Selectivity and Promiscuity of the First and Second PDZ Domains of PSD-95 and Synapse-associated Protein 102*

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PDZ domains typically interact with the very carboxyl terminus of their binding partners. Type 1 PDZ domains usually require valine, leucine, or isoleucine at the very COOH-terminal (P0) position, and serine or threonine 2 residues upstream at P−2. We quantitatively defined the contributions of carboxy-terminal residues to binding selectivity of the prototypic interactions of the PDZ domains of postsynaptic density protein 95 (PSD-95) and its homolog synapse-associated protein 90 (SAP102) with the NR2b subunit of the N-methyl-D-aspartate-type glutamate receptor. Our studies indicate that all of the last five residues of NR2b contribute to the binding selectivity. Prominent were a requirement for glutamate or glutamine at P−3 and for valine at P0 for high affinity binding and a preference for threonine over serine at P−2, in the context of the last 11 residues of the NR2b COOH terminus. This analysis predicts a COOH-terminal (E/Q)(S/T)V motif for the first two PDZ domains of PSD-95 and SAP102. A survey of the human genome sequences for proteins with a COOH-terminal (E/Q)(S/T)V motif yielded 50 proteins, many of which have not been previously identified as PSD-95 or SAP102 binding partners. Two of these proteins, brain-specific angiogenesis inhibitor 1 and protein kinase Cα, co-immunoprecipitated with PSD-95 and SAP102 from rat brain extracts.

Postsynaptic density protein 95 (PSD-95)1 (1), also known as synapse-associated protein 90 (SAP90) (2), is a neuronal protein that is specifically enriched at the postsynaptic densities of dendritic spines (3). PSD-95 is a member of a family of proteins that includes SAP102 (4), SAP97 (hDlg) (5, 6), and PSD-93 (Chapsyn-110) (7, 8). All members of this family share the same general structure: three PSD-95/Dlg/Zo-1 (PDZ) domains, one Src homology 3 domain, and one guanylate kinase homology domain. All of these domains mediate protein-protein interactions. Therefore, PSD-95 and related proteins are thought to function as structural scaffolds that assemble protein complexes, thereby facilitating signal transduction. NR2b subunits of the NMDA-type glutamate receptor (9) and Shaker-type K+ channels (10) were the first proteins described to bind to PSD-95. These interactions occur via the first two PDZ domains of PSD-95. Additional neuronal proteins that have subsequently been characterized as interacting with the PDZ domains of PSD-95 include neuronal nitric-oxide synthase (7, 11), CRIPT (12), SynGAP (13, 14), Citron (15), and two isoforms of the plasma membrane Ca2+ pump (PMCA2h and -4b) (16) (see Table I). In addition, a number of novel PDZ domain-containing proteins have been described that are also localized to the synapse, including MAGI-1 (17), S-SCAM (MAGI-2) (18), GRIP (19), ABP (GRIP2) (20), CASK (LIN-2) (21), and PICK1 (22).

PDZ domains are unique among protein-protein interaction domains, because the proteins that bind to PDZ domains generally do so by their very COOH-terminal residues. The subunits of the Shaker K+ channel and NMDA receptor that bind to the first two PDZ domains of PSD-95, SAP102, and PSD-93 have the sequence E(S/T)V/(N)Motif, which is conserved among all vertebrates (19). The C-terminal domain of PSD-95 and a peptide ligand derived from the very COOH terminus of CRIPT (TKNYKQTSV) has been solved (23). This work showed that the very COOH-terminal valine and the threonine two positions upstream (the 0- and −2-positions P0 and P−2, respectively) form crucial interactions in the PDZ domain binding pocket. Thus, the minimal consensus sequence for binding to the PDZ domain has been defined as an (S/T)XV motif, where X can represent any residue. However, it is clear that other residues must contribute to specificity for a given PDZ domain, because various proteins and ion channels that have an (S/T)V motif do not bind any of the PDZ domains under conditions in which the other ligands do. Examples include the neuronal inwardly rectifying K+ channels Kir3.2 and Kir3.3 (COOH-terminal sequence is in both cases ESKV (24)); the Na+ channel Na1.5 (ESIV (25)), which is present not only in muscle but also brain (26); and diacylglycerol kinase ζ (ETAV (27)). Furthermore, the β3 adrenergic receptor does not interact with the first two PDZ domains of PSD-95 although it does conform to the (S/T)XV motif (SKV (28)). This receptor does, however, bind to the third PDZ domain of PSD-95 (28). This PDZ domain possesses a binding preference that is quite different from the first two PDZ domains (e.g. see Ref. 12) of PSD-95 and does not interact with NR2 subunits (9, 29) or Shaker-type K+ channels (10). Similarly, Neulin carries the sequence TRK at its COOH terminus but only interacts with the third and not the first two PDZ domains of PSD-95 (30).

PDZ domains can be broadly divided into several categories, based on their general ligand specificity. Type I PDZ domains,

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*This work was supported by National Institutes of Health (NIH) Research Grant R01-NS05563 and American Heart Association Established Investigator Award 0040151N (to J. W. H.). 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.

‡Supported in part by NIH Training Grant AG00213.

§Supported by NIH Training Grants AG00213 and DK07759.

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1The abbreviations used are: PSD, postsynaptic density protein; SAP, synapse-associated protein 90 (SAP90) (1), also known as synapse-associated protein 90 (SAP90) (2), is a neuronal protein that is specifically enriched at the postsynaptic densities of dendritic spines (3). PSD-95 is a member of a family of proteins that includes SAP102 (4), SAP97 (hDlg) (5, 6), and PSD-93 (Chapsyn-110) (7, 8). All members of this family share the same general structure: three PSD-95/Dlg/Zo-1 (PDZ) domains, one Src homology 3 domain, and one guanylate kinase homology domain. All of these domains mediate protein-protein interactions. Therefore, PSD-95 and related proteins are thought to function as structural scaffolds that assemble protein complexes, thereby facilitating signal transduction. NR2 subunits of the NMDA-type glutamate receptor (9) and Shaker-type K+ channels (10) were the first proteins described to bind to PSD-95. These interactions occur via the first two PDZ domains of PSD-95. Additional neuronal proteins that have subsequently been characterized as interacting with the PDZ domains of PSD-95 include neuronal nitric-oxide synthase (7, 11), CRIPT (12), SynGAP (13, 14), Citron (15), and two isoforms of the plasma membrane Ca2+ pump (PMCA2h and -4b) (16) (see Table I). In addition, a number of novel PDZ domain-containing proteins have been described that are also localized to the synapse, including MAGI-1 (17), S-SCAM (MAGI-2) (18), GRIP (19), ABP (GRIP2) (20), CASK (LIN-2) (21), and PICK1 (22).

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Selectivity of PDZ Domains

Proteins that have been observed to associate with the PDZ domains of PSD-95 (as well as related proteins that do not associate with PSD-95 PDZ domains) are listed along with their COOH-terminal PDZ-domain binding consensus sequence. Residues highlighted in boldface indicate optimal residues for that position for binding to PDZ1 and -2 of PSD-95. Relative affinities (for binding different PDZ domains or relative to similar proteins) are also listed. These are denoted by (+), ÷, or + in order from weakly to strongly binding. See Discussion for full details and citations. ND, not determined. Nedasin was not tested for PSD-95 binding; given are interactions with SAP102 (last line).

| Protein | Position | PSD-95 | No binding detectable |
|---------|----------|--------|-----------------------|
| NR2a    | 10 9 8 7 6 5 4 3 2 1 0 | PDZ1 | PDZ2 | PDZ3 | PDZ ND |
| KS2C6  | Y E K L S S S I L E S E + + + | + | + | + | + |
| KS2d   | S A H F S L E S E V + + + | + | + | + | + |
| Kv1.1   | C V N K S K L L T D V (+) (+) (+) | (+) | (+) | (+) | (+) |
| Kv1.2   | Y V N I T K M L T D V (+) (+) (+) | (+) | (+) | (+) | (+) |
| Kv1.3   | Y V M K K I K I T D V (+) (+) (+) | (+) | (+) | (+) | (+) |
| Shaker  | N A M A V S I E T T D V + + + | + | + | + | + |
| Kir2.1  | E P R P L R R E S E E I + + + | + | + | + | + |
| Kir2.3  | D N I S Y R R E S A K + + + | + | + | + | + |
| Kir2.2  | D V A N L E N E S K V + + + | + | + | + | + |
| Kir2.3  | C L P P P E S E S K V + + + | + | + | + | + |
| B1AR    | G R Q G F S S E S K V + + + | + | + | + | + |
| B2AR    | G R N C N T N D S P L X X X | X | X | X | X |
| PMCA4b  | D S P L H S L E T S V + + + | + | + | + | + |
| PMCA2b  | G S P I H S L E T S L + + + | + | + | + | + |
| Magu1-1 | H H T H S Y I E T T H + + + | + | + | + | + |
| CRIP2   | L D T K N Y K Q T S V + + + | + | + | + | + |
| Citrin  | Q V N K V W D Q S S V (+) + (+) | (+) | (+) | (+) | (+) |
| Sema4c  | L P D S N P E E S S V + + + | + | + | + | + |
| Sema4f  | A P L A T C D E T S I + + + | + | + | + | + |
| SynGAP  | S F P P W V Q Q T R V + + + | + | + | + | + |
| ErbB4   | P P P Y R H R N T V V + + + | + | + | + | + |
| Neurogln| P H P H S H S T T T R V + + + | + | + | + | + |
| Nedasin | K K Q V V F P S S S V + + + | + | + | + | + |

