ1–3}$, and $SAP102_{PDZ1–3}$ (Fig. 2*B*). 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 AMPA receptors with the full-length SAP97 and with the PDZ1–3 segment and the PDZ2 domain but not with the PDZ1 and PDZ3 domains. No binding was observed to the unique N-terminal region (residues 2–100) of SAP97 (Fig. 3*A*). Interestingly, as judged by the intensity of the eluted GluR-A immunoreactivity, binding to the single PDZ2 domain was as strong as to the PDZ1–3 segment, although in both cases the binding was somewhat weaker than that observed with the full-length SAP97 (Fig. 3*A*). Because the functional activity of the single PDZ domains may be affected by their expression as GST fusion proteins, we also determined the binding of His-tagged single PDZ domains (solubilized from overexpressing *E. coli* cultures) to GST fusions of the CTDs of GluR6, GluR-A, -B, and -D, a C-terminal segment of NR2A, and CRIPT. As shown in Fig. 3*B*, the PDZ1 and PDZ3 domains bound selectively to GluR6, and CRIPT, respectively, whereas the PDZ2 domain bound to NR2A and to GluR-A. The relatively low amounts of His-immunoreactive PDZ2 in the eluates may suggest that singly expressed PDZ2 is less stable than PDZ1 and PDZ3. None of the single PDZ domains bound to GST alone or to the CTDs of GluR-B and GluR-D (Fig. 3*B*). The observed domain specificity agrees well with what is reported for GluR6, CRIPT, and NR2A (8, 11, 16). Thus, all three separate domains are capable of specific interactions. Hence, our results indicate 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. 4*A*). The C-terminal amino acid sequence of GluR-A (-ATGL) conforms to the consensus motif for type I PDZ domain interactions, -(T/S)*X*Φ, 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 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. 4*A*). Adding an extra alanine after Leu-907 (*XA908*, Fig. 4*A*) 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. 4*A*).

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

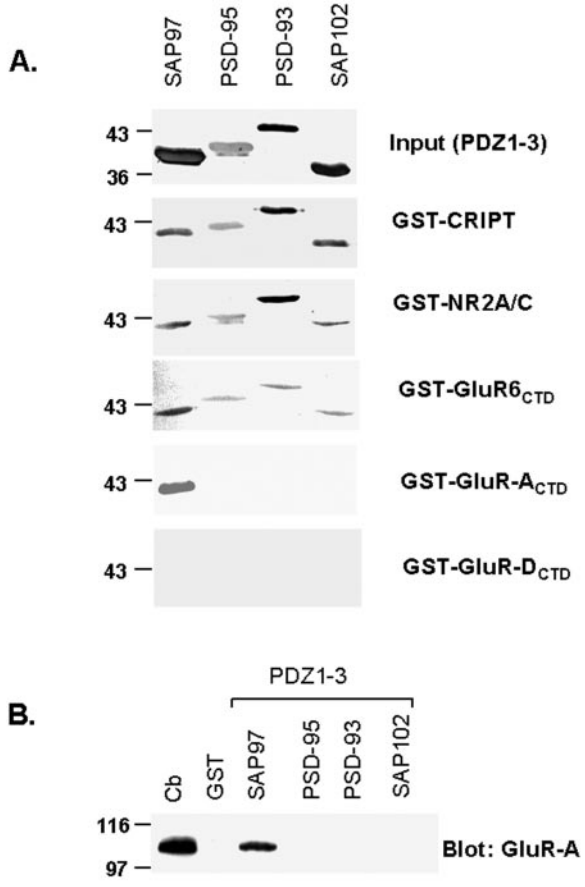

FIG. 2. **GluR-A interacts selectively with SAP97 PDZ domains.** *A*, binding of His-tagged PDZ1–3 segments of SAP97, PSD-95, PSD-93, and SAP102 to the CTDs of GluR6, GluR-A, and GluR-D, a C-terminal segment of NR2A, and CRIPT was determined in a GST pull-down assay. Anti-His immunoblots of the input (*top panel*) and the eluates are shown. The GST fusion protein is indicated on the *right*. *B*, binding of rat cerebellar AMPA receptors to GST-fused PDZ1–3 segments of the indicated synaptic MAGUK proteins. Anti-GluR-A immunoblot of the input (rat cerebellar detergent extract, *Cb*) and the eluates are shown.

