A novel determinant of PSMD9 PDZ binding guides the evolution of first generation of super-binding peptides

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**Abstract**

PDZ domain is one of the most widespread protein interaction domains found in nature. Due to their integral role in numerous biological functions, their ability to act as scaffolds for signal amplification, and occurrence of mutations linked to human diseases, PDZ domains are attractive therapeutic targets. Based on the differential binding affinities of select C-terminal peptides of the human proteome to one such PDZ domain (PSMD9) and by exploring structure-activity relationship, we design and convert a low affinity tetrapeptide (~439µM) to a tight binding sequence (~5µM). The peptide inhibits PSMD9-hnRNPA1 interactions which are critical in basal and stimulus induced NF-κB signaling and a potential therapeutic target in cancers including chemo or radiation-induced therapy resistance. Extensive application of computer modelling including ligand mapping and all atom molecular dynamic simulations help us rationalize the structural basis for the huge differences in binding affinity and inform us about per residue contribution to binding energy. Our findings are in accord with the classical preference of (PSMD9) PDZ domain for C-terminal sequences that contain hydrophobic residues at the P0 (C-terminal) position. In addition, for the first time, we identify a hitherto unknown occupancy for cysteine at the P-2 position, that drives high-affinity interaction in a PDZ domain.

**Introduction**
All cellular processes are driven by the complex interplay of protein-protein interactions (PPIs), carefully orchestrated to function in a spatiotemporal manner. These interactions are governed by protein interaction domains which bind to target proteins via specialized binding interfaces. The interfaces have evolved to ensure high degree of specificity for a target protein and at the same time malleable enough to recognize a variety of interacting partners. In majority of domains involved in protein-protein interactions, the binding site is well-studied as it is often the target for designing small molecule or peptide-based inhibitors of the PPIs. Many inhibitors that block signaling cascades capitalize on these conserved regions or “hotspots” of binding sites. One such conserved well studied protein interaction domain is the ubiquitous PDZ domain (PDZ is an acronym of PSD-95, Discs-large, ZO-I), found in many synaptic junctions, where signaling molecules are concentrated. PDZ domains predominantly bind to the C-termini of interacting partner proteins although some of them bind to internal sequences. Typically this domain is characterized by a conserved fold comprising of six beta sheets (β1-β6) capped by two alpha helices (α1 and α2) (Figure S2). Since PDZ domains dictate the direction and amplitude of signaling cascades by acting as scaffolds recruiting other proteins, mutations in this domain are associated with diseases such as cancer and neurodegenerative disorders. All the above mentioned properties make PDZ domains attractive therapeutic targets. However, the development of inhibitors to target PDZ-peptide interactions with specificity is a challenge because a single PDZ domain can recognize a wide variety of client proteins, and many of them have very similar binding interfaces. This demands an in-depth investigation of the target binding interface at high resolution to understand the exquisite specificity.

Previously, using a C-terminal peptide library representing the human proteome, we identified several novel interacting partners of PSMD9. Two of these proteins were of special interest -
hnRNPA1 (carrying the GRRF motif at the C-terminus) and growth hormone (carrying the SCGF motif at the C-terminus). The binding affinities of these two C-terminal peptides to PSMD9 were dramatically different (K_D of GRRF was ~ 600µM and of SCGF was ~ 8µM).

Natural variants of the GRRX series (also from the human proteome) carrying a hydrophobic residue (X=I, L or C) at the P0 position retained binding to PSMD9. However, a mutant peptide, GRRG (G at position P0), neither bound to PSMD9 nor was able to inhibit the binding of hnRNPA1 to PSMD9. These results established that like other classical PDZ domains, PDZ domain of PSMD9 preferred a hydrophobic residue at the P0 position. In sharp contrast, the mutant SCGG peptide was as good as the WT peptide SCGF in inhibiting the binding of GH to PSMD9.

These observations posed an interesting puzzle. Two peptides, each carrying the same bulky hydrophobic residue at P0, which is the hallmark of peptide recognition by PDZ domain proteins, show ~70-fold difference in binding affinity. Additionally, this C-terminal hydrophobicity appears to be critical for the weak binder but not for the tight binder. This prompted us to ask the following questions: what contributes to the binding affinity of SCGF to the PSMD9 PDZ domain? What limits the binding affinity of GRRF? And finally, is it possible to characterize the role of each residue in binding to enable the conversion of the poor binding GRRF to a tight binder, which could compete more effectively with its natural counterpart in hnRNPA1, thereby inhibiting PSMD9-hnRNPA1 interaction?