found on PSD-95 and its homologs, bind (S/T)V(X/V/I/L) COOH termini, whereas type II PDZ domains (e.g. the fourth and fifth PDZ domain of GRIP and the PDZ domain of CASK), bind an Φ-X-Φ motif, where Φ represents a hydrophobic residue (preferably tyrosine or phenylalanine at P') (31, 32). A third type of PDZ domain present in neuronal nitric-oxide synthase shows a preference for aspartate at P' (31, 32). A third type of PDZ domain present in neuronal nitric-oxide synthase shows a preference for aspartate at P' (31, 32). A third type of PDZ domain present in neuronal nitric-oxide synthase shows a preference for aspartate at P' (31, 32). A third type of PDZ domain present in neuronal nitric-oxide synthase shows a preference for aspartate at P' (31, 32).
polarimetric peptide binding assays allowed us to quantitatively determine the effects of amino acid substitutions at various positions along the peptide sequence on PDZ domain binding. We were surprised by a strong, often severalfold preference at the very COOH termini of their interaction partners. However, residues of the COOH-terminal (S/T) motif for a V consensus sequence located at the very COOH termini of their interaction partners. Bulk sequencing of peptides that bound to PDZ domains during screening of the resulting clones for correct orientation of the inserts. All vectors were confirmed by DNA sequencing with the AmpliTaq system (PerkinElmer Life Sciences). Peptide fusions were expressed and purified as described (41, 42) with the following modifications. The vector DNA was electrotransferred into the E. coli strain BL21-DE3 cells. 50-ml cultures were grown overnight, diluted 1:10 with LB medium, and incubated until the cultures reached an A600 of 1.0 (2–4 h). Fusion protein expression was induced with isopropyl-1-thiogalactopyranoside (100 \mu M, 2 h). Cells were harvested, resuspended in TBS (10 mM Tris-Cl, pH 7.4, 150 mM NaCl), and cleared by ultracentrifugation (40,000 rpm, 186,000 g, 1 h). Sarkosyl was neutralized by adding 2–4% (final concentration) Triton X-100 from a 20% stock solution. Lysates were incubated with TBS and eluted by adding 15 mM glutathione in 150 mM NaCl, 50 mM Tris-Cl, pH 8. The fusion proteins were dialyzed against TBS and subsequently quantified by determining A280 and, in parallel, by the bicinchoninic acid assay (Pierce) and a Coomassie assay (Pierce) in microtiter plate format. The purity and quality of all fusion proteins were evaluated by SDS-polyacrylamide gel electrophoresis and detection by staining with Coomassie Brilliant Blue and by immunoblotting with anti-GST antibodies to ensure minimal degradation. Purified GST fusion proteins usually did not show contamination with other proteins by Coomassie Brilliant Blue staining and were discarded otherwise.

**Peptide Synthesis**—Peptides based on the NRR2 COOH terminus and the COOH termini of other potential PDZ ligands were synthesized manually using a MultiPep Peptide Synthesis kit according to the manufacturer’s instructions (Chiron-Mimotopes) (44). This method utilizes standard solid phase peptide synthesis with Fmoc-protected amino acids, followed by deprotection and cleavage of the peptide from the solid-phase support with trifluoroacetic acid. We typically synthesized peptides using a 96-well plate array of polylysine pins serving as the solid phase support. After cleavage, peptides were cleaned with two ether/petroleum ether extractions to remove leftover protecting groups.

The remaining trifluoroacetic acid was eliminated by dissolving the peptide in water/acetate acid/acetoniitride followed by lyophilization. Peptides were dissolved in water and stored frozen until used. All peptides contained at their NH2 termini a lysine, followed by a serine-lysine spacer. The NH2-terminal lysine residue was modified with a biotin or a fluorescein tag (Fmoc-biotinyl-lysine and Fmoc-fluorescein-sulfonyl-lysine were from AnaSpec). The NH2-terminal tag-KSG sequences were followed by 11 residues corresponding to the wild type (WT) or modified COOH termini of NR2b and of other PDZ-binding proteins. Selected peptides were analyzed by mass spectrometry, and all of the peptides were quantified with a BCA assay.

**Production of GST Fusion Proteins**—GST fusion proteins of the first and third PDZ domain of PSD-95, cDNA sequences encoding human PSD-95 residues 82–202 (PDZ1) and 344–443 (PDZ2); residue numbers refer to full-length human PSD-95 sequence as given in Ref. 48) were excised with EcoRI from corresponding GAD10 vectors, which contained the respective sequences that had originally been inserted into the GAD10 EcoRI cloning sites (10) (kindly provided by Morgan Sheng, MIT, Cambridge, MA). The latter two protein sequences are completely identical to residues 39–159 and 301–400 encoding the first and third PDZ domain of rat PSD-95, respectively. DNA fragments were purified by agarose gel electrophoresis and ligated into EcoRI-digested pGEX4T-1 (Amersham Biosciences) before screening the resulting clones for correct orientation of the inserts. All vectors were confirmed by DNA sequencing with the AmpliTaq system (PerkinElmer Life Sciences). Peptide fusions were expressed and purified as described (41, 42) with the following modifications. The vector DNA was electrotransferred into the E. coli strain BL21-DE3 cells. 50-ml cultures were grown overnight, diluted 1:10 with LB medium, and incubated until the cultures reached an A600 of 1.0 (2–4 h). Fusion protein expression was induced with isopropyl-1-thiogalactopyranoside (100 \mu M, 2 h). Cells were harvested; resuspended in TBS (10 mM Tris-Cl, pH 7.4, 150 mM NaCl); digested with lysozyme; supplemented with 10 mM EDTA, 15 mM dithiothreitol, and protease inhibitors (0.2 mM phenylmethanesulfonyl fluoride, 1 \mu M pepstatin A, 0.1 \mu M aprotinin, 1 \mu M leupeptin, 1 \mu M aprotinin, and 1 \mu M leupeptin) (final concentration) sarkosyl (43), followed by sonication. Lysates were clarified by ultracentrifugation (45-Ti rotor, 40,000 rpm, 186,000 g, 1 h). Sarkosyl was neutralized by adding 2–4% (final concentration) Triton X-100 from a 20% stock solution. Lysates were incubated with glutathione-Sepharose (Amersham Biosciences) overnight at 4 °C. Resins were washed with TBS and eluted by adding 15 mM glutathione in 150 mM NaCl, 50 mM Tris-Cl, pH 8. The fusion proteins were dialyzed against TBS and subsequently quantified by determining A280 and, in parallel, by the bicinchoninic acid assay (Pierce) and a Coomassie assay (Pierce) in microtiter plate format. The purity and quality of all fusion proteins were evaluated by SDS-polyacrylamide gel electrophoresis and detection by staining with Coomassie Brilliant Blue and by immunoblotting with anti-GST antibodies to ensure minimal degradation. Purified GST fusion proteins usually did not show contamination with other proteins by Coomassie Brilliant Blue staining and were discarded otherwise.

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PSD-95 and SAP102 bound with higher affinity than the first were determined. We found that the second PDZ domain of aggregate FP of the solution increased. The measured values fluorescent peptide bound to PDZ fusion proteins increased, the rotational mobility of this fluorescent peptide decreased when 100 nM solutions of fluorescein-labeled peptides containing the by FP.

It is interesting to note that SAP102 had slightly higher affinity for main binding to their respective ligands (12, 56). It is also given PDZ domain can be quite flexible. For example, serine at the -2-position (P^-2) can usually be substituted by threonine as is the case for Kv1.4 (10), and isoleucine or leucine can often replace valine at P^0 (51–55). Our goal was to quantify sequence selectivity of the first two PDZ domains of PSD-95 and SAP102 for target binding using defined peptides. We determined binding constants by measuring FP of fixed concentrations of fluorescein-labeled peptides with increasing concentrations of PDZ domains. Sequence selectivity indicated by these in-solution measurements was corroborated by an ELISA-styled assay with biotinylated peptides, which were attached to streptavidin-coated plates and incubated with increasing concentrations of PDZ domains.

Constant concentrations of the NR2b WT peptide were incubated with increasing concentrations of purified GST-PDZ fusion proteins, and FP was determined with a Victor® V plate reader (Fig. 1). The peptide contained the 11 COOH-terminal residues of NR2b and the linker sequence KSG at the NH2 terminus with the NH2-terminal lysine fluorescein tagged. The rotational mobility of this fluorescent peptide decreased when bound to a PDZ fusion protein. Therefore, as the proportion of fluorescent peptide bound to PDZ fusion proteins increased, the aggregate FP of the solution increased. The measured values could be fitted to saturation curves from which the \( K_D \) values were determined. We found that the second PDZ domain of PSD-95 and SAP102 bound with higher affinity than the first PDZ domain (0.91 versus 2.3 \( \mu \text{M} \) for PSD-95; 0.64 versus 1.41 \( \mu \text{M} \) for SAP102). These values are in good agreement with those obtained earlier for in-solution interaction assays for PDZ domain binding to their respective ligands (12, 56). It is also interesting to note that SAP102 had slightly higher affinity for the NR2b WT peptide than PSD-95 for corresponding PDZ domains.