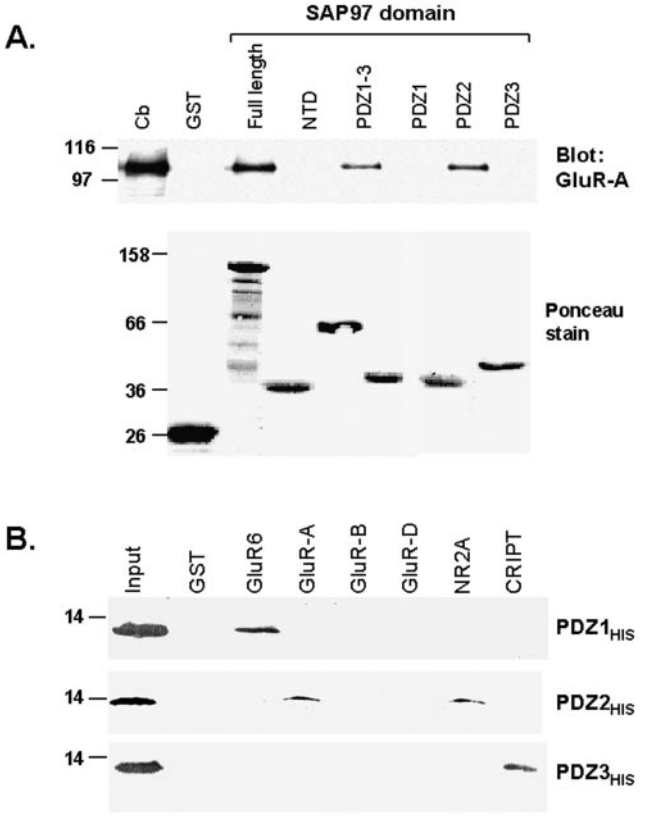

FIG. 3. **Interaction of single PDZ domains of SAP97 with GluR-A.** *A*, binding of rat cerebellar AMPA receptors to GST-fused full-length SAP97 and the indicated SAP97 domains, was determined in a GST pull-down assay. The *upper panel* is an anti-GluR-A immunoblot of the input (*Cb*) and the eluates, whereas the *lower panel* shows the corresponding protein stained eluates. *B*, GST pull-down showing the interaction of single His-tagged PDZ1, PDZ2, and PDZ3 domains of SAP97 to the indicated GST fusion proteins. Anti-His immunoblots of the eluates are shown.

the binding to $SAP97_{PDZ1–3}$ (Fig. 4*B*). 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. 5*A*). 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 $SAP97_{PDZ1–3}$, whereas the wild-type CTD did not show any binding under the same conditions (Fig. 5*B*). Surprisingly, in further analyses, the mutated GluR-D CTD displayed the same SAP97 selectivity as GluR-A CTD, having no interaction with $PSD\text{-}95_{PDZ1–3}$, $PSD\text{-}93_{PDZ1–3}$, or $SAP102_{PDZ1–3}$ (Fig. 5*B*). 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

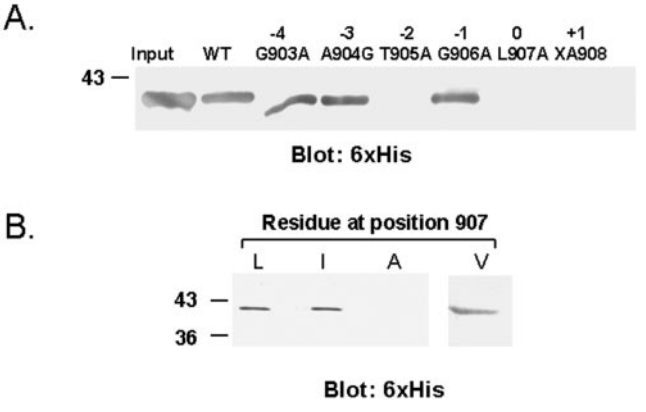

FIG. 4. **Role of the C-terminal PDZ binding motif.** *A*, GST pull-down analysis of the effect of mutations within the C-terminal 5 residues of GluR-A upon SAP97 interaction. Binding of His-tagged $SAP97_{PDZ1–3}$ to wild-type GluR-A CTD and to the indicated point mutants was determined by anti-His immunoblotting of the glutathione eluates. Position "0" refers to the C-terminal residue, "+1" to an extra residue added to the C terminus, "−1" to the penultimate C-terminal residue, etc. *B*, GST pull-down analysis of the effects of substitutions at the GluR-A C-terminal residue on the binding of His-tagged $SAP97_{PDZ1–3}$.

