The PDZ Domain of SAP90

CHARACTERIZATION OF STRUCTURE AND BINDING*

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The structural features of the PDZ1 domain of the synapse-associated protein SAP90 have been characterized by NMR. A comparison with the structures of the PDZ2 and PDZ3 domains of SAP90 illustrates significant differences, which may account for the unique binding properties of these homologous domains. Within the postsynaptic density, SAP90 functions as a molecular scaffold with a number of the protein-protein interactions mediated through the PDZ1 domain. Here, using fluorescence anisotropy and NMR chemical shift analysis, we have characterized the association of PDZ1 to the C-terminal peptides of the GluR6 subunit of the kainate receptor, voltage-gated K⁺ channel Kv1.4, and microtubule-associate protein CRIP1, all of which are known to associate with SAP90. The latter two, which possess the consensus sequence for binding to PDZ domains (T/S/V-X-V-oh, have low micromolar binding affinities (1.5–15 μM). The C-terminus of GluR6, RLPGRKTMA-oh, lacking the consensus sequence, binds to PDZ1 of SAP90 with an affinity of 160 μM. The NMR data illustrate that although all three peptides occupy the binding groove capped by the GLGF loop of PDZ1, specific differences are present, consistent with the variation in binding affinities.

Glutamatergic synapses express three types of ionotropic glutamate receptors, N-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid, and kainate, which are responsible for the majority of excitatory signals in the central nervous system (1, 2). Clustering of these transmembrane receptors at synapses is essential for efficient signal transduction complexes, thus coupling receptors to downstream signaling processes (6–11).

The PSD95/SAP90 protein is a member of the membrane-associated guanylate kinase family and contains three PDZ domains (PDZ1, PDZ2, and PDZ3), a Src homology 3 domain, and an inactive guanylate kinase domain (12–14). These domains are involved in the recognition of different membrane receptors, cytoskeleton, and intracellular components of the signaling machinery (15). It is known that SAP90 binds the NR2 subunits of N-methyl-D-aspartate receptors through the first two PDZ domains (16, 17). SAP90 binds directly to the cytoskeleton via the microtubule-associated protein CRIP1 (18) and modulates the cytoskeletal architecture and synaptic structure through interactions mediated by the guanylate kinase domain (19–22).

Recently, SAP90 has been found to modulate the electrophysiological properties of kainate receptors (23, 24). In particular, the KA2 subunit of the kainate receptor interacts with both the Src homology 3 and guanylate kinase domains of SAP90, whereas the GluR6 subunit binds specifically to the PDZ1 domain (23). The last four residues of the GluR6 C-terminus (E-T-M-A-oh) were shown to be responsible for the association. This motif presents a variation of the previously defined consensus sequence for binding to PDZ domains (e.g. T/S-X-V-oh) (5).

The PDZ domain consists of ~90 residues and is the most abundant constituent of the superfamily of PSD proteins, to which PSD95/SAP90 belongs. The tertiary fold of PDZ domains typically exhibits a six-stranded, antiparallel β-barrel flanked by two helices (4, 25). The C-terminal tails of the target molecules fit into a hydrophobic groove and usually form an additional β strand in the PDZ β-sheet (4). PDZ domains have been divided into two classes, according to the nature of their ligands. The first class binds the consensus sequence (S/T)²⁻X¹⁻(V/L)²⁻, and the second class prefers the (F/V)²⁻X¹⁻(F/V/A)²⁻ motif; the preference for the X¹⁻ position, when present, is not easily generalized (5). Usually the C-terminal carboxylate group of the target protein is directly involved in the binding and fits into the loop formed by the first two β strands of the PDZ domain (4, 26–28).