Substantially lower \( K_D \) values have been observed by solid phase-based assays including surface plasmon resonance measurements (30, 57). However, these solid phase measurements were based on interactions of immobilized peptides carrying respective COOH-terminal SXV motifs with recombinant proteins that contained more than one PDZ domain. Accordingly, these solid phase assays may not reflect the true value for a single PDZ domain-target interaction because one protein may simultaneously bind two or more of the immobilized peptides, thereby dramatically reducing its off-rate and causing rebinding and avidity effects (58, 59). Similar considerations apply for experiments with single PDZ domains of SAP102 expressed as GST fusion proteins (4) because the GST moieties may dimerize and thereby result in avidity effects in solid phase assays (58). To avoid these problems, we primarily worked with peptides in solution. In our solid phase assays, we used GST fusions with a single PDZ domain, and the wells were coated with our peptides at low density.

We tested peptides derived from proteins that were initially identified as binding partners for one or more of the three PDZ domains of PSD-95 and were either strong or weak interactors (Fig. 2A). Highest affinities were observed for binding of the second PDZ domains of PSD-95 and SAP102 to peptides derived from established binding partners determined by FP. Fluorescein-tagged peptides with the COOH-terminal sequences of different binding partners of PSD-95 and SAP102 (A) and chimeric peptides carrying various COOH-terminal portions of GluR1 and NR2b (B; residues derived from NR2b are underlined) or GluR2 were titrated with purified GST fusion proteins of PSD-95 and SAP102 PDZ1 or -2 domains (the identity of the PDZ domain is given at the top of each column). NR2bD4 refers to a peptide that was NH2-terminally shifted by 4 positions away from the COOH terminus, eliminating the COOH-terminal ESDV sequence. FP measurements were performed, and \( K_D \) values were calculated as described under “Experimental Procedures.” To facilitate reading of the relative binding affinities, \( K_D \) values were normalized to the WT NR2b \( K_D \), which was set to equal 1, and the inverses of the normalized values were plotted as bars. Numbers beside the bars give the actual \( K_D \) values (in \( \mu \text{M} \), not determined). The peptide sequence is listed in the far right column. \( K_D \) values greater than the 10-fold \( K_D \) for WT NR2b are generally listed in the chart as 0, because accurate values for peptides with weak affinity were difficult to determine. Each assay was usually done in quadruplicate.
to the (S/T)XV motif. Several pieces of evidence suggest that the NR1 C2' region can associate with PSD-95 and SAP102, although it is unknown whether this interaction is mediated by the first two or rather the third PDZ domains of these two proteins (9, 61, 62). The low affinities observed for the Kv1.1 and NR1 C2'-derived peptides may therefore reflect that binding of C2' to the first two PDZ domains is not as firm as for the other interactions described above and may require addition interactions for stabilization. PSD-95 and SAP102 may form interactions to NR1 in parallel to those with NR2 subunits in the NMDA receptor complex, thereby increasing the stability of the complex. However, we cannot rule out the possibility that Kv1.1 and NR1 does not at all associate with one of the first two PDZ domains of PSD-95 or SAP102 in vivo. Removal of the last 4 residues of NR2b by shifting the peptide sequence 4 positions toward the NH2 terminus exposes another sequence at the very COOH terminus that constitutes an (S/T)XV motif with isoleucine substituting for valine at the COOH terminus. However, this peptide did not show detectable binding at all.

We also analyzed the interactions to the first two PDZ domains of PSD-95 and SAP102 with the COOH termini of Neur-oligin and CRiPT, two proteins that bind to the third PDZ domain of PSD-95. As expected (12, 30), the Neur-oligin peptide did not show any detectable binding; however, the CRiPT peptide exhibited relatively strong binding to the second, although not the first, PDZ domains of PSD-95 and SAP102. In fact, the COOH-terminal CRiPT sequence comes close to the optimal binding sequence for the first two PDZ domains of PDS-95 and SAP102 (see below), and the original work on CRiPT described a weak interaction of this protein with the second, although not the first, PDZ domain of PSD-95 in the yeast two-hybrid system (12). In summary, the results described in this and the previous paragraphs correspond well to earlier qualitative and semi-quantitative observations and afford a reliable survey of binding constants. Accordingly, our assay is well suited to determine the contribution of certain residues at the COOH terminus of NR2b for PDZ domain binding by comparing the resulting affinity values of the various peptides with alterations in their sequences. The COOH termini of GluR1 (TGL) and GluR2 (VKI) subunits of the AMPA-type glutamate receptors constitute consen-sus sequences for binding type I and type II PDZ domains, respectively. GluR1 has been shown to be associated with SAP97, which, like PSD-95, contains type I PDZ domains, but no interaction with PSD-95 or SAP102 was detectable (41, 50). GluR2 binds to type II PDZ domains present in GRIP1 and -2 (19, 20). Peptides derived from these COOH termini did not show detectable binding (Fig. 2B), further confirming the specificity of our assays. We produced chimeric peptides based on the GluR1 COOH-terminal sequence. Starting at the very COOH-terminal position P0, we replaced an increasing number of GluR1 residues with those of NR2b. Little specific binding was detectable until at least the last 4 GluR1 residues (i.e. P3–P0) were replaced with those from NR2b (Fig. 2B). Substituting positions P–4 and P–5 further improved binding of the peptides, especially to the PDZ1 domains, which required these substitutions for detectable peptide interactions. Of note, binding by PDZ2 of PSD-95 and SAP102 was not very sensitive to substituting isoleucine for glycine at P–4 in these experiments, in contrast to those with single point mutations described below. This difference may reflect that contributions of each position may be influenced by the context of the other positions, including those upstream of P–4, although these latter positions appear to have little influence on binding when probed by single point mutations (see below).

**Contribution of the Residues of the Very COOH Terminus of NR2b to Binding to the First Two PDZ Domains of PSD-95 and SAP102**—To evaluate the role of each position at the COOH terminus of NR2b in PDZ binding, we systematically introduced single point mutations in this sequence and determined the KD values of the resulting peptides by FP (Fig. 3). We also evaluated the relative contributions of each position in solid phase assays with the corresponding biotinylated peptides anchored at streptavidin plates. The density of streptavidin in the plate wells was low, a characteristic of the plates we used that helped to avoid avidity and rebinding effects. For most biotinylated peptides, the solid phase binding assays were performed at single concentrations of the PDZ fusion proteins (Fig. 4), but for selected peptides, titration curves with increasing amounts of the PDZ fusion proteins were obtained (Fig. 5). These solid phase assays corroborated the relative contribution of each residue to PDZ binding (compare Fig. 3 with Figs. 4 and 5). The apparent KD values calculated from the solid phase titration assays were generally higher than those for the FP measurements, usually by a factor of 2–4. This increase in the...
that the serine (NR2 subunits) or threonine (Kv1.4) at Pfying PDZ domain interactions with target proteins, indicated
and Kv1.4 COOH termini, which were the first studies identi-
corroborates the latter.

However, the ranking order from these solid-phase titration
assays agrees very well with that obtained by FP and therefore
Therefore, the solid phase

The presence of valine versus
leucine or isoleucine at P0 may therefore be crucial in deter-
making the specificity of a COOH-terminal interaction with a
PDZ domain, with valine increasing the selectivity for PSD-95
and SAP102.

initial binding reaction of the purified PDZ fusion proteins with the
immobilized peptides. Those steps are performed after the re-
moval of unbound PDZ domains and take a minimum of 1.5 h.
If longer washing periods are applied, the apparent KD values
increase further (data not shown). Therefore, the solid phase
titation assays provide not true but only "apparent" KD values.
However, the ranking order from these solid-phase titration
assays agrees very well with that obtained by FP and therefore
corroborates the latter.

Initial experiments on the interaction of PSD-95 with NR2 and
Kv1.4 COOH termini, which were the first studies identi-
ifying PDZ domain interactions with target proteins, indicated
that the NR2 subunits or threonine (Kv1.4) at P0 and
the valine at P0 are the most crucial residues and cannot
effectively be replaced by alanine, hence the reference to the
"IST/TXV" motif. Later studies on other type I and also type II
PDZ domains indicated that valine at P0 can be replaced by
other hydrophobic residues including leucine, isoleucine,
and phenylalanine. The presence of valine versus
leucine or isoleucine at P0 may therefore be crucial in deter-
making the specificity of a COOH-terminal interaction with a
PDZ domain, with valine increasing the selectivity for PSD-95
and SAP102.