Materials and Methods:

Cloning, expression and purification of PDZ domain

PDZ domain was amplified from PSMD9-pRSETA plasmid and cloned in His-tagged expression vector, pETyong between BamHI and EcoRI restriction sites. For protein expression, a single
colony of PDZ-pETYong in BL21 Codon Plus cells (Agilent) was inoculated in 5ml of Luria broth with 50µg/ml of kanamycin and 34µg/ml of chloramphenicol. The overnight starter culture was inoculated in 1liter LB kanamycin-chloramphenicol-medium and culture was allowed to grow till O.D. reached 0.4, after which IPTG was added to a final concentration of 100µM. The culture was grown till O.D. reached 1.5. Cells were harvested by centrifugation at 5000rpm. The induced cell pellet was resuspended in lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 10mM imidazole, 0.1% Triton-X 100, 10% glycerol, 5mM beta-mercaptoethanol, 1X protease inhibitor). Purification of PDZ domain was carried out by nickel affinity (Ni-IDA) chromatography (Clontech, Takara) followed by gel filtration chromatography on HiLoad 16/600 Superdex200 column (GE Healthcare).

Peptide synthesis and purification

Peptides were commercially synthesized from APS Lifetech. Peptides were synthesized with N-terminal biotin tag (Biotin-KGG-XXXX-OH, where XXXX corresponds to the peptide sequence, -OH is the carboxy terminus) and purified to 99% purity by HPLC. Lyophilized peptides were reconstituted to obtain a concentration of 25mM in 100% DMSO (molecular biology grade, Sigma), dispensed in 10µl aliquots and stored at -20°C. Cysteine-containing peptides were stored in DMSO containing 1mM DTT.

Site-directed mutagenesis:

Amino acid substitutions were carried out by site-directed mutagenesis using a modified protocol from Stratagene QuikChange mutagenesis kit. Complementary primers were synthesized, each carrying the mutation in the center, flanked on both sides by unmodified nucleotide sequence (described in Table S1, supplementary section). The PCR amplified products were subjected to DpnI digestion to remove parental DNA and transformed in E.coli DH5α. Plasmids were isolated
using the plasmid isolation kit (Sigma GenElute kit) and sequenced (Eurofins Genomics India Pvt Ltd).

**ELISA for protein-peptide interaction and inhibition**

ELISA for protein-peptide interaction and inhibition were performed as described previously.

**DTNB labelling of protein**

Purified PDZ domain (72µM) was incubated with 20 fold molar excess (1440µM) of DTNB (5,5′-dithio-bis-[2-nitrobenzoic acid]) (Invitrogen) or Ellman’s reagent in 50mM Tris, 150mM NaCl, pH 8.5 for one hour at 25°C under dark conditions. DTNB reacts with the free thiol converting it to 2-nitro-5-thiobenzoate (TNB−), which is ionized to TNB2− dianion, a yellow colored compound. The excess DTNB was removed by desalting over PD10 desalting column (G25 Sephadex, GE Healthcare). The absorption spectrum of labelled protein was monitored from 280nm to 700nm and the extent of labelling was calculated from the absorbance at 412nm and molar extinction coefficient of DTNB.

**Glutathione modification of protein**

Purified PDZ domain was incubated with a 20-fold molar excess of glutathione in 50mM Tris, 150mM NaCl, pH 8.5 for one hour. The excess unreacted glutathione was removed by desalting over a PD10 column (G25 Sephadex, GE Healthcare). The extent of glutathione modification was checked by incubating glutathione-modified PDZ domain with excess of DTNB, which would react with free cysteines, if available and monitoring the absorption spectrum from 280nm to 700nm.

**Homology Modelling**

There is currently no crystal structure available for the PDZ domain of PSMD9 protein. Hence the three-dimensional atomic structure of this domain was constructed using comparative modelling.
methods using the program Modeller (version 9.12) as described earlier. The sequence used for
the modelling the PDZ domain of PSMD9 and the corresponding secondary structural regions are
highlighted (Figure 3) Nas2 and PSMD9 share 42% sequence identity and 64% sequence similarity
in their PDZ domains. The crystal structure of Nas2 PDZ domain from yeast (PDB ID 40O6),
the ortholog of mammalian PSMD9, was used as the template for modelling the PDZ domain of
PSMD9.