An alternative PDZ binding mode involves an internal motif, usually a β-finger, associating with the binding pocket of the PDZ domain. This has been characterized for the binding of the neuronal nitric oxide synthase-PDZ domain to the α-synthrophin-PDZ domain (28) and to the PDZ2 domain of SAP90 (29). The PDZ domain of neuronal nitric oxide synthase inserts its C-terminal β-finger into the canonical synthrophin and SAP90 PDZ binding pocket, replacing the C-terminal tail of the receptor with a β turn.
Here, we describe the structural features of the PDZ1 domain of SAP90. The conformation is compared with those reported for PDZ2 and PDZ3 of SAP90, illustrating important differences and similarities to these homologous protein domains. Using NMR chemical shift variation and fluorescence anisotropy, the association of the C-terminal peptides of GluR6, CRIPt, and the voltage-gated K⁺ channel Kv1.4 to the PDZ1 domain of SAP90 is characterized.

TABLE I

Structural statistics for a family of 28 structures

| Distance restraints | Value |
|---------------------|-------|
| Intraresidue (i = j = 0) | 233 |
| Sequential (|j| = 1) | 233 |
| Medium range (2 < |j| < 4) | 25 |
| Long range (|j| > 4) | 86 |
| Hydrogen bonds | 30 |
| Total | 607 |

| Dihedral angle restraints |  |
|---------------------------|---|
| Φ | 70 |
| Ψ | 49 |
| Total | 168 |

Mean r.m.s.d* deviations from the experimental restraints and covalent geometry

| Distance restraints (Å) | 0.094 ± 0.002 |
| Bond (Å) | 0.0078 ± 0.0003 |
| Angles (°) | 0.89 ± 0.04 |
| Impropers (°) | 0.85 ± 0.08 |

Mean energies (kcal mol⁻¹)

| | Value |
|---|-------|
| E_{NOE}b | 49 ± 4 |
| E_{dih}b | 58 ± 3 |
| E_{repel} | 66 ± 4 |

Ramachandran plot (residues 1–89)

| Residues in the most favorable regions (%) | 70.1 |
| Additional allowed regions (%) | 24.7 |
| Generously allowed regions (%) | 5.2 |

Atomic rms differences* (better defined regions)d

| Backbone heavy atoms (Å) | 0.87 |
| Heavy atoms (Å) | 1.0 |

Atomic rms difference for the overall structure

| Backbone | 1.3 |
| Heavy atoms | 2.0 |

* rmsd, root mean square deviation; rms, root mean square.

b Force constants of 75 kcal mol⁻¹ Å⁻² and 200 kcal mol⁻¹ rad⁻² were applied using square well NOE and dihedral angles penalty functions.

c The precision of the atomic coordinates is defined as the average r.m.s. difference between the 28 final structures and the mean coordinates of the protein.

d The unstructured portions of the C-terminal tail and the β1/β2 loop were not considered.
**EXPERIMENTAL PROCEDURES**

**Protein Overexpression and Purification**—The SAP90-PDZ1 domain was prepared by cloning a PCR-amplified DNA fragment encoding residues 60–150 of rat SAP90 into the NcoI/BamHI sites of PET 11d (Novagen). This construct contains a C-terminal hexahistidine tag, so that a 96-amino acid protein is produced. Synthesis of recombinant protein in BL21 (DE3)pLysS strain of bacteria was induced when the optical density reached 0.4 by 1 mM isopropyl-

**NMR Spectroscopy**—All samples for the NMR experiments were prepared at a protein concentration of 1.0 mM in an aqueous 10 mM phosphate buffer (10% D2O) with 150 mM NaCl at pH 6.8 (direct meter reading). The spectra were collected at 25 °C and 35 °C on a Bruker Avance 600 spectrometer using a triple resonance probe equipped with triple-axes gradients. All spectra were processed with NMRPipe (30) and analyzed using Sparky 3 (as provided by T. D. Goddard and D. G. Kneller, University of California San Francisco). The sequential assignment of the 1H and 15N resonances was obtained by inspection of three-dimensional HNHA, 1H-15N NOESY-HSQC, and 1H-15N TOCSY-HSQC experiments as well as two-dimensional 1H-15N TOCSY-HSQC experiments. The NOESY-type experiments were collected with mixing times ranging between 80 and 120 ms, whereas both DIPSI and MLEV pulse trains were implemented for TOCSY experiments, typically for 40 ms.