NR2 subunits, Kv1.1, and Kv1.4, carry either aspartate or
glutamate at P-1. Substituting glutamate for aspartate in the
NR2b peptide sequence did not cause big changes in affinities
for PDZ1 or -2 of PSD-95 and SAP102. Glutamine-substituted
peptides bound equally well, and so did asparagine-substituted
ones, at least for PDZ domain 95/1 in the solid phase single
concentration survey assay (Fig. 4). However, asparagine
substitution substantially reduced binding to the other three PDZ
domains in that assay (we did not perform FP assays for this
substitution). Serine showed a profile similar to that of aspar-
agine, with nearly unaltered binding affinity for PDZ domain
102/2, which exhibited a

![Figure 4](http://www.jbc.org/)

![Figure 5](http://www.jbc.org/)

![Figure 6](http://www.jbc.org/)
serine and threonine at this position form a hydrogen bridge with the histidine residue at the first position of the aB helix (see Fig. 8), which is conserved in type I PDZ domains (see Table III). The -2-position of NR2b and of other NR2 subunits has a serine. Replacing this serine with threonine as present in Kv1.4 and Kv1.1 resulted in a substantial increase in binding affinities for all four PDZ domains (Figs. 3 and 5). As expected, an alanine replacement caused a strong decrease in binding to most PDZ domains, although some binding in the low micromolar range was observed for 102/2 (Fig. 3; see also Fig. 4).

Peptides with the bulky tryptophan at this position did not show binding (Fig. 4). Accordingly, serine at P-2 can be replaced by threonine, which increases binding, but other residues are not well tolerated.

Glutamate is conserved at the -3-position in NR2 subunits and Kv1.4, but this position was initially considered less critical for PDZ binding, in part because Kv1.1, although a weaker interactor than Kv1.4 (10) (see also Fig. 2), carries a hydrophobic residue at this position and because substituting glutamine for glutamate in Kv1.4 did not alter its interaction with PSD-95 (9, 10). We found that only glutamate or glutamine at P-3 allows for high affinity interaction with PDZ1 and -2 of PSD-95 and SAP102. Other substitutions including those with aspartate, serine, threonine, alanine, leucine, phenylalanine, or lysine substantially reduced or eliminated binding. The fact that aspartate but not glutamine substitutions decreased binding indicates that the length of the side chain but not the negative charge is critical at this position for interaction with our PDZ domains. Both the \(\text{c}_{-3}\) of the glutamate residue and the amide group at the \(\text{a}_{-3}\) of the glutamine residue are capable of forming hydrogen bonds, and this ability seems to be important (see below).

Like the \(\text{a}_{-1}\)-position, the \(\text{a}_{-1}\)-position appears to tolerate a variety of residues. NR2a and -2b, NR2c and Kv1.1, and Kv1.4 and NR1 have isoleucine, leucine or valine, respectively, at this position, and replacing the valine in Kv1.4 with either arginine or tryptophan did not alter PSD-95 binding in yeast two-hybrid interaction assays (10). In addition, more recently identified binding partners for PDZ1 and -2 of PSD-95 include Krr2.1 and -2.3 (24, 51), and ErbB4 (63), which possess an arginine at that position.

Our peptide binding assays confirm that P-3 of NR2b is quite flexible and accepts not only hydrophobic residues (e.g., leucine; Figs. 4 and 5) but also lysine, which carries a positive charge similar to arginine. However, glycine substitution resulted in a strong loss of binding, indicating that the removal of a side chain does disturb binding. This position is, therefore, not completely neutral. In fact, alanine, methionine, and aspartate substitutions also strongly reduce binding. Collectively, these data indicate that P-4 accepts various hydrophobic and positively charged residues, but the presence of other residues including glycine, alanine, methionine, or aspartate selects against binding to PDZ1 and -2 of PSD-95 and SAP102.

Positions -5 through -10 generally accepted most substitutions, suggesting that they are less critical in determining PDZ domain interactions. However, aspartate was not well tolerated at P-5 (Figs. 3 and 4). Replacing tyrosine with an aspartate at P-10 resulted in a strong increase in binding affinity for the second PDZ domains of PSD-95 and SAP102 in the FP assay (Fig. 3) but not the solid phase assay (Fig. 4). It is quite possible that this position is too close to the plate surface to be fully accessible in the solid phase assay for interaction with the PDZ domain and may, therefore, only show an effect in the FP assay. These results suggest that selected positions past the P-4 may make some, although quite limited, contributions to PDZ domain interactions.

**Affinities of Peptides for the Third PDZ Domains of PSD-95 and SAP102**—We also tested the CRIPT- and Neuroligin-derived peptides along with those corresponding to the COOH termini of the various glutamate receptor subunits and Kv1.4 in binding assays with the third PDZ domains of PSD-95 and SAP102. The CRIPT peptide exhibited by far the highest affinity for both PDZ domains with a \(K_D\) in the range of 3 \(\mu\)M for PDZ3 of PSD-95 as determined by titration of FP (Fig. 6A). Neuroligin binding was also saturable but showed a much higher \(K_D\) of 28 \(\mu\)M in this assay (Fig. 6A), whereas the NR2b wild-type peptide does not show specific binding for PDZ3 (Fig. 6B). For nearly all of the peptides based on the NR2b COOH-terminal sequence, FP and the solid phase assay indicated little to no specific binding (data not shown). However, the NR2c peptide consistently showed distinguishable binding above background (Fig. 6B). In fact, FP titration indicated that association of this peptide with PDZ3 is saturable with a \(K_D\) around 25 \(\mu\)M (Fig. 6A). This finding opens up the possibility that the COOH terminus of NR2c may not only interact with the first two but also the third PDZ domains of PSD-95 and SAP102. Although the affinity is relatively low for the latter interactions, they may help stabilize association of NR2c with the first two PDZ domains of PSD-95 and SAP102. In a similar way, CRIPT may not only interact with the third but also with the second and perhaps first PDZ domains of PSD-95 and SAP102. The idea of combining weak and strong COOH-terminal interactions with PDZ domains also receives support by...
early findings that NR2 subunits and Kv1.4 interact most robustly with the second PDZ domains of PSD-95 and SAP102 and more weakly with the first PDZ domains (10, 29). Because each NMDA receptor and each Kv1.4 channel possesses several subunits with appropriate COOH-terminal SXV motifs, simultaneous interactions of one receptor or channel complex with several PDZ domains appears likely.

Survey of the Available Human Genome Sequences for Potential Binding Partners for the First Two PDZ Domains of PSD-95 and SAP102—To obtain an impression of how many potential binding partners with a COOH-terminal (E/Q)(S/T)XV motif exist, we screened proteins as predicted by the currently available draft version of the human genome for this motif. We did not use any restrictions at the –1-position and –4-position. We identified 54 proteins and divided them into four categories (Table II). Group 1 contains all proteins with a COOH-terminal (E/Q)(S/T)XD/E/Q/NV sequence. Of note, nearly all proteins in that group carry a hydrophobic or positively charged residue at P–4, although this had not been a criterion for grouping those proteins together. We predict that these proteins are likely to bind strongly to the first two PDZ domains of PSD-95 and SAP102. In fact, five of the 14 members of this group had been identified as binding partners (in boldface type at the top of the list in Table II), and we present evidence that at least one member of the brain-specific angiogenesis inhibitor family (BAI1) does also interact with PSD-95 (see below). Group 2 proteins differ from group 1 by having a residue different from the binding-promoting aspartate, glutamate, asparagine, or glutamine at P–1 but carrying an advantageous aliphatic or positively charged residue at P–4 (i.e. isoleucine, leucine, valine, arginine, or lysine). Only two of the 16 proteins in this category are actually known to interact with the first two PDZ domains of PSD-95 or SAP102 (PMCA4b and CRIP7). Group 3 proteins have no favorable residue at either P–1 or P–4, and only two of 20 proteins have been shown to bind to PSD-95 or SAP102. Finally, group 4 contains proteins with a lysine or an arginine at P–1, which inhibit binding to our PDZ domains. Three of the four proteins, Kir3.2, Kir3.3 (24), and the β1-adrenergic receptor (28), have actually been tested for binding to PDZ1 or -2 of PSD-95, with negative results. In the same studies, parallel pull-down experiments resulted in interactions that serve as positive controls (the inward-rectifying K+ channels Kir2.1 and Kir2.3 bound to PSD-95 in Ref. 24, presumably via the second PDZ domain (51), and the β1-adrenergic receptor bound to the third, although not the first or second, PDZ domain of PSD-95 in Ref. 28). These results confirm our finding that a lysine and perhaps an arginine are inhibiting binding to the first two PDZ domains of PSD-95 and SAP102.