Peptide Docking

The 3D structures of the complexes of PDZ-GRRF and PDZ-SCGF were generated as described
earlier. Using the same protocol, 3D models of PDZ complexed with variants of GRRF and SCGF
peptides were generated. The 3D structures of the linear conformations of the peptides were
generated using the Xleap module in AMBER. The fully extended peptide was energy
minimized in an implicit solvent using the Sander module in AMBER. Then the minimized
peptide in its extended conformation was docked with the refined model of the PDZ domain of
PSMD9 protein. Peptide docking was carried out with two different docking programs
HADDOCK and ATTRACT. For docking of peptides into the canonical pocket of PDZ
using HADDOCK, a binding site was defined using residues Gly215, Cys216, Asn217, Ile 218
and Gln181 based on the available crystallographic data of other PDZ–peptide complexes. No such
constraints were used for running ATTRACT (completely blind docking was performed).

Molecular Dynamics Simulations

MD simulations were carried out on the apo-PDZ domain and the predicted PDZ-peptide
complexes. In addition, MD simulations were carried out for several mutated PDZ proteins and
PDZ-peptide complexes and the mutated structures were generated by replacing (mutating) the
respective residues in Pymol. The Xleap module was used to prepare the system for the MD
simulations. Hydrogen atoms were added and the N-terminus of the GRRF peptide was capped with the residue ACE. All the simulation systems were neutralized with appropriate numbers of counter ions. The neutralized system was solvated in an octahedral box with TIP3P \(^{15}\) water molecules, leaving at least 10 Å between the solute atoms and the borders of the box. MD simulations were carried out with the Sander module of the AMBER 16 package in combination with the ff14SB force field \(^{16}\). All MD simulations were carried out in explicit solvent at 300K. During all the simulations the long-range electrostatic interactions were treated with the particle mesh Ewald \(^{17}\) method using a real space cut off distance of 9 Å. The settle \(^{18}\) algorithm was used to constrain bond vibrations involving hydrogen atoms, which allowed a time step of 2 fs during the simulations. Solvent molecules and counter ions were initially relaxed using energy minimization with restraints on the protein and peptide atoms. This was followed by unrestrained energy minimization to remove any steric clashes. Subsequently the system was gradually heated from 0 to 300 K using MD simulations with positional restraints (force constant: 50 kcal mol\(^{-1}\) Å\(^{-2}\)) on protein and peptides over a period of 0.25 ns allowing water molecules and ions to move freely followed by gradual removal of the positional restraints and a 2ns unrestrained equilibration MD simulation at 300 K. The resulting systems were used as starting structures for the respective production phase of the MD simulations. For each case, three independent (using different initial random velocities) MD simulations were carried out starting from the well equilibrated structures. Each MD simulation was carried out for 100ns and conformations were recorded every 4ps. Simulation trajectories were visualized using VMD \(^{19}\) and figures were generated using Pymol\(^{14}\).

**MMPBSA Calculations**
The MMPBSA (Molecular Mechanics Poisson–Boltzmann Surface Area) methodology has been widely used to investigate the docking of ligands to receptors \(^{20-22}\). We applied it to calculate the binding free energies between the PDZ and peptides. Five thousand conformations were extracted from the last 50 ns of the MD simulations of each PDZ-peptide complex. The MMPBSA calculations were carried out after removing the water molecules and the counter ions. Binding free energies were calculated using the single trajectory method, based on the assumption that the bound and unbound conformations of the protein and peptide are quite similar. In this protocol, the isolated conformations of the peptide and the PDZ domain were extracted from the corresponding PDZ-peptide complexes. For each conformation, the binding free energy (\(\Delta G_{\text{bind}}\)) of the peptide to the protein was calculated as follows:

\[
\Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{receptor}} + G_{\text{peptide}})
\]

(1)

The binding free energy is estimated as a sum of three terms:

\[
\Delta G_{\text{bind}} = \Delta G_{\text{MM}} + \Delta G_{\text{sol}} - T\Delta S
\]

(2)

where \(\Delta G_{\text{MM}}\) is the change in the molecular mechanics energy upon complexation in the gas phase, \(\Delta G_{\text{sol}}\) is the change in solvation free energy and \(T\Delta S\) is the change of conformational entropy associated with ligand binding. The entropy term (\(-T\Delta S\)) was not computed and hence not included in the binding energy values.