Titrations of the PDZ1 protein with peptide ligands were performed by adding solid aliquots of the peptides to 300 μM solutions of the protein until saturation was reached. The 1H and 15N resonance variations were followed at 35 °C by collecting HSQC experiments. Combined chemical shift perturbations were calculated using the following equation:

\[
\Delta \delta_{em} = \left( \delta_{em} - \langle \delta_{em} \rangle \right) \quad (\text{Eq. 1})
\]

with a scaling factor (α) of 0.17 (31).

**Structure Calculations**—Interproton upper bound restraints of 2.2, 3.8, and 5.0 Å were obtained by sorting two-dimensional NOEASY and three-dimensional NOEASY-HSQC peaks according to their relative intensity. Pseudo-atom corrections were applied for methyl groups and for unresolved methylene protons (32). Dihedral angles were constrained to reproduce the coupling constants measured in HNHA experiments using the Karplus equation.

Additional dihedral angle restraints were obtained by backbone chemical shift analysis using TALOS (33). Amide protons involved in intramolecular hydrogen bonding were identified by inspection of the intensity of the amide/water exchange peaks in three-dimensional NOEASY- and TOCSY-HSQC experiments. Hydrogen bond restraints were introduced, taking into account these data, the presence of secondary structure from chemical shift deviations from random coil values (34), and the proximity of a donor based on the NOEs. Structures were calculated using the program CNS (35), employing a torsion angle simulated annealing protocol following previously published procedures (36). Floating chiralities were used for the methylene and methyl groups resolved but not diastereotopically assigned. In the final minimization step, a force constant of 75 Kcal mol⁻¹ Å⁻² was applied for distance constraints (including the hydrogen bond constraints), 200 Kcal mol⁻¹ rad⁻² was applied for dihedrals restraints, and 1 Kcal mol⁻¹ Hz⁻¹ was applied for coupling constants. A total of 100 structures was calculated. The coordinates have been deposited in the Protein Data Bank (accession number 1KEF).

**Fluorescence Anisotropy**—Steady-state fluorescence spectra were collected on a SPEX 1681 Fluorolog (Edison, NJ) spectrophotometer using a 450-W xenon lamp for excitation and a cooled photomultiplier tube for detection. Polarized spectra were collected in L-format using Glan-Thompson polarizers (polarization > 200:1). Excitation spectra were collected by observing the fluorescence emission at 530 nm while varying the excitation wavelength between 450 and 500 nm. Emission spectra were collected by exciting the sample at 492 nm and observing the emission between 510 and 600 nm. Typically, an increment of 2 nm was implemented. The total intensity (S) and the anisotropy (r) were calculated according to the equations:

\[
S = I_{w} + 2g_{ab}I_{wb}
\]

\[
r = (I_{w} - g_{ab}I_{wb})/S
\]

where \(I_{w}\) indicates the fluorescence intensity (signal/reference — background/reference) observed with the excitation polarizer in the x orientation and the emission polarizer in the y orientation. Correcting for lamp intensity fluctuations using the spectrometer reference channel had no effect on the anisotropy values. The emission polarization bias (g-factor) was calculated using horizontally polarized excitation (37).

Changes in the steady-state fluorescence anisotropy in a system undergoing binding equilibrium can theoretically reflect changes in global or local rotational mobility or in fluorescence lifetime or quantum yield. If the fluorophore can exist in either a free form (F) or bound to the protein (B), the observed anisotropy is given by

\[
r = r_{F}f_{F} + r_{B}f_{B}
\]

where \(r_{F}\) and \(r_{B}\) are the anisotropies of the free and bound forms. For the case in which quantum yield and fluorescence lifetime of the flu-
orophore are not affected by binding, the quantities \( f_p \) and \( f_b \) are the fraction of the total fluorophore present in the free and bound form.