A comparison with Table I, which lists all known interaction partners of the first two PDZ domains of PSD-95 and SAP102 with COOH-terminal SXV motifs, indicates that Table II identifies most proteins with COOH-terminal (E/Q)(S/T)XV sequences (NR2a to -d, Kv1.4, Kir3.2, 3.3, β1 adrenergic receptor, PMCA4b, CRIP7, Citron, and Sema4c in Table I). Maguini and SynGAP also possess COOH-terminal (E/Q)(S/T)XV sequences (Table I) but were not identified, because the sequences for these two proteins predicted from the human genome projects only show versions with truncated COOH termini, perhaps due to inaccuracies in the draft sequence (64). Kv1.1, Kv1.2, Kv1.3, Kir2.1, Kir2.3, β2-adrenergic receptor, PMCA2h, ErbB4, Neda1, and Neureilgin (Table I) do not conform to the (E/Q)(S/T)XV motif and were not detected in our screen, which is, therefore, not complete in terms of binding partners for the first two PDZ domains of PSD-95 and SAP102. However, most of the latter proteins are known or predicted to interact with these PDZ domains more weakly than proteins with a COOH terminus that matches the (E/Q)(S/T)XV sequence (e.g. Kv1.4 and PMCA4b bind much stronger than Kv1.1–3 and PMCA2b, respectively; see “Discussion”).

Not all of the proteins in Table II are actually potential binding partners of PSD-95 and SAP102 because their expression patterns do not overlap. For example, PSD-95 and SAP102 are only detectable in brain, whereas the PDZ binding kinase is abundant in testis and placenta and weakly expressed in heart but not brain (65, 66). Tyrosine-related protein 1 is involved in melanin syntheses and restricted to melanocytes. It binds to the PDZ domain of GIPC (67), but due to its restriction to melanocytes it would not constitute an interaction partner for PSD-95 or SAP102 in neurons. The Na+ channel Na1.5 was originally described as muscle-specific and interacts in these tissues with the PDZ domains of syntrophins (68). Recently, limited expression of Na1.5 in the brain has been described (26), but PDZ-domain interaction assays for a Na1.5-derived peptide were negative (25), suggesting that Na1.5 does not interact with PSD-95 or SAP102 even if co-expressed in some neurons. Another example of a protein that has a COOH-terminal (E/Q)(S/T)XV motif and is present in neurons but does not appear to interact with PSD-95 is diacylglycerol kinase ζ, which binds to the PDZ domains of syntrophins but not of PSD-95 (27).

Interaction of Brain-specific Angiogenesis Inhibitor BAI1 with PSD-95—Many proteins revealed by our proteomic search have not been considered in terms of association with PSD-95. We were able to obtain antibodies for three of these proteins (BAI1, PKCa, and frizzled 2) and evaluated those proteins for co-immunoprecipitation with PSD-95 and SAP102 from rat brain extracts. Antibodies against BAI1 (48) recognize three bands in brain extract, all of which migrate in the range around 150 kDa; the lower two bands form a doublet, which is often not resolved (Fig. 7A and data not shown). These bands are enriched after immunoprecipitation with the same antibodies (Fig. 7A), suggesting that all three polypeptides are specifically recognized by the antibody and represent various isoforms of BAI1. BAI1 immunoprecipitates also contained PSD-95, but SAP102 was never detectable in the BAI1 complex (Fig. 7A). To ensure that the BAI1-PSD-95 complex was present in the intact brain and did not form during homogenization, we homogenized parallel samples in a 50-fold larger buffer volume than under our standard conditions before collecting crude membranes by ultracentrifugation from both conditions. Subsequent immunoprecipitation resulted in comparable immunoreactivity for BAI1-associated PSD-95 (Fig. 7A). We did observe coprecipitation of BAI1 and PSD-95 and a lack of coprecipitation of BAI1 and SAP102 not only after extraction with 1% deoxycholate but also after extraction with either 1% Triton X-100 or 1% SDS (data not shown). Together with an analogous finding for PMCA2b, which also selectively binds to PSD-95 but not SAP102 (52), our observations that PSD-95, but not SAP102, is associated with BAI1 in the brain indicate that PSD-95 and SAP102 may play different roles in their cellular context by interacting with distinct, although partially overlapping, protein pools. BAI1-related BAI2 and BAI3 also have a COOH-terminal SXV motif (YQTFE) with a hydrophobic residue at P–4 and a glutamate at P–1 and carry an acidic residue at P–10. Because these residues at these positions increase binding of our NR2b-derived peptides to the first two PDZ domains of PSD-95, it is likely that BAI2 and -3 are also interaction partners for PSD-95.

In parallel with BAI1, we immunoprecipitated the Wnt receptor frizzled 2 (69–71). The frizzled 2 antibody recognized one major band in brain extract of the expected molecular mass by immunoblotting (Fig. 7B). Immunoprecipitation with this
We performed a BLAST search for COOH-terminal (E/Q)(S/T)XV-containing proteins. The search was restricted to the working draft sequence of the human genome protein products. For each match, the GenBank accession number is given, along with the COOH-terminal consensus sequence (residues that potentially increase affinity for PSD-95 PDZ binding are in boldface type) and if there is evidence whether (+) or not (−) the protein (or its mRNA) is expressed in the brain (−) or not (−) indicates that there is no evidence for brain expression, Y−/− indicates that expression in the brain is low and spatially narrow). The entries are grouped (1 through 4) in order from the highest predicted affinity group to the lowest. Protein names in boldface type indicate confirmed association with PSD-95 PDZ domains.

| GenBank™ | Brain | Protein | Position |
|----------|-------|---------|----------|
| NP_000824 | + NR2a | Y K K M P S I E S D V |
| NP_000825 | + NR2b | Y E K L S I E S D V |
| NP_000826 | + NR2c | W R R I S S L E S E V |
| NP_000827 | + NR2d | S A H F S L E S E V |
| NP_002224 | + Voltage-gated K⁺ channel Kv1.4 | C S N A K A V E T D V |
| NP_002234 | + Inwardly rectifying K⁺ channel Kir1.3 | L R T L L Q S N V |
| NP_062455 | + Rho guanine exchange factor 3 (ARHGEF3) | C G N S R H G E S N V |
| NP_055265 | + Rho guanine exchange factor 16 (ARHGEF16) | R M E R L R V E T D V |
| NP_001693 | + Brain-specific angiogenesis inhibitor 1 | G Q D I D L Q T E V |
| NP_001694 | + Brain-specific angiogenesis inhibitor 2 | E P P D G D F Q T E V |
| NP_001695 | + Brain-specific angiogenesis inhibitor 3 | D V Q E D F Q T E V |
| NP_006662 | + Solute carrier family 1 (glutamate transporter) member 7 | T I Q I S L E T N V |
| NP_016361 | ? Hypothetical protein FLJ14050 | R A R G P R E S E V |
| NP_060962 | - PDZ-binding kinase; spermatogenesis-related protein | A H I V E A L E T D V |

**Group 2. (S/T/V/K/R)/(E/Q)(S/T/XV) (X = any except D/E/Q/N/K/R)**

| +/− | Sodium channel Nav1.5 α subunit (cardiac) | P S P D R D R E S I V |
| NP_000325 | − Sodium channel Nav1.4 α subunit (skeletal muscle) | T V R P G V K E S L V |
| NP_009138 | − Dual specificity phosphatase 10 (MKPS) | T P K L M G V E T V V |
| NP_000034 | − Apoptosis (APO-1) antigen 1 (Fas antigen) | S N F R N E I Q S L V |
| NP_037359 | − Breast cell glutaminase | A L S K E N L E S M V |
| NP_060171 | − Hypothetical protein FLJ20185 (ARHGAP8) | L E G S G G R Q S V V |

| + | Plasma membrane Ca²⁺ ATPase PMCA4b | D S S L Q S L E T S V |
| NP_054890 | + CRIP1 | L D T K N Y K Q T S V |
| NP_092592 | + Receptor-type protein-tyrosine phosphatase, -1 | G N A E S L E S |
| NP_057240 | + Myotubulin related protein 2 (dual-specificity phosphatase) | A Q C V T P V Q T V V |
| NP_002728 | + PKCa | Q F V H P I L Q S A V |
| NP_004708 | + Diacylglycerol kinase, α | V I G H E D L E T A V |
| NP_057438 | ? Organic anion transporter OAT-E | S A T D S Q L Q S S V |
| NP_005493 | ? ATP-binding cassette, subfamily A member 1 | L Q D E K V K E S Y V |
| NP_057204 | ? Putative ring zinc finger protein NY-REN-43 | V L A S G N R E S V |
| NP_002368 | ? MAS1 oncogene (angiotensin receptor) | N C N T V V T V E T V V |

**Group 3. X(E/Q)(S/T)/X(V (X = any except I/L/V/ K/R, X = any except D/E/Q/N/K/R)**

| + | Semaphorin 4c | L P D S N P E E S S V |
| NP_060259 | + Citron | Q V N K V W D Q S S V |
| NP_045786 | + G protein-coupled receptor 45 (orphan) | V Y V C N E N Q S A V |
| NP_009158 | + Rho GTPase-activating protein 6; rhoGAPX-1, isoform 1 and 4 | D N P D A L P E T L V |
| NP_003402 | + Monocarboxylate acid transporter, member 1 | E G G P K E E S P V |
| NP_004198 | + Monocarboxylate acid transporter, member 3 | G E V V H T P E T S V |
| NP_002832 | + Receptor-type protein-tyrosine phosphatase, γ | S D F P A E S M E S L V |
| NP_003637 | + Diacylglycerol kinase, α | M I Q E E D Q E T A Y |
| NP_003496 | + frizzled 1 (Wnt receptor) | L T N S K Q G E T T V |
| NP_001457 | + frizzled 2 | L T N S R H G E T T V |
| NP_036325 | + frizzled 4 | V K P G K G S T E T V |
| NP_003498 | + frizzled 7 | L S H S S K G E T A V |
| NP_067679 | + KIAA0626 gene product | K N T C I I Y E S H V |
| NP_060710 | + Neph-1 | Q R F Q Q R M Q T H V |
| NP_003227 | ? Transforming growth factor, α | R T A C C H S E T V V |
| NP_076941 | ? Hypothetical protein MGC3103 | G S A E R L E E S S V |
| NP_057580 | ? Hypothetical protein | L K K V A E T P V |
| NP_110920 | − Nucleosomal binding protein 1 | D G K K E E P Q S I V |
| NP_062550 | − Class-I MHC-restricted T cell-associated molecule | E K H I Q V P E S I V |
| NP_000541 | − Tyrosinase-related protein 1 | E K L Q N F P Q S V |