The molecular mechanics free energy (\(\Delta G_{\text{MM}}\)) is further split into Van der Waals (\(\Delta G_{\text{vdw}}\)) and electrostatic (\(\Delta G_{\text{ele}}\)) energies:

\[
\Delta G_{\text{MM}} = \Delta G_{\text{ele}} + \Delta G_{\text{vdw}}
\]

(3)

The solvation free energy \(\Delta G_{\text{sol}}\) arises from polar (electrostatic) solvation free energy (\(\Delta G_{\text{PB}}\)) and nonpolar solvation free energy (\(\Delta G_{\text{SA}}\)) as in eq 4:

\[
\Delta G_{\text{sol}} = \Delta G_{\text{PB}} + \Delta G_{\text{SA}}
\]

(4)
ΔG_{PB} is computed by solving the linearized Poisson–Boltzmann (PB) equation using Parse radii and a solvent probe radius of 1.4 Å. In our calculations, the dielectric constant was set to 1.0 for the interior of the solutes and 80.0 for the solvent. ΔG_{SA} was determined using a solvent accessible surface area (SASA)–dependent term as in eq 5:

\[ ΔG_{SA} = γ \times \text{SASA} + β \]  

where γ is the surface tension proportionality constant and was set to 0.00542 kcal/(mol·Å^{-2}), and β is the offset value, which was set to 0.92 kcal/mol here.

Per-residue Decomposition

In order to detect the "hot spot" residues, the effective binding energies were decomposed into contributions of individual residues using the MMGBSA energy decomposition scheme. The MMGBSA calculations were carried out in the same way as in the MMPBSA calculations. The polar contribution to the solvation free energy was determined by applying the Generalized Born (GB) method (igb = 2) \(^{42}\), using mbondi2 radii. The non-polar contributions were estimated using the ICOSA method \(^{42}\) by a solvent accessible surface area (SASA) dependent term using a surface tension proportionality constant of 0.0072 kcal/mol Å\(^2\).

Results:

PDZ is the C-terminal peptide recognition domain in PSMD9

In our previous study \(^7\), we had generated a structural model of the PDZ domain of PSMD9 (as there was no structural data available) using the solution NMR structure of the PDZ2 domain of harmonin (PDB ID 2KBS with bound peptide) as template \(^23\); PDZ domain of PSMD9 shared 33% sequence identity and 55% sequence similarity with PDZ2, the highest sequence identity amongst all the PDZ domains with resolved structures at that time. The domain boundaries of the PDZ
domain of PSMD9 predicted from this model lie between residues 108-195, similar to the annotation found in UniProt (O00233). However, the construct of the PDZ domain generated using these domain boundaries failed to express in *E. coli*. Later, the crystal structure of Nas2 PDZ domain from yeast, the ortholog of mammalian PSMD9, was reported (PDB ID 40O6); \(^9\). Using this structure as template, we revisited the domain boundaries of the PDZ domain of PSMD9 and designed a new construct of PDZ domain spanning residues 121-223. This PDZ domain was found to be soluble, expressed in a stable form, and could be purified to homogeneity (Figure S1). We tested the ability of the isolated PDZ domain to bind to the two differential binders- the GRRF and SCGF tetrapeptides identified earlier during screening \(^7\). Like full length PSMD9, the PDZ domain was found to bind to GRRF with low affinity (\(K_D\) 439.3±62.94\(\mu\)M), and to SCGF with high affinity (\(K_D\) 11.06±0.75\(\mu\)M); additionally, the affinities were remarkably close to those observed with the full length PSMD9 (Figure 1A and 1B). These results indicate that the PDZ domain essentially accounts for the entire C-terminal peptide binding potential of PSMD9.

