Targeting the PDZ Domains of Molecular Scaffolds of Transmembrane Ion Channels

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ABSTRACT

The family of multidomain proteins known as the synaptic associated proteins (SAPs) act as molecular scaffolds, playing an important role in the signaling and maintenance of several receptors and channels. The SAPs consist of 5 individual protein domains: 3 PDZ (PSD95, Disc Large, Zo1) domains, an SH3 domain, and an inactive guanyl kinase (GK) domain. The 3 PDZ domains bind the C-termini of specific receptors and channels, leading to the transient association with cytoskeletal and signaling proteins. Molecules targeting specific domains of the SAPs may provide a novel route for the regulation of channel and receptor function. Here we describe a structural-based approach for the development of such inhibitors for the PDZ domains of SAP90. The high sequence homology of the 3 domains has necessitated targeting regions outside the canonical binding pocket. The structural features of the PDZ domains with the C-termini of different receptors (GluR6), channels (Kv1.4), and cytoskeletal proteins (CRIPT) provide insight into targeting these regions.

KEYWORDS: PDZ domains, synaptic associated proteins, PSD95, SAP90, glutamate receptors, molecular scaffold, NMR, ligand binding

INTRODUCTION

PDZs (PSD95, Disc Large, Zo1) are small globular domains composed of ~90 amino acids exhibiting high sequential homology and functioning as protein-protein interaction modules. Typically PDZs adopt a β -sandwich fold including 2 α -helices and 2 β -sheets composed of 5/6 β -strands and recognize the C-termini of their protein targets, see Figure 1. The binding contact occurs in a hydrophobic cleft

delimited by the second α -helix, the second β strand, and the first loop. The ligand is normally inserted as an additional β -strand, in an anti-parallel fashion with respect to the second β -strand (β 2), with the C-terminus inserted into a characteristic pocket formed by the β 1/ β 2 loop. The most relevant side chains for PDZ selectivity are located at the 0 and -2 positions (employing a numbering scheme with the C-terminus 0, and proceeding -1, -2, toward the N terminus), which has been used for defining different classes of PDZ domains. However, all 6 C-terminal residues can play a role in modulating the binding affinity.¹ In some cases a contribution from residues located up to position -10 has been reported.¹

The multidomain protein SAP90 (synapse-associated protein 90) contains 3 N-terminal PDZ domains followed by an SH3 and an inactive GK domain. SAP90, also known as PSD95 (post synaptic density protein-95) is concentrated in the post synaptic density of glutamatergic neurons and is one member of a super family of PDZ-containing proteins, including SAP97/hdlg, SAP102, and Chapsyn110/PSD93,²⁻⁵ that play an important role in coupling the membrane ion channels with their signaling partners. The first 2 PDZ domains (PDZ1 and PDZ2) are responsible for the recognition of ion channels. For example, the PDZ1 domain selectively couples with the GluR6 subunit of kainate receptors,⁶ while both PDZ1 and PDZ2 are capable of binding to receptors⁷ and potassium channels.⁸ The third PDZ domain (PDZ3) interacts preferentially with cytoskeleton proteins like CRIPT⁹ or cell surface proteins, like neuroligins.¹⁰ Furthermore, the PDZ2 domain is capable of binding the neuronal nitric oxide synthase (nNOS) protein,¹¹ catalyzing the synthesis of nitric oxide (NO). In this later case the SAP90 PDZ2 recognizes its partner through an internal motif located on a β -finger region of the nNOS protein.¹² The SAP90 PDZ domains, together with the GK domain, can bind several proteins (SynGAP,¹³ SPAL,¹⁴ and MAGUIN-1¹⁵) involved in the mitogen-activated protein kinase (MAPK) signaling cascade.¹⁶

Despite the different binding affinities, the 3 PDZ domains of SAP90 share a high sequence homology; binding C-termini with a hydrophobic residue at position 0 and serine/

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Figure 1. Secondary structure elements of the PDZ1 of SAP90, illustrating the basic fold and the nomenclature of the secondary structural elements.

threonine in position -2 (defined as class I PDZ domains). Some differences outside these classification sites have been noted. The PDZ1 and PDZ2 domains prefer negatively charged residues in position -1 and -3 ,¹ while CRIPT,⁹ binding to PDZ3, has serine and glutamine at -1 and -3 , respectively. Here, we describe our efforts to develop structural insight into the specificity illustrated by the PDZ domains of SAP90. Using high-resolution nuclear magnetic resonance (NMR), we characterize the structure of different receptors (GluR6), channels (Kv1.4), and cytoskeletal proteins (CRIPT), while associated with PDZ1 of SAP90. Based on these structural features, coupled with results previously reported for the PDZ domains of SAP90,¹⁷⁻¹⁹ several sites for development of PDZ domain-specific molecules are identified. A cyclic peptide, incorporating a side-chain to side-chain lactam-bridged system, targeting one site is described.