**Group 4. (E/Q)(S/T)/(K/R)/V**

| NP_002231 | + Inwardly rectifying K⁺ channel, Kir3.2 | D V A N L E N E S K V |
| NP_004374 | + Inwardly rectifying K⁺ channel, Kir3.3 | C L P P E S E S K V |
| NP_006875 | + β₂-adrenergic receptor | C B P G F A S E S K V |
| NP_065110 | − Cysteinyl leukotriene CysLT2 receptor | V S V W L R K E T R V |

**Selectivity of PDZ Domains**

**TABLE II**

*Human proteomic search for proteins containing PSD-95 PDZ binding consensus sequence (E/Q)(S/T)XV*

| GenBank™ | Brain | Protein | Position |
|----------|-------|---------|----------|
| NP_000824 | + NR2a | Y K K M P S I E S D V |
| NP_000825 | + NR2b | Y E K L S I E S D V |
| NP_000826 | + NR2c | W R R I S S L E S E V |
| NP_000827 | + NR2d | S A H F S L E S E V |
| NP_002224 | + Voltage-gated K⁺ channel Kv1.4 | C S N A K A V E T D V |
| NP_002234 | + Inwardly rectifying K⁺ channel Kir1.3 | L R T L L Q S N V |
| NP_062455 | + Rho guanine exchange factor 3 (ARHGEF3) | C G N S R H G E S N V |
| NP_055265 | + Rho guanine exchange factor 16 (ARHGEF16) | R M E R L R V E T D V |
| NP_001693 | + Brain-specific angiogenesis inhibitor 1 | G Q D I D L Q T E V |
| NP_001694 | + Brain-specific angiogenesis inhibitor 2 | E P P D G D F Q T E V |
| NP_001695 | + Brain-specific angiogenesis inhibitor 3 | D V Q E D F Q T E V |
| NP_006662 | + Solute carrier family 1 (glutamate transporter) member 7 | T I Q I S L E T N V |
| NP_016361 | ? Hypothetical protein FLJ14050 | R A R G P R E S E V |
| NP_060962 | - PDZ-binding kinase; spermatogenesis-related protein | A H I V E A L E T D V |
antibody, but not with a control antibody, resulted in the same band, indicating that this antibody effectively immunoprecipitated frizzled 2 under our conditions. However, we never observed coprecipitation of either PSD-95 or SAP102 with frizzled 2, whether membrane extracts were prepared with 1% deoxycholate, Triton X-100, or SDS (Fig. 7A and data not shown). The COOH-terminal sequence of frizzled 2 matches that of frizzled 1 (GETTV) and is very similar to the COOH termini of frizzled 4 (SETVV) and frizzled 7 (GETAV). All of these sequences predict at best weak interactions with the first two PDZ domains of PSD-95 or SAP102 (group 3 in Table II). Our negative results suggest that frizzled 2 and its homologs do not bind to PSD-95 or SAP102 in vivo.

Interaction of PKCs with PSD-95 and SAP102—We performed similar experiments looking for co-immunoprecipitation of PKCs with PSD-95 and SAP102. PKCa is listed in group 2 (Table II) with an unfavorable alanine at P−1 but a beneficial leucine at P−3 in its COOH-terminal sequence (LQSAV), suggesting an intermediate affinity for the first two PDZ domains of PSD-95 and SAP102. PKCa has been shown earlier to bind with its COOH terminus to the PDZ domain of PICK1 (22). Both PSD-95 and SAP102 coprecipitated with PKCa (Fig. 7C).

As in the previous experiments (Fig. 7A), immunoprecipitations with appropriate control antibodies were negative for PSD-95 and SAP102, indicating the specificity of the immunoprecipitations. Similar results were obtained when 1% Triton X-100 or 1% SDS instead of the routinely employed deoxycholate was used for solubilization of the membrane fractions (data not shown). Comparable amounts of PSD-95 and SAP102 coprecipitated with PKCa whether the brain tissue was homogenized in the standard volume or at a 50-fold higher dilution, indicating that PKCa was associated with PSD-95 and SAP102 in vivo before homogenization of the brain tissue (Fig. 7C). The novel finding that BAI1 and PKCs are associated with PSD-95 testifies to the utility of our proteomic approach in defining the potential pool of interaction partners for various PDZ domains.

DISCUSSION

Our results demonstrate proteins with COOH-terminal (E/Q)S/T/XV sequences possess the potential for stable interactions with the first two PDZ domains of PSD-95 and SAP102. This potential is further increased if P−1 is aspartate, glutamate, asparagine, or glutamine and if P−4 is a hydrophobic residue including leucine, isoleucine, valine, or perhaps tryptophan, lysine, or arginine (10). Aspartate and perhaps glutamate at P−1 may also foster binding to the first two PDZ domains of PSD-95 and SAP102. Nevertheless, the 0-, −2-, and −3-positions are highly sensitive to the precise residue present and are most crucial in determining the selectivity of the NR2b COOH terminus for binding to the first two PDZ domains of PSD-95 and SAP102. We found a severalfold preference of these PDZ domains for valine at P0 over the related amino acids leucine and isoleucine, for threonine at P−2 over serine, and for glutamate and glutamine at P−3 over aspartate. Accordingly, even conservative substitutions at these positions such as serine for threonine or aspartate for glutamate affect binding. As discussed in the following paragraphs, these findings are in good agreement with published results on proteins that bind to these PDZ domains (see Table I).

The first identified binding partners for PSD-95 were the NR2b subunits and Kv1.4. They not only match the (E/Q)S/T/XV motif, they also possess an aspartate or glutamate at P−1 and an isoleucine, leucine, or valine at P−4. They are therefore likely to be among the strongest interactors in vivo. Kv1.1, Kv1.2, and Kv1.3 also have (S/T/X)V motifs and hydrophobic residues at P−4 and aspartate at P−1. However, they possess hydrophobic residues at P−3, which should strongly reduce binding to the first two PDZ domains of PSD-95 and SAP102 (Figs. 3–5). In fact, PSD-95 association with Kv1.1, Kv1.2, or Kv1.3 appears to be much weaker than with Kv1.4 in a yeast two-hybrid assay and a clustering assay in mammalian cells (10, 72). In a clustering assay, Shaker, a Drosophila homolog of the Kv1 subfamily, exhibits an interaction with PSD-95 that is as strong as the interaction between Kv1.4 and PSD-95 (72). In contrast to Kv1.1, Kv1.2, and Kv1.3, Shaker perfectly matches positions 0 through −3 of the Kv1.4 COOH terminus, and P−4 carries an isoleucine. These findings corroborate our consensus sequence for binding to the first two PDZ domains of PSD-95 and SAP102.

The next two proteins in Table I are the inwardly rectifying K+ channels Kir2.1 and Kir2.3 (24, 51). Both proteins have an isoleucine rather than a valine at P0, which should strongly reduce binding affinity for those PDZ domains. However, they carry an arginine at P−4, an aspartate or glutamate, respectively, at P−2, and, in the case of Kir2.1, a glutamate at P−1. According to our analysis, these residues help to strengthen the interaction with our PDZ domains and may promote stable binding even if the COOH-terminal position is not valine but the related isoleucine. Kir3.2 and Kir3.3 do conform to the generic (S/T/X)V motif, but a careful analysis indicates that they do not bind to PSD-95 or SAP102 (24). The lack of detectable interaction is probably due to the presence of the positively charged lysine at P−1, which inhibits binding to our NR2b-
derived peptides at this position. Similarly, the β₂ adrenergic receptor carries an ESXV motif with lysine at P⁻¹ and does not appear to interact with either the first or the second PDZ domain of PSD-95 (28); it does bind to the third PDZ domain of PSD-95 (28), indicating that the third PDZ domain is quite different from the first two PDZ domains. The β₂ adrenergic receptor with the COOH-terminal DSPL sequence binds to the PDZ domain of the Na⁺/H⁺ exchange regulatory factor (53) but does not seem to associate with PSD-95 (28), probably because of the COOH-terminal leucine without compensatory residues at the −1, −4-, or −10-position.