**Figure 1.** Comparison of the binding affinities of C-terminal peptides to PSMD9 and PDZ domain. The interaction of PSMD9 and PDZ domain with C-terminal tetrapeptides GRRF (C-terminus of hnRNPA1) (1A) and SCGF (1B) (C-terminus of growth hormone) was tested by ELISA. The dissociation constant was calculated to be 758.2±116.2\(\mu\)M for the PSMD9-GRRF interaction and
439.3±62.94μM for PDZ-domain-GRRF interaction. The dissociation constant was calculated to be 10.30±1.35μM for PSMD9-SCGF interaction and 11.06±0.75μM for PDZ domain-SCGF interaction. Data was collected from sample run in duplicates and represented as mean±SEM (standard error of mean (n=2)). The data was fit to one site-specific binding in GraphPad Prism software.

**Designing a super binding peptide**

As described in the introduction, the high affinity interaction of SCGF and the inability of SGGF but not SCGG mutant peptide to inhibit the growth hormone-PSMD9 interaction implied a hitherto unreported role of the cysteine at the P-2 position in defining the binding affinity of the peptide SCGF. We therefore asked whether replacing the arginine at position P-2 in GRRF with cysteine would convert the low affinity sequence (GRRF) to a tight binding motif GCRF. Indeed, peptide GCRF (K_{D}: 5.66±0.62μM) bound with almost 100-fold greater affinity than GRRF and the affinity was marginally better than that of SCGF (Table 1). This high affinity interaction driven by cysteine seems position-specific as the affinity of the peptide GRRC with a P0 cysteine is only a 10-fold higher than that of GRRF (Table 1). Further, replacing cysteine at P-2 in peptide SCGF to SGGF resulted in complete abrogation of peptide binding (Table 1) emphasizing the importance of cysteine at position P-2 in imparting high affinity for the peptide to the PDZ domain of PSMD9. Thus, with the newly engineered soluble PDZ domain, and design based on knowledge, we converted a very weak binding peptide (GRRF) to a tight binder (GCRF), and identified a unique position-specific determinant which confers high affinity to the peptide that binds PSMD9 PDZ domain.

**Table 1.** Binding affinities of C-terminal peptide variants to wild type and mutant PDZ domains:

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Protein</th>
<th>K_{D} (μM)±SEM (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>Motif</td>
<td>Form</td>
<td>Affinity (µM±SEM)</td>
</tr>
<tr>
<td>---------</td>
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<td>-------------------</td>
</tr>
<tr>
<td>GRRF</td>
<td>PDZ WT</td>
<td>439.3±62.94</td>
</tr>
<tr>
<td>SCGF</td>
<td>PDZ WT</td>
<td>11.06±0.75</td>
</tr>
<tr>
<td>GCRF</td>
<td>PDZ WT</td>
<td>5.66±0.62</td>
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</tr>
<tr>
<td>GRRRC</td>
<td>PDZ WT</td>
<td>71.65±9.05</td>
</tr>
<tr>
<td>SCGG</td>
<td>PDZ WT</td>
<td>44.03±7.19</td>
</tr>
<tr>
<td>SGGF</td>
<td>PDZ WT</td>
<td>No binding</td>
</tr>
<tr>
<td>GCGF</td>
<td>PDZ WT</td>
<td>8.89±0.82</td>
</tr>
<tr>
<td>SCGF</td>
<td>C216G mutant</td>
<td>12.86±0.99</td>
</tr>
<tr>
<td>SCGF</td>
<td>F162G mutant</td>
<td>19.9±0.71</td>
</tr>
<tr>
<td>SCGF</td>
<td>Q181G mutant</td>
<td>17.24±1.07</td>
</tr>
<tr>
<td>SCGF</td>
<td>I218G mutant</td>
<td>21.45±2.03</td>
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<tr>
<td>SCGF</td>
<td>Glutathione</td>
<td>9.94±0.64</td>
</tr>
<tr>
<td>SCGF</td>
<td>DTNB modified</td>
<td>7.5±0.66</td>
</tr>
<tr>
<td>GCRF</td>
<td>DTNB modified</td>
<td>13.4±1.17</td>
</tr>
</tbody>
</table>

Binding affinities of PDZ domain (wild type, mutant and modified) and peptides. Values are represented in µM±SEM (n=2) Data was computed from one site-specific binding model using GraphPad Prism software