The binding models used to fit the data were derived from the following equilibrium

\[ F + P = B \] (Eq. 5)

and, in the presence of a competitor,

\[ C + P = D \] (Eq. 6)

where \( F \) and \( B \) are the fluorescent states, \( C \) and \( D \) are the unbound and bound nonfluorescent competitor, respectively, and \( P \) is the unbound protein. For the case of Eq. 5, the equilibrium concentration of \( B \) may be solved for analytically in terms of the initial concentrations of the reagents from the quadratic equation below.

\[ B^2 - B(f_b + f_0 + K_D) + f_b f_0 = 0 \] (Eq. 7)

The solution to the simultaneous equilibrium of Eqs. 5 and 6 results in a similar cubic equation, which was solved numerically using previously published algorithms \( (37) \). The latter model implies that the competition between fluorescent and nonfluorescent peptide is direct.

Titration experiments were performed by adding the PDZ1 protein to solutions containing a fixed amount of both PDZ1 and a fluorescent binder (normally 100 nm for the fluorescent binder and 10 \( \mu M \) for the protein). These latter experiments are particularly useful for determining the binding characteristics of compounds with weak affinity, given that very few intra- and inter-residue NOEs are observed.

The NMR-derived tertiary structure consists of two \( \alpha \)-helices and six \( \beta \) strands forming an antiparallel, \( \beta \)-sandwich. The \( \beta \)-sheets are composed of four \((\beta_1, \beta_6, \beta_4, \) and \( \beta_5)\) and three \((\beta_2, \beta_3, \) and the N-terminal portion of \( \beta_4 \) strands, respectively (see Fig. 3). The angle between the two \( \beta \)-sheets is \( \sim 60^\circ \), with the helices located at the corners of the sandwich. The loops interconnecting the secondary structure elements are relatively well ordered, with the exception of the \( \beta_1/\beta_2 \) loop, for which very few intra- and inter-residue NOEs are observed.

The structural statistics for a family of 28 structures, shown in Fig. 2. The 28 structures were selected from the 100 calculated by requiring no NOE violations greater than 0.3 \( \AA \), no dihedral angles violations greater than 0.3\(^\circ\), and coupling constants violation greater than 2 Hz.

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The general folding of PDZ1 closely resembles the shape of the PDZ2 domain of SAP90 \( (31) \). In particular, the structural motif of the variable \( \beta_2/\beta_3 \) loop is very similar in the two molecules and seems to represent a unique characteristic of the PDZ
domains of SAP90. Excluding the β2/β3 loop, the structural features of the PDZ1 domain observed here are similar to those of the functionally unrelated NHERF-PDZ1 domain, suggesting that this three-dimensional arrangement is widely preserved in nature.

Closer inspection of the structure shows that the hydrophobic residues Leu7, Leu14, Phe16, Ile18, Ile33, Ile35, Val73, and Leu76, most of which are highly conserved, cluster together in the region between β2 and β3, forming the characteristic binding pocket of PDZ domains. A serine (Ser17) occupies the second position of the β2 strand, a position that is often important for selectivity in ligand binding (18, 31). The conserved His69 is located at the beginning of the α2-helix, with the side chain projecting toward the hydrophobic binding pocket. Similar to what is observed for the PDZ2 domain of SAP90, the side chain of an asparagine (Asn24) is in the middle of the β2/β3 loop in proximity to the side chain of His69 (31). In the same loop, His76 represents another conserved feature, adopting a position similar to that of His182 of the PDZ2 domain of SAP90 and Asn102 of the α1-syntrophin PDZ domain (28, 31). In both of these other proteins, these residues are directly involved in the binding of their targets. Finally, positively charged amino acids are found at key positions at the beginning of the β1/β2 loop (Arg9), in the β3 strand (Lys37) just below the second position of the β2 strand, and at the end of the α2-helix (Lys77).

Peptide Binding to the PDZ1 Domain of SAP90—The association of peptides containing the C termini of GluR6, Kv1.4, and CRIPT was examined. The binding affinity of these peptides for the PDZ1 domain was measured by fluorescence anisotropy using both direct titration of the fluorescein-labeled compounds and competition experiments (see Figs. 4 and 5). All of the peptides display ligand-specific, saturable binding to the PDZ1 domain of SAP90. Importantly, the fluorescence intensities of the peptides are only slightly altered by the addition of the protein, indicating that the fluorescein is not directly interacting with the PDZ1 domain and therefore is not affecting ligand binding. Indeed, the x-ray structure of the PDZ3 domain of SAP90 bound to the C terminus of CRIPT indicates that the N terminus of the peptide is removed from the protein, projecting out into the solvent (4).