METHODS

The 3 peptides were chosen based on their differing specificities for the PDZ domains of SAP90: GluR6 binds solely to PDZ1,²⁰ Kv1.4 displays affinity for both PDZ1 and PDZ2,²¹ and CRIPT binds selectively to PDZ3.⁹ The C-terminus of the GluR6 subunit of the kainite receptor was examined as a 15-residue peptide (His⁻¹⁴-Thr⁻¹³-Phe⁻¹²-Gln⁻¹¹-Asp⁻¹⁰-Arg⁻⁹-Arg⁻⁸-Leu⁻⁷-Pro⁻⁶-Gly⁻⁵-Lys⁻⁴-Glu⁻³-Thr⁻²-Met⁻¹-Ala⁰-OH). The Kv1.4 was examined as Tyr⁻⁵-Lys⁻⁴-Glu⁻³-Thr⁻²-Asp⁻¹-Val⁰-OH, and was previously shown to bind tightly to PDZ1 of SAP90 (Table 1).¹⁹ CRIPT, a cytoskeleton molecule that has been reported to target preferentially the PDZ3 domain, was examined as a 9-residue peptide⁹ (Thr⁻⁸-Ly⁻⁷-Gln⁻⁶-Tyr⁻⁵-Lys⁻⁴-Gln⁻³-Thr⁻²-Ser⁻¹-Val⁰-OH).

The structures of the PDZ1 peptide complexes were determined by high-resolution NMR. The samples containing 1.0 mM ¹⁵N,¹³C-PDZ1 and 4.0 mM peptide were prepared in 10 mM phosphate buffer, 150 mM NaCl at a pH of 6.8. The ¹H and ¹⁵N assignments were determined previously¹⁹; the ¹³C assignments were determined employing standard methods.²² Several 2- and 3-dimensional ¹⁵N and ¹³C filtered experiments were used to isolate the peptide resonances and to measure intramolecular and intermolecular nuclear Overhauser enhancements (NOEs) involving the peptide amide protons.

The structure refinement was achieved using dihedral angle constraints derived from TALOS²³ and a torsion angle simulated annealing using CNS.²⁴ For the resolved diastereotopic groups floating chirality was applied. Extensive molecular dynamics (MD) simulations were performed using the GROMACS package,^{25,26} using a cubic box of $6.5 \times 6.5 \times 6.5$ nm³ containing ~8500 molecules of water. The NOE-derived interproton distance restraints were applied with a constant of 10 kJ mol⁻¹ Ala⁻², and Arg⁻⁶ averaging was implemented on chemical equivalent and overlapped protons. The distance restraints were removed after 600 ps and the system was therefore freely evolving during the remaining 1.0 ns.

Table 1. Peptide Sequences and Binding Affinities at PDZ1¹⁹ and Relative PSD 95 PDZ Specificity

Peptide	Selectivity	Sequence	K _D (μM)
Kv1.4 (potassium channel)	PDZ1 and PDZ2	YKETDV-OH	1.5
CRIPT (microtubule associated protein)	PDZ3 (lower affinity to PDZ1 and PDZ2)	TKNYKQTSV-OH	15.0
GluR6 (kainite receptor)	PDZ1	HTFNDRRLPGKETMA-OH	160.0

RESULTS

The resulting structures of the complexes formed between the PDZ1 of SAP90 and the C-termini of GluR6, Kv1.4, and CRIPT are shown in Figure 2. The structure of the PDZ1-Kv1.4 is delineated by an extremely large number of NOEs, both intramolecular and intermolecular. Interestingly, no intermolecular NOEs could be observed for the Lysecon^{d-4} side chain, while the Tyr⁻⁵ aromatic ring is close to A80 methyl group. Noticeably, the β -protons of the N85 residue are close in space to the Tyr⁻⁵ H α proton. In all of the refined structures, the isopropyl group of Val⁰ projects into a deep hydrophobic cavity between the β 2 strand and the α 2 helix formed by L75, F77, I79, and L137. The side

chain of Thr⁻² is located on the same side of the strand respect to Val⁰. While the hydroxide hydrogen bonds with H130, the methyl group forms van der Waals contacts with the methyls of I79 and V134. On the opposite face of the strand, the side chain of Glu⁻³ is extended over the β -sheet surface and salt bridges with K98 on the β 3 strand. The Lysecon^{d-4} side chain appears relatively flexible, however in many NMR-derived structures the positively charged amino group is a short distance from the D84 carboxylic acid located on the top of the β 2/ β 3 loop. The aromatic ring of Tyr⁻⁵ is accommodated in a pocket formed by the end of the β 2 strand and the β 2/ β 3 loop.