Non covalent nature of the PDZ domain-SCGF motif interaction
The binding experiments with designed peptide variants suggested that the presence and precise positioning of cysteine in the peptides can drive high affinity interactions and can be used to generate tight-binding peptides. Since the free thiol group of cysteine has the propensity to form a disulfide bond with a neighboring cysteine, we tested the possibility that the high affinity of the cysteine peptides for PSMD9 PDZ may be due to disulfide bond formation between cysteine residue in the peptide with the cysteine residue of the PDZ domain. In our structural model, the \( \beta_5 \) sheet which forms the floor for the peptide interactions, harbors a cysteine (Cys 216). We modified this singular cysteine by reaction with the Ellman’s reagent, DTNB. Approximately 80% of the PDZ domain was modified at the end of two-hour incubation. This modification had no effect on the binding affinity of SCGF (\( K_D = 11.06 \pm 0.75 \mu M \) for wild type PDZ domain versus \( 7.5 \pm 0.66 \mu M \) for the thiol modified PDZ) (Table 1). In addition, we mutated the cysteine in the PDZ domain to a glycine (C216G) and found that the mutation did not alter peptide binding (\( K_D = 12.86 \pm 0.99 \mu M \) (Table 1). Together, these results provide unambiguous evidence for the role of cysteine in driving high-affinity interaction of the peptides with the PSMD9 PDZ domain through non-covalent interactions.

Inhibition of protein-protein interaction by designed peptides

Once we defined a tight binder of the PSMD9 PDZ domain, we investigated whether this motif could inhibit the interaction of PSMD9 with hnRNPA1. Both SCGF and GCRF inhibited PSMD9-hnRNPA1 interaction. The IC\(_{50}\) values were found to be 3.4\( \mu M \) for GCRF (Figure 2) which is very similar to that of SCGF (5.5\( \mu M \)) but several fold more potent than the parent peptide GRRF (758.2\( \mu M \)) \(^7\). We next aimed to understand the structural basis for these differences in the affinities of the various peptides. This information would define the future strategy for the design of peptide
mimetics or small molecule inhibitors of I\( \kappa \)B\( \alpha \) degradation and NF-\( \kappa \)B signaling, via inhibition of the PDZ domain of PSMD9.

**Figure 2.** Inhibition of PSMD9-hnRNPA1 interaction by C-terminal peptides SCGF (blue) and GCRF (red). The IC\(_{50}\) of SCGF was 5.5\( \mu \)M and GCRF was 3.4\( \mu \)M. Data was collected from sample run in duplicates and is represented as mean \( \pm \) SEM (n=2). The data was fitted to dose response for inhibition with variable slope (four parameters) in GraphPad Prism software.

**Homology model of PSMD9 PDZ domain**

Equipped with the successful demonstration of the near complete overlap between peptide binding to PSMD9 and the engineered PDZ domain, homology models were generated using the Nas2 PDZ domain (~42% sequence identity and 64% sequence similarity) structure as template; this contrasts with the older model\(^{23}\) where solution NMR structure of PDZ2 domain of harmonin was used as template. The model adopts a fold consisting of five \( \beta \)-sheets and two \( \alpha \)-helices (Figure 3A). The peptide binding pocket is very similar in both models, except that in the new model the floor of the binding groove is now formed by \( \beta 5 \) sheet instead of the typical \( \beta 2 \) sheet observed in
most PDZ domain structures. The binding site is primarily hydrophobic and is complemented by a small positive potential, arising from a charge cluster located at one end of the binding groove (Figure 3B). Detailed comparison of the old and new homology models of PSMD9 are discussed in the supplementary section (Figure S3)

![Sequence of the PDZ domain of PSMD9 with secondary structures mapped onto it.](image)

**Figure 3.** Sequence of the PDZ domain of PSMD9 with secondary structures mapped onto it. (A) Cartoon representation (B) electrostatic surface representation calculated using APBS where the blue and red refer to positive (+5kcal/mol) and negative (-5kcal/mol) potentials respectively of the homology model of the PDZ domain of PSMD9.