The plasma membrane Ca²⁺-ATPase PMCA4b has a COOH-terminal interaction sequence that is predicted to allow for high affinity interaction: threonine at P⁻², the hydrophobic leucine at P⁻⁴, and aspartate at P⁻¹⁰. It strongly binds to PDZ1 and -2 of PSD-95 and SAP102 (16, 52). PMCA2b also binds to the first two PDZ domains of PSD-95, but these interactions are much weaker than those observed with PMCA4b (16), and no binding could be observed between PMCA2b and SAP102 (52). This binding behavior would be predicted from our findings that the leucine at the 0-position of PMCA2b should strongly reduce binding to PDZ1 and -2 of PSD-95 and SAP102. Maguin-1 matches the (E/Q)/S/T/XV motif, co-immunoprecipitates with PSD-95 from rat brain extracts, and binds to one or more PDZ domains of PSD-95 (73). Although the exact PDZ domains that interact with Maguin-1 have yet to be defined, based on our analysis it could bind to the first two domains because it has not only the (E/Q)(S/T) domain but also an isoleucine at P⁻⁴. However, the histidine at P⁻¹ is partially positively charged and may potentially be inhibitory for the binding to the first two PDZ domains and perhaps direct it to the third PDZ domain.

CRIPT also possesses that (E/Q)/S/T/XV motif and a lysine at P⁻⁴, which, like isoleucine at this position, increases binding to the first two PDZ domains of PSD-95 and SAP102. Although it was originally described mainly as a binding partner for the third PDZ domain of PSD-95 (12), CRIPT did interact with the second PDZ domain in the original yeast two-hybrid assays (12). A peptide derived from the COOH terminus of CRIPT in our titration assays with Kᵡ values between 1 and 2 μM to the second PDZ domains of PSD-95 and SAP102, although binding to the first PDZ domains was either very weak or not detectable (Fig. 2). We propose that the COOH terminus of CRIPT strongly interacts not only with the third but also the second PDZ domains of PSD-95 and SAP102. This binding pattern would explain why application of a membrane-permeable peptide derived from the CRIPT COOH terminus effectively dispersed PSD-95 from the postsynaptic site (74).

Similar to CRIPT’s QTSV COOH-terminal sequence, Citron has the sequence QSSV at its COOH terminus and binds preferentially to the third and possibly also to the second PDZ domain of PSD-95 (15, 75). It carries aspartate at P⁻⁴, in contrast to CRIPT’s lysine, and P⁻² is a serine rather than a threonine; accordingly, we predict that Citron binds in vivo much more weakly than CRIPT to the second PDZ domain of PSD-95. Indeed, Citron’s interaction with PDZ2 of PSD-95 appears to be much less favorable than with PDZ3 (15). The Citron-related COOH-terminal sequence EESSV is present in the semaphorin Sema4C and predicts a modest affinity for the first two PDZ domains of PSD-95 and SAP102. Sema4C co-immunoprecipitates with PSD-95 from rat brain and binds to a fusion protein containing the first two PDZ domains of PSD-95 (76). Another semaphorin, Sema4f, which has also recently been shown to interact with the second PDZ domain of PSD-95, possesses an advantageous threonine at P⁻² but a disadvantageous isoleucine at P₀ (77). SynGAP is a Ras-GTPase-activating protein initially identified in a subcellular fraction enriched with postsynaptic structures (13) and also by a yeast two-hybrid screen with the third PDZ domain of SAP102 (14). SynGAP does match the (E/Q)/S/T/XV motif but does carry an arginine at P⁻¹. Because P⁻¹ does not tolerate a lysine well, we would expect that an arginine at this position also inhibits binding to the first two PDZ domains of PSD-95 and SAP102. However, we have not tested this prediction, and it is possible that P⁻¹ does accept an arginine.

Yeast two-hybrid interaction assays have suggested that SynGAP binds not only to the third but also to the first two PDZ domains (14). However, the interaction between the first two PDZ domains and SynGAP was not confirmed by other tests. Because the yeast two-hybrid system does result in a substantial portion of false positive results, it is quite conceivable that SynGAP may interact with the third but not the first two PDZ domains of PSD-95 and SAP102 in intact neurons, as would be predicted by our studies.

Having an asparagine at P⁻³, the neuregulin receptor ErbB4 does not conform to the (E/Q)/S/T/XV motif but does interact with the first two PDZ domains of PSD-95 and SAP102 (63, 78). ErbB4 does have an arginine at P⁻⁴, which, like lysine (Figs. 3–5), may foster binding to the first two PDZ domains of PSD-95 (10). We hypothesize that the conservative, although not ideal, substitution of a glutamate or glutamine at P⁻³ by an asparagine can be counterbalanced by arginine at P⁻⁴, thereby permitting the PDZ domain interaction. Neurologin is another protein that has a generic SXV motif but does not match the (E/Q)/S/T/XV sequence. It has a threonine at P⁻³ and an arginine at P⁻¹, which may exert a negative effect at this position. In agreement with our data, no binding to the first two PDZ domains of PSD-95 was detectable in earlier studies, although it does interact with the third PDZ domain of PSD-95 (30).

Finally, affinity chromatography with recombinant SAP102 protein identified one of the COOH-terminal splice variants of a novel protein, Nedasin, as a binding partner for the first two PDZ domains of PSD-95 and SAP102. Although it does have a hydrophobic residue at P⁻⁴, it does have a hydrophobic residue at P⁻⁴, which may in part compensate for the lack of the more optimal glutamate/glutamine at P⁻³.

In summary, the 20 proteins that have been shown to bind with a COOH-terminal (S/T/X)V motif to the first two PDZ domains of PSD-95 or SAP102, 11 have an (E/Q)/S/T/XV sequence and should interact with these PDZ domains in a stable manner and with high affinity (Table I). The other nine binding partners have variations either at the 0- or 3-position that are predicted to weaken the interactions and at the same time residues at the −1- or −4-position and in some cases also at the −10-position that strengthen the interactions and perhaps compensate for the variations at the 0- and −3-positions. Of these nine proteins, only a few have been shown to actually interact with PSD-95 and SAP102 by coimmunoprecipitation experiments from the brain. We conclude that there is a substantial amount of flexibility in the COOH-terminal binding sequences of ligands of the first two PDZ domains of PSD-95 and SAP102. Most proteins with the optimal (E/Q)/S/T/D/E/Q/S/N/V sequence have been shown or are likely to interact with those PDZ domains (see Table II and Fig. 7). Deviation from this sequence often results in weaker interactions. Many of the weaker interactions may have an auxiliary character by acting primarily in concert with other interactions within a protein complex, thereby contributing to the overall binding of two or more proteins in the context of multiple protein-protein interactions.

The third PDZ domains of PSD-95 and SAP102 possess bind-
ing requirements that are quite different from the first two PDZ domains. Many of the proteins that bind to the first two PDZ domains do not effectively interact with the third, including NR2 subunits and the various Shaker-type and inwardly rectifying K⁺/H₁₁₀₀¹ channels (Table I). Furthermore, the wild-type NR2b COOH-terminal peptide as well as peptides based on the wild-type NR2b sequence with single amino acid residue substitutions (see Figs. 3 and 4) do not exhibit any specific binding to PDZ3 of PSD-95 and SAP102 (data not shown). Other proteins, including the α₁-adrenergic receptor and Neuroligin, associate with the third rather than the first two PDZ domains of PSD-95 or SAP102 (Table I). A sequence alignment of the PDZ domains from PSD-95 and SAP102 (Table III, lower part) shows that PDZ1 and -2 have a substantially higher degree of homology among themselves than with the PDZ3 domains, although all of them are type I PDZ domains. This study focused on the interaction between NR2b and PDZ1 and -2 of PSD-95 and SAP102. The exact determinants of the specificity for the third PDZ domain could not be explored within this study, because it would require a completely different set of peptide libraries. However, it appears that no single amino acid substitution of the NR2b consensus sequence will allow binding to PDZ3, since none of the peptides listed in Fig. 3 and 4 showed specific binding to either of the third PDZ domains of PSD-95 or SAP102.

To evaluate our results with respect to available structural information, we have modeled the binding of an NR2b-based peptide in the second PDZ domain of PSD-95. Our approach is based on the NMR solution structure of PSD-95 PDZ2 (80) and the co-crystal structure of PSD-95 PDZ3 with a peptide based on the CRIP1 COOH terminus (23). The peptide IESDV was modeled in the same β-strand conformation as the CRIP1 peptide KQTSV (23) and manually docked into the binding pocket using Sybyl (Tripos). Some visualization was also performed with WebLab Viewer Lite (Accelrys) (Fig. 8). Stabilizing hydrogen-bonding interactions are expected based on this theoretical approach that closely match the interactions observed in the co-crystal structure of PDZ3 and CRIP1 (23). Table III (upper section) summarizes residues in PDZ2 important for binding to NR2b, based on previous structural and these modeling data as well as our peptide binding assays.