The association of the PDZ1 domain of SAP90 with the C-terminal peptides was also monitored by NMR. For all three peptides, titration to the PDZ1 in solution induced a significant perturbation of the backbone 1H and 15N chemical shifts of several residues, as reported for the three peptides in...
Fig. 6. In the titration of the CRIPTr- and Kv1.4-derived peptides, the bound and unbound forms of PDZ1 produced two distinct sets of NMR resonances simultaneously detectable in the HSQC spectra. This is typical of slow kinetic rates ($k_{on}/k_{off}$) for the complex formation equilibrium and is often associated with tight binding. The binding of the GluR6-derived peptide, on the other hand, leads to a single set of resonances representing a population-weighed average of the bound and free forms of SAP90-PDZ1 (see Fig. 7). Furthermore, the change in the chemical shifts is, in general, much less pronounced than that observed for the titration of the other two peptides. These data are consistent with the binding affinities obtained from the fluorescence anisotropy measurements.

To probe the association of the three peptides to PDZ1, the chemical shift perturbation was mapped onto the three-dimensional structure of PDZ1 determined here. The low binding affinities prevented the measurement of direct interactions between the peptides and the PDZ1 domain (i.e. which specific residue of the ligand residue is leading to the perturbation of chemical shift). It must be noted that chemical shifts are very sensitive to changes in chemical environment that may be due to direct interaction with the ligand or could be induced by changes in protein structure. Here, we do not observe differences in the NOE pattern of the PDZ1 domain upon ligand titration. Additionally, the results of the chemical shift perturbation for the three peptides present a general binding motif that is very similar to that reported in the complex of the PDZ3 domain of SAP90 with the C terminus of CRIPTr (4).

Based on the chemical shift perturbation, all three peptides bind to the same region of the PDZ1 domain. Residues at the end of the $\beta1/\beta2$ loop and the $\beta2$ strand (region Gly15–Ile18) are the residues most affected by the titration of the peptides. Similarly, the resonances of the amino acids Thr22–Asn24 in the middle of the $\beta2/\beta3$ loop are significantly perturbed, including a large change of the side chain of Asn24. Smaller chemical shift variations involving the $\alpha1$-helix and the C-terminal end of the $\alpha2$-helix are also observed. Notably, the variation of the chemical shifts of the $\beta3$ strand is different for the three peptides examined. In fact, Ile28, located in $\beta3$, is affected by the addition of the peptide derived from Kv1.4 and GluR6, but not from CRIPTr.

**DISCUSSION**

Previous structural studies of the domains of SAP90 have provided insight into many aspects of its biological function. Specifically, it has been observed that the dimensions of the hydrophobic cavity within the ligand binding pocket determine the selectivity toward leucine or valine at the C terminus of the ligand (39, 40). Likewise, the side chain of the second residue in the $\beta2$ strand dictates the preference of the $-1$ position of the ligand. For example, a serine residue in the PDZ1 (and PDZ2) domain of SAP90 is known to stabilize, through hydrogen bonding, the acidic amino acids aspartate and glutamate (18, 41); a hydrophobic residue at this position would induce a preference for other hydrophobic residues at the $-1$ position of the ligand (41–44).

In the structure of the PDZ1 domain of SAP90 presented here, the hydrophobic pocket for the C-terminal residue generated by the side chains of Leu14 and Phe16 is small, consistent with a preference for valine (over leucine or isoleucine) at position 0 of the C terminus. Indeed, both Kv1.4 and CRIPTr possess valine at the C terminus. In contrast, the C terminus of GluR6 is an alanine, which would not be expected to adequately fill this binding pocket. At the second position of the $\beta2$ strand, serine 17 would be expected to stabilize the binding of peptides containing hydrophilic or charged amino acids at the $-1$ position by the formation of hydrogen bonds. Both Kv1.4 and CRIPTr fulfill such criteria (aspartate and glutamine, respectively), whereas GluR6 (with a methionine at position $-1$) does not. Additionally, this methionine would not be expected to be solvent-exposed.