The structure of the PDZ1-CRIPT complex is similarly defined by a large number of NOEs, despite a lower binding affinity and therefore faster exchange rate of the ligand. Defining the mode of ligand binding there are intermolecular NOEs involving the methyl groups of both Val⁰ and Thr⁻² with the β 2 strand (F77, I79) and the α 2 helix (V134, L137, K138). The side chains of Ser⁻¹ and Gln⁻³ have NOEs with residues located on the β 2 strand and on the β -sheet surface opposite to the α 2 helix. The Lys in position -4 does not give detectable NOEs, while Tyr⁻⁵ gives several NOEs with the methyl of A80. No other intermolecular NOEs are detected for the remaining residues of the CRIPT peptide. The backbone of the CRIPT peptide encompassing residues Val⁰ and Thr⁻² is well defined and shows the classical β -sheet insertion structural motif. Starting from Gln⁻³ the backbone is less ordered and moves away from the β 2 strand by flanking the β 2/ β 3 loop. The NOEs detected between the Tyr⁻⁵ side chain and the methyl of Ala⁸⁰ places the aromatic ring at the conjunction of the β 2 strand with the β 2/ β 3 loop. Although the side chains of both Lysecon^{d-4} and Lysecon^{d-7} appear totally unordered, the backbone conformation would allow the positioning of the positively charged amino groups close to the negative charges located on the β 2/ β 3 loop.

The structure of the GluR6-PDZ1 complex is defined by a much smaller number of NOEs, consistent with the weaker binding affinity. Indeed, no intermolecular NOEs are observed for Ala⁰. Met⁻¹ is well defined with backbone NOEs with S78 and I79 and side chain interactions with L75 or I100 located on the β 3 strand. The Thr⁻² methyl group gives NOEs with V134 on the α 2 helix and with I79 on the β 2 strand. Finally the Glu⁻³ side chain interacts with A80 and T97 on the β 2 and β 3 strand, respectively. A univocal family of structures for the GluR6/PDZ1 can be obtained if hydrogen bond restraints are introduced into the structure refinement calculation (Figure 2, bottom). The variation in the chemical shifts of the amide protons in the carboxylic binding loop suggest the formation of hydrogen bonds with the C-terminus of GluR6. Similarly, the shifts of I79 can be explained with a hydrogen bond with the carbonyl of Thr⁻². The resulting structures are similar to

Figure 2. Stereo views showing the NMR structures of the PDZ1 domain bound to Kv1.4 peptide (top), CRIPT (middle), and GluR6 (bottom). The conformers are superimposed for best fit of N, C α , and carbonyl atoms. All the ligand's heavy atoms are shown for the fragment encompassing residues 0 and -5; carbons are depicted in dark green, oxygen in red, nitrogen in blue, sulfur in yellow. In the protein portion, only the superimposed atoms are shown as light green.

PDZ1-Kv1.4 complex, where the C-terminal 3 residues are extending the β -sheet, with interactions to the $\beta 2$ strand. The next 3 residues, Gly⁻⁵-Lys⁻⁴-Glu⁻³, extend toward the $\beta 2/\beta 3$ loop. The remaining residues, further toward the N terminus, are unordered.

DISCUSSION

The different binding affinities previously measured for the 3 ligands can be directly correlated to the quantity of the NMR restraints, and therefore to the resolution of the 3-dimensional models of the complexes. The best binder, the Kv1.4-derived peptide, leads to the better-defined structure, while a lower resolution is observed for the CRIPT and finally for the GluR6 ligands (see Figure 2). Nevertheless, some common features can be extrapolated by comparing the 3 models. All of them in fact exhibit a similar extended conformation in the fragment encompassing positions 0 and -3. The peptides enhance the β -sheet surface in correspondence of the $\beta 2$ strand in an antiparallel fashion (Figure 3). The carboxylic C-terminal end enters the $\beta 1/\beta 2$ loop by hydrogen bonding the amides of residues L75, G76, and F77.