**Ligand mapping MD simulations**

The homology model was subjected to all atom molecular dynamics (MD) simulations. MD simulations show that the overall structure of the apo PDZ domain was stable with RMSD ~4.5Å (Figure 4A). The α2 helix which is part of the α/β binding groove is flexible in the apostate (Figure 4B and 4C), resulting in deformations and instabilities in the α/β binding pocket. The binding site
adopts a closed conformation with both α helix (α2) and β strand (β5) assuming a collapsed state (Figure 4D) to occlude the ‘active site’ residues as can be seen from the reduced distance between α2 and β5 (Figure 4D). In order to open this hydrophobic pocket, we decided to carry out ligand mapping simulations, which are techniques to enhance sampling and access to buried/cryptic pockets. These simulations were carried out with benzene molecules added to the solvent. No unfolding of the PDZ domain was observed during the ligand mapping simulations (Figure S4). As speculated, the benzene molecules occupy the hydrophobic peptide binding groove, preventing the formation of the closed state. Additionally, the presence of the benzene molecules in the peptide binding groove also resulted in a widening of the groove by ~3Å as compared to the standard MD simulations of the apoprotein (Figure 4D) in aqueous environment. Further, the conformations sampled were clustered to identify conformational ‘substates’ that we use for docking the peptides.
**Figure 4.** (A) RMSD (black, red and green correspond to three triplicates) (RMSD was calculated by superimposing all the residues of sampled structures on to the starting structure of the simulation). (B) RMSF of the conformations of PDZ domain of PSMD9 in its apoprotein (C) Cartoon representation of apo-PDZ domain colored according to the flexibility with blue to red corresponding to low to high flexibility. (D) Distance between the α2 and β5 in the binding groove of the conformations sampled during apo (black) and ligand mapping (red) simulations.

**Docking of GRRF and SCGF peptides to the PDZ domain**

For the PDZ domain-peptide docking, two different docking algorithms/programs (Haddock and ATTRACT) were used. Both programs predicted that both the peptides, GRRF and SCGF, would bind at the binding groove between α2 and β5 of the PDZ domain (Figure 5). Upon visual inspection of all the docked poses, a peptide-protein complex similar to PDZ-peptide complexes with phenylalanine at the P0 position was chosen. In this conformation, the peptide binds in an extended manner, antiparallel to β5 and engages in a large number of polar and hydrophobic interactions that extend the beta sheet by an additional strand (Figure 5) as seen with a typical PDZ domain-C-terminal peptide interaction. The hydrophobic side chain of phenylalanine at the P0 position of the peptide buries into the hydrophobic pocket formed by Pro147, Ala148, Phe162, Ile176, Val180, Leu201, Leu203, Gly215 and Cys216 (Figure 5). The peptide further interacts with the beta sheet mainly through backbone/side chain hydrogen bonds with residues Gly215, Cys216, Asn217 and Ile218 of β5-strand of the PDZ domain (Figure 5). Some of these interactions were similar to that proposed in the Nas2 interactions with the C-terminus of the archaeal ATPase.
Figure 5. Cartoon representation of the PDZ domain of PSMD9 in complex with the (A) GRRF and (B) SGCF peptide. Important residues in the binding site are shown in stick representation. The peptide is bound in canonical mode and shown in stick representation (labelled in red). Hydrogen bonds are highlighted in black dashed lines. (C, D) 2D diagram of PDZ–peptide (GRRF(C), SCGF(D)) residue contacts calculated using Ligplot²⁸.