From an examination of the binding pocket of the PDZ1 domain of SAP90, we postulate that the long, hydrophobic side chain of the methionine at position $-1$ occupies the binding pocket of valine (Val$^0$) of the consensus sequences. With the $-1$ methionine occupying the hydrophobic pocket, the negatively charged C terminus of GluR6 would project away from the binding pocket, not forming hydrogen bonds to the GLGF loop as observed in the x-ray structure of PDZ3 of SAP90 (4). Such an arrangement is similar to the $\beta$-finger binding motif of neuronal nitric oxide synthase. The carboxylate of Ala$^0$ could easily reach the positively charged residues near the binding pocket (e.g. Arg$^3$, Arg$^{37}$, and Lys$^{37}$); the small size of the side chain of the alanine side chain would not be expected to interfere with this interaction. In support of this model, we observed that titration of the C terminus of GluR6 causes a perturbation of the chemical shift of the side chain of Lys$^{37}$, located in $\alpha2$ of PDZ1. During the binding of the GluR6 peptide, only the C-

![Diagram of the residues of the PDZ1 domain of SAP90 contributing to the binding of the C terminus of GluR6.](image)

The residues displaying chemical shift changes (backbone $^1$HN and $^{15}$N, as defined in the text) greater than 0.15 ppm are highlighted.

![Amino acid sequence alignment of the three SAP90 PDZ domains.](image)

Identical residues are marked with *asterisks*. Colons and semicolons indicate conserved and semi-conserved substitutions, respectively (45). The secondary structure of PDZ1 as determined in this study is reported above the sequence.
terminal portion of the α2-helix (including Lys77) is affected; the remaining portion of the helix, including His69, is not altered (see Fig. 8). The observation that His69 is not involved in the binding of the GluR6 C terminus is quite unique. In all other class I PDZ domains studied to date, the side chain of this highly conserved histidine, located at the beginning of the α2-helix, contributes to ligand specificity by hydrogen bonding to the serine/threonine hydroxyl group at the −2 position of the ligand (4, 27). Instead, for the PDZ1 domain examined here, we observe that Asn24, whose side chain is close in space to His69, is affected by the binding of the peptides (all three of them), as indicated by large chemical shift changes of both the backbone and the side chain amide group. We therefore postulate that the threonine residue at the −2 position (all three peptides contain a Thr−2) is interacting with Asn24 upon binding to the PDZ1 domain and not His69. Interestingly, in the examination of the PDZ2 domain of SAP90, both the asparagine in the β2/β3 loop and the histidine in the α2 helix are affected by the binding of the neuronal nitric oxide synthase-binding protein CAPON, suggesting a slightly different binding mode between the two PDZ domains (31).

We have previously shown that SAP90 binds to kainate receptors through a specific interaction between the C-terminal tail of GluR6 and the PDZ1 domain of SAP90 (the C terminus of GluR6 displayed little affinity for PDZ2 or PDZ3 of SAP90) (23, 24). Interestingly, a structural comparison of the canonical binding regions of the PDZ1 and PDZ2 domains of SAP90 reveals a striking similarity. The protein folds are comparable, and most of the residues forming the binding pockets are identical. This may indicate that the observed specificity displayed by the PDZ domains is a result of the tetrameric structure of the kainate receptor (multiple copies of the C termini of the individual subunits) at the postsynaptic membrane. Alternatively, the β2/β3 loop, which is divergent between PDZ1 and PDZ2 (see Fig. 9) and varies in the number of prolines and charge, with PDZ1 containing two additional aspartate residues may play a role in the specificity. Importantly, the β2/β3 loop of the PDZ2 domain of SAP90 contributes directly to the binding of the neuronal nitric oxide synthase-PDZ domain through an unusual interaction with a strand of the neuronal nitric oxide synthase β-finger (41). In a similar fashion, residues of the C terminus of GluR6 beyond the tetrapetide may be interacting with the β2/β3 loop, accounting for the binding specificity observed in the in vitro assays. Current mutational studies in our laboratories are probing this issue.

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