At the -4 position the peptide backbones deviates away from the $\beta 2$ strand and interacts with the $\beta 2/\beta 3$ loop. The importance of this loop has been clearly demonstrated: the deletion of the loop in both PDZ1 and PDZ2 diminish the binding affinity to the Kv1.4 potassium channel.²¹ The 3 ligands examined have different preferences for the 3 PDZ domains of SAP90 and a closer look of the sequences of the $\beta 2/\beta 3$ loop illustrates several important features (Figure 4) that may account for the range of binding affinities.

Figure 3. Superposition of the last 6 C-terminal residues of Kv1.4 (dark green), CRIPT (blue), and GluR6 (magenta). The binding pocket of the PSD95/SAP90 PDZ1 domain is shown in light green.

PDZ1	GGT DN PHI GDD PSI
PDZ2	GGV GN QHI PGD NSI
PDZ3	GGED GEGI
	***. * ** . * **

Figure 4. Comparison of the $\beta 2/\beta 3$ loop of the PDZ1, PDZ2, and PDZ3 domains of SAP90.

We previously reported that the PDZ2 domain closely resembles the PDZ1 domain in the canonical ligand-binding domain¹⁹ and even the shape of the $\beta 2/\beta 3$ loops is similar. As the N85 residue is conserved in both domains, and its side chain undergoes chemical shift changes upon titration of the PDZ2 domain with the CAPON peptide,¹⁸ it is probable that this residue mediates the ligand β -strand extension also in the PDZ2 domain. In the structure of PDZ2 from hPTP1 interacting with the C-terminus of the guanine nucleotide exchange factor RA-GEF-2,²⁷ an asparagine residue in the $\beta 2/\beta 3$ loop interacts a Asp⁻⁵ of the ligand. In contrast, the PDZ3 domain of SAP90 lacks 5 residues in the $\beta 2/\beta 3$ loop. Comparing our findings here with the x-ray structure of the CRIPT/PDZ3 complex demonstrates complete agreement for the C-terminal 4 residues (positions 0 to -3). Unfortunately, in the crystal the peptide does not diffract N-terminal of position -4, indicating that residues preceding this position are unordered in the crystal. In our NMR-derived structure of CRIPT bound to PDZ1, Tyr⁻⁵-Lys⁻⁴ interact with the $\beta 2/\beta 3$ loop, particularly the abundant negatively charged residues within the loop. Given that these negative charges are not maintained in the PDZ2 of SAP90, in future design efforts we have targeted this region as a mode for enhancing the specificity for PDZ1.

In both structures of Kv1.4 and CRIPT bound to PDZ of SAP90, the side chain of the -3 residue projects out toward the $\beta 3$ strand, placing a Glu⁻³ (Kv1.4) or Gln⁻³ (CRIPT) outside the canonical binding pocket. Comparison of the sequences of the 3 PDZ domains of SAP90 indicates an important difference in this region. Both PDZ1 and PDZ2 have a lysine (K98 and K193 for PDZ1 and PDZ2, respectively), while PDZ3 contains a phenylalanine, F340, at this position. One mode to target this difference is afforded by cyclization of the side chains at positions -1 and -3.²⁸ Previously we examined the cyclic peptide version of CRIPT, Tyr-Lys-c[Lys-Thr-Glu(β Ala)]-Val-OH, while bound to the PDZ1 domain of SAP90 by NMR (Figure 5).²⁹ The results from these studies clearly indicate that the lactam bond formed by the cyclization extends outside the canonical binding pocket to interact with the residues of the $\beta 3$ strand. Currently, we are modifying the linker to target the differences in the PDZ1/PDZ2 and PDZ3 domains of SAP90 in an effort to develop an analog with enhanced specificity for the different domains.

Figure 5. Structure of the cyclic peptide, Tyr-Lys-c[(-Lys-Thr-Glu(β Ala)-)-Val-OH], while bound to PDZ1 of SAP90. All of the residues in which intermolecular NOEs have been measured are shown in light green. The interaction of the cyclic, lactam ring system with K98 is illustrated.

CONCLUSIONS

In conclusion we have solved the structure of PDZ1 of SAP90 bound to 3 peptides related to the C-terminus of Kv1.4, CRIPT, and GluR6. Our results allow for the detailed characterization of the structural determinants modulating affinity for the PDZ1 domain. Comparison of these data with the other known structures of the PDZ2 and PDZ3 of SAP90 provides several leads for the development of analogs with enhanced PDZ domain specificity.

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