The free carboxyl terminus of valine at P⁰ is predicted to form hydrogen bonds with the carbonyl oxygens of Leu₁⁷⁰, Gly₁⁷¹, and Phe₁⁷² in the highly conserved GLGF backbone, in agreement with the PDZ3-CRIPT peptide structure (23) (Fig. 8 and data not shown; see Table III for an alignment of these residues with other PDZ domains). The valine hydrophobic side chain is oriented into a hydrophobic pocket lined with side chains from residues in the GLGF motif (Phe₁⁷² and Ile₁⁷⁵) and B helix (Val₂²⁹ and Leu₂₃₂; Ile₁⁷⁵ and Leu₂₃₂ are not depicted in Fig. 8, but see Ref. 23). Substitutions of other hydrophobic residues (alanine, leucine, and isoleucine) have a detrimental effect on binding to the second PDZ domain (Fig. 3). Perhaps the conformation of the residues that line the hydrophobic pocket is such that only valine at P⁰ has the right side chain length that allows a good fit. Alanine would be too small to fill the volume, creating energetically unfavorable unfilled space. Although leucine and isoleucine can be accommodated in this position to some degree, their longer side chains might be too large to fit in the pocket as well as valine’s side chain does, decreasing the overall affinity of the corresponding peptides for the PDZ domains.

Analogous to the –1-position serine side chain in the crystal of PDZ3 and the CRIP1 peptide (23), the P⁻¹ asparagine side chain of the NR2b peptide is oriented in the β-strand such that its carboxyl side chain points out of the PDZ binding pocket. In contrast to predictions based on the co-crystal structure for PDZ3-CRIPT peptide (23), the carboxyl oxygen of the asparagine residue side chain may participate in hydrogen bonding interactions with the hydroxyl side chain of Ser²¹⁷² (Fig. 8;
see also Ref. 80) or the amine hydrogens of Lys\textsuperscript{193} of the second PDZ domain (Fig. 8; although they may not form simultaneously, all three hydrogen bonds are indicated). It is possible that Lys\textsuperscript{193} is not readily available for formation of a hydrogen bond with the aspartate at P\textsuperscript{-1}, because it may be tied up in a hydrogen bond with the glutamate or glutamine at P\textsuperscript{-3} (see below). The residue that corresponds to Ser\textsuperscript{173} in PDZ2 is conserved in all PDZ1 and -2 domains of PSD-95 and of its homologs SAP97, SAP102, and PSD-93 and also in the NR2-interacting PDZ5 domains of MAGI-1, -2, and -3 (Table III). However, this residue is an asparagine in PDZ3 of PSD-95 (Asn\textsuperscript{326}) and its homologs (Table III). When Asn\textsuperscript{326} is mutated to a serine, a peptide derived from the COOH terminus of Kv1.4 that normally binds only to PDZ1 and -2 can now also bind to the third PDZ domain (12). The potential hydrogen bonding interactions with Ser\textsuperscript{173} may explain the preference for residues with negatively charged, electron-donating side chains (aspartate, glutamate) at P\textsuperscript{-1}. Positively charged (lysine) or hydrophobic residues substituted at P\textsuperscript{-1} greatly reduced affinity for the PDZ domain.

The CRIPT/PDZ3 co-crystal structure predicted that the hydroxyl side chain of the threonine at P\textsuperscript{-2} forms hydrogen bonding interactions with the nitrogen of the imidazole side chain of His\textsuperscript{372}, which is conserved in most type I PDZ domains (e.g. Table III). The -2-position of wild-type NR2b is a serine, which according to our modeling can also interact with the corresponding His\textsuperscript{325} in PDZ2 (Fig. 8). In our binding studies, we observed that substituting the P\textsuperscript{-2} serine with a threonine results in an ~2-fold increase in affinity. One potential explanation for this result is that the hydrophobic Val\textsuperscript{229} in PDZ2, which is one \(\alpha\)-helical turn away from His\textsuperscript{325}, lines the binding pocket of and is in close proximity to the residue at P\textsuperscript{-2} of the ligand (Fig. 8). The interaction of the NR2b S2T ligand in the binding pocket of the PDZ domain may be stabilized by hydrophobic interactions between the methyl group of the threonine side chain and the hydrophobic side chain of Val\textsuperscript{229}. Because serine lacks this methyl group, the interactions of the wild-type NR2b peptide would be weaker. Val\textsuperscript{229} is conserved in PDZ1 and -2 of PSD-95 and its homologs; it is isoleucine in PDZ5 of MAGI-1, -2, and -3 (Table III). However, this position carries an alanine in PDZ3 of the PSD-95 homologs (Table III), which may provide less if any hydrophobic interactions with the threonine side chain.

The carboxyl side chain of glutamate at P\textsuperscript{-3} can form a hydrogen bond with Thr\textsuperscript{192} or with Lys\textsuperscript{193} of PDZ2 (Fig. 8; we show both potential hydrogen bonds, although only one of the two may exist at any given time). Our results indicate a substantially reduced affinity when this glutamate is replaced by aspartate but not when it is substituted by glutamine. Glutamine possesses the length and the potential to form hydrogen bonds with Thr\textsuperscript{192} or with Lys\textsuperscript{193} comparable with glutamate but does not carry a negative charge. Aspartate does have a negative charge, but it is shorter than glutamine. Our findings, therefore, indicate that the main factor for strong binding to PDZ2 of PSD-95 is the side chain length and the capability to form a hydrogen bond with either Thr\textsuperscript{192} or Lys\textsuperscript{193}. It does not appear to be the negative charge of the glutamate, although the latter may stabilize binding by electrostatic interactions with Lys\textsuperscript{193}. Such considerations may especially be true for ligand binding to PDZ1 of PSD-95, in which case changing glutamate to a glutamine at P\textsuperscript{-3} results in a substantial reduction in affinity (Fig. 3). Thr\textsuperscript{192} and Lys\textsuperscript{193} are conserved in all PDZ1 and -2 domains of the PSD-95 homologs (Table III). However, the PDZ5 domains of MAGI-1, -2, and -3 have leucine and arginine at the positions homologous to Thr\textsuperscript{192} and Lys\textsuperscript{193}, respectively, and all PDZ3 domains of PSD-95 homologs have serine (Ser\textsuperscript{319}) and phenylalanine at the corresponding positions (Table III). The glutamine residue at P\textsuperscript{-3} of the PDZ3 ligand CRIPT can form a hydrogen bridge with Ser\textsuperscript{319} and also with Asn\textsuperscript{326} (23). However, the high affinity PDZ3 ligand Neuroligin has threonine rather than glutamate or glutamine at P\textsuperscript{-3}, indicating that PDZ3 of PSD-95 and its homologs have requirements different from those of PDZ1 or -2 for strong binding.

The -4-position of the ligand is at the border of the PDZ binding pocket proper. The CRIPT/PDZ3 co-crystal was unable to resolve the structure at and beyond this position in the CRIPT peptide (23). However, the NMR structure of the \(\alpha\)-syntrophin PDZ domain in complex with a peptide derived from the COOH terminus of syntrophin’s binding partner Na\textsubscript{v}1.4 (KESLV) indicates that Asp\textsuperscript{143} forms a salt bridge with lysine at P\textsuperscript{-4} (81) (Asp\textsuperscript{143} corresponds to Glu\textsuperscript{226} in PSD-95 PDZ2;
Table III). Mutating Asp143 to glycine nearly abolished the binding of this peptide to syntrophin's PDZ domain (25). Our interaction assays with PDZ2 of PDZ-95 and SAP102 indicate a preference for either hydrophobic or positively charged residues at P-1. Aspartate is not tolerated at this position. The situation appears to be different for PDZ-95-PDZ1, which shows reduced affinity for the lysine-substituted peptide, and for SAP102-PDZ1, which tolerates an acidic aspartate substitution at this position in our fluorescence polarization assays (Fig. 3).

For SAP102-PDZ1, which tolerates an acidic aspartate substitution at this position in our fluorescence polarization assays (Fig. 3). In fact, the aspartate at this position in our fluorescence polarization assays (Fig. 3) shows reduced affinity for the lysine-substituted peptide, and for SAP102-PDZ1, which tolerates an acidic aspartate substitution at this position in our fluorescence polarization assays (Fig. 3). Staudinger, J. L., Olsson, E. N. (1997) J. Biol. Chem. 272, 9827–9832.

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Acknowledgments—We thank Dr. Mary C. Horne (University of Iowa, Iowa City, IA) for useful discussions and comments and much helpful advice on molecular biological techniques, Dr. S. Ramaswamy (University of Iowa) for advice on the structural modeling, Dr. Craig C. Garner (University of Alabama, Birmingham, AL) for providing the pGEX plasmids for expression of the second PDZ domain of PDZ-95 and all three PDZ domains of SAP102 as GST fusion proteins, Dr. Morgan Sheng (MIT, Cambridge, MA) for supplying the GAD10 vectors that contained the first and third PDZ domain of human PSD-95, Dr. Kyung Keun Kim (Chonnam National University, Kwangju, South Korea) for antibodies against BA11, and Dr. Randall T. Moon (University of Washington, Seattle, WA) for antibodies against frizzled 2.

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Selectivity and Promiscuity of the First and Second PDZ Domains of PSD-95 and Synapse-associated Protein 102
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J. Biol. Chem. 2002, 277:21697-21711.
doi: 10.1074/jbc.M112339200 originally published online April 5, 2002

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