and GluR-DΔP902 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. 4*A*), our results strongly suggest that upstream elements shared by the

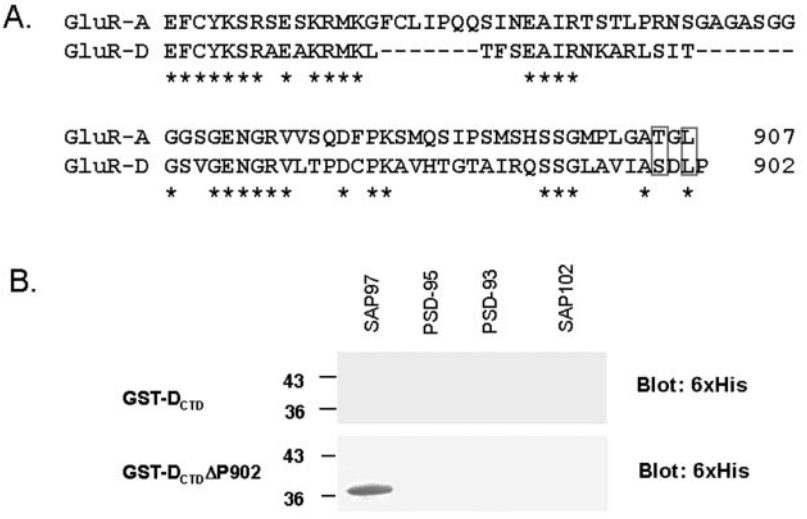

FIG. 5. **Selective binding of SAP97 to GluR-D ΔP902 mutant.** *A*, alignment of the C-terminal domains of GluR-A and GluR-D. PDZ binding motifs of GluR-A and GluR-DΔP902 are *boxed*. The *asterisks* indicate identical residues. *B*, GST pull-down analysis of the binding of the PDZ1–3 segments of SAP97, PSD-95, PSD-93, and SAP102 to the C-terminal domains of GluR-D and GluR-DΔP902 mutant. Anti-His immunoblots of the eluates are shown.

two subunits play a role in SAP97 binding. Further inspection of the C-terminal sequences of GluR-A and GluR-D reveals several regions of sequence identity (Fig. 5*A*). 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. 6*A*, SAP97$_{PDZ1–3}$ bound to the last 11 residues (897–907) of GluR-A (A-11$_{SSG}$) but not to a mutant peptide in which the tripeptide was replaced by a trialanyl sequence (A-11$_{AAA}$). 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 S898R, S898E, and S898I. 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-DΔP902 by three alanines also abolished the binding of SAP97 PDZ domains (Fig. 6*B*).

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-A(L907A), or GluR-A$_{His}$. 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. 7*A*, *upper panel*). A direct anti-FLAG immunoblot of the cell homogenates showed that all constructs were expressed at similar levels (Fig. 7*A*, *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. 7*B* 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. 7*B* and data not shown). In conclusion, association with recombinant SAP97 does not appear to have any striking effects on the cellular distribution of luR-A in transiently transfected cells.

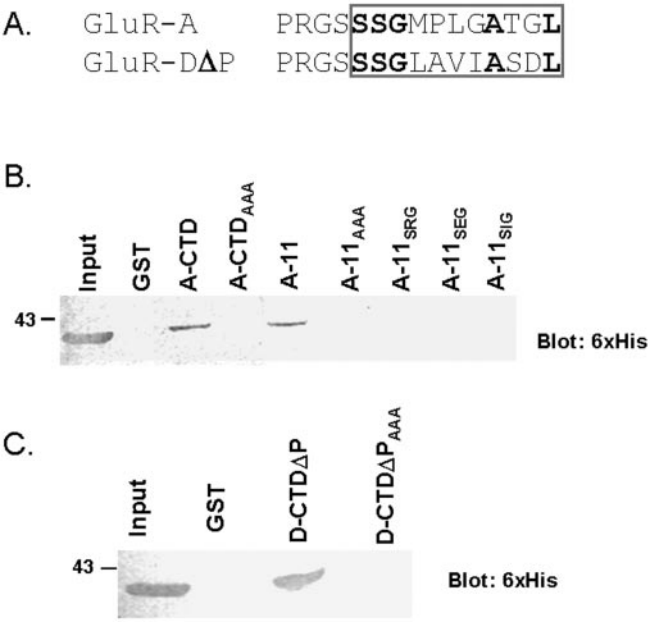

FIG. 6. **Importance of the SSG sequence for the SAP97 interaction.** *A*, the 11-mer peptides (*boxed*) of GluR-A and GluR-DΔP902 fused to GST. The C-terminal residues of GST encoded by the vector are also indicated. *B*, binding of His-tagged SAP97$_{PDZ1–3}$ to wild-type and mutated GST-fused C-terminal domains (*A-CTD*) and 11-mer C-terminal peptides (*A-11*) of GluR-A. Mutations introduced into wild-type SSG sequence are indicated. An anti-His immunoblot of the eluates is shown. *C*, binding of His-tagged SAP97$_{PDZ1–3}$ to the C-terminal domain of GluR-DΔP902 carrying a either "wild-type" SSG sequence (*D-CTDΔP*) or a trialanyl mutation (*D-CTDΔP$_{AAA}$*). An anti-His immunoblot of the eluates is shown.

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 CRIPT 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

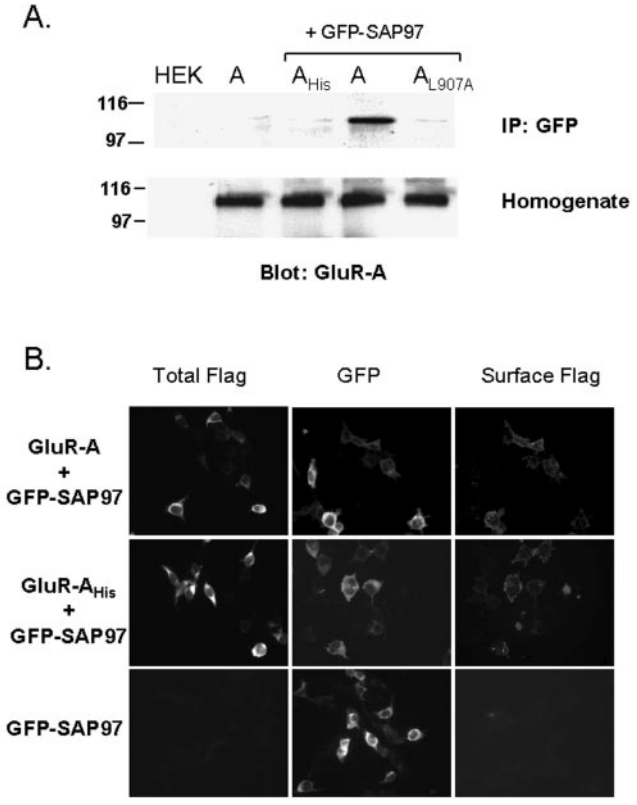

FIG. 7. **Interaction between SAP97 and GluR-A in HEK293 cells.** *A*, immunoprecipitation analysis of GluR-A C-terminal mutants with SAP97. HEK293 cells were transiently transfected for the expression of FLAG-tagged GluR-A constructs, which had a wild-type C terminus (*A*), His-tagged C terminus ($A_{His\text{His}}$), or the C-terminal point mutation L907A (A_{L907A}) either alone or together with GFP-tagged SAP97, as indicated. GFP was immunoprecipitated from detergent extracts prepared from the transfected cells. Anti-FLAG immunoblots of the GFP immunoprecipitates and of the detergent extracts ("homogenates") are shown. *B*, HEK293 cells were transfected with GFP-fused SAP97 either alone or together with FLAG-tagged GluR-A or GluR-A_{HIS} (carrying a C-terminal His_6 tag). Cells were fixed and stained with anti-FLAG IgG followed by anti-mouse-Cy3 with ("*Total*" staining) or without ("*Surface*" staining) prior to permeabilization by Triton-X-100. GFP fluorescence corresponding to the surface-stained cells is also shown.

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-DΔP902. 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, 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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