Molecular dynamics simulations of PDZ-peptide complexes

To evaluate the stability of the predicted binding mode of the GRRF and SCGF peptides, the PDZ-peptide complexes were subjected to MD simulations. The overall structure of the protein–peptide docked models remained stable, with both the protein and the peptide remaining bound within RMSD of ~4Å against the docked models (Figure 6A, B). In these complex simulations, the PDZ
domain displayed reduced flexibility, as the presence of the peptide stabilizes the binding groove
and the nearby loop, attenuating the conformational flexibility seen in the apo PDZ domain. The
peptide in its bound state was stabilized by various interactions, and also shows reduced flexibility,
with the N-terminal region being wobbly as compared to the C-terminal region (Figure 6C). The
C-terminal phenylalanine of the peptide remains deeply buried in SCGF but partially buried in
GRRF within a hydrophobic crevice located at one end of the α2-β5 binding groove (Figure 5).
The peptides further interact with the terminal β5 sheet mainly through backbone/side chain
hydrogen bonds with residues Gly215, Cys216, Asn217 and Ile218 of the β5-strand and Gln181
from the α2-helix of the PDZ domain (Figure 5A and 5B). In the case of SCGF, the P-2 cysteine
(i.e., second from the N-terminus, third from C-terminus) occupies the hydrophobic binding pocket
(formed by Ile159, Phe162, Leu173, Ile176 and Ile218) and the shorter hydrophobic side chain of
P-2 cysteine remains buried in the pocket. In contrast, the side chain of arginine at P-2 position in
the GRRF peptide does not occupy the binding pocket and is exposed to solvent, interacting with
the α2-helix. The longer side chain of P-2 arginine further prevents the side chain of P0
phenylalanine from being deeply buried into the hydrophobic pocket. The buried side chains of
P-2 cysteine and P0 phenylalanine provide tight packing for the bound SCGF peptide, which is
not possible in the case of GRRF due to the tendency of P-2 arginine to remain exposed to the
solvent. This is further evident from the interaction analysis of PDZ-GRRF and PDZ-SCGF
complexes. In the case of the PDZ-SCGF complex, the backbone of bound peptide is involved in
five hydrogen bond interactions with the backbone of the strand β5, with three of these hydrogen
bonds (N217-C2, C216-F0, G215-F0) preserved for > 90% of the simulation time, and the other
two preserved (I218-S3, Q181-F0) for ~50% of the simulation time. However, in the PDZ-GRRF
complex, only three backbone-backbone hydrogen bond interactions were observed between the
peptide and PDZ, and only one interaction (I218-R2) was long-lived, (75% of the simulation time), while the other two hydrogen bonds (I218-G3, C216-F0) were less stable (< 40% of the simulation time). The considerable differences in the number of bonds formed and the extent of tight packing between the two peptides SCGF and GRRF with PDZ could readily account for their dramatically different binding affinities. In summary, MD simulations provide a plausible model underlying the observed affinity differences between the SCGF and GRRF peptides, and a likely role of the P-2 Arginine (vis a vis cysteine) which was not evident from the biochemical experiments.

**Determination of the binding affinity of PDZ – peptide complexes**

We next analyzed the energetics of peptide interactions with the PDZ domain. In general, the interaction energies of the peptides with the PDZ domain are favorable ($\Delta G$ between -17.5 kcal/mol to -27.2 kcal/mol; Table 2) (Figure 6D), however the SCGF peptide showed better binding ($\Delta G$ -27.2 kcal/mol) compared to GRRF ($\Delta G$ -17.5 kcal/mol). This is in good agreement with the experimental data ($K_D$ of GRRF is ~440µM and of SCGF is ~11 µM). Further analysis from the models revealed that the P0 phenylalanine makes a significant contribution to the binding of both peptides, more so in the case of GRRF (as seen in experiments), highlighting the importance of hydrophobicity (Figure 6E). In the case of GRRF, the rest of the peptide residues do not contribute favorably to the binding free energy. In contrast, all the residues of SCGF contribute favorably to its binding, with P-2 cysteine making significant contributions ($\Delta G$~ -4.5 kcal/mol; Figure 6E). From the PDZ domain, only residues from the $\alpha2/\beta5$ binding groove appear to contribute to the binding energetics, with the major contributions ($\Delta G \leq -3.0$ kcal/mol; Figure S5) coming from the residues of the $\beta5$ strand (Figure S5).
Figure 6. RMSD (RMSD was calculated by superimposing all the residues of sampled structures onto the starting structure of the simulation) of the peptide bound PDZ domain (A) and bound peptide (B), RMSF (C) of the conformations of GRRF (black) and SCGF (red) peptides in complex with PDZ, from the corresponding complex simulations. (D) Calculated MMPBSA binding free energies ($\Delta G_{\text{bind}}$) for PDZ-GRRF (black) and PDZ-SCGF (red) (E) Decomposition of binding free energy on per residue basis for all the four residues in the GRRF (black) and SCGF (red) peptides from the corresponding complex simulations.

Deciphering the role of C-terminal hydrophobicity in mediating affinity:

Extensive structural studies have indicated that peptide binding to PDZ domains is mediated predominantly by the terminal four residues of the peptide that interact directly with the binding
groove in the PDZ domains\textsuperscript{29}. As mentioned earlier, the binding groove in PSMD9 PDZ is unusual and is characterized by a cyclic permutation in which $\beta_5$ forms the floor instead of $\beta_2$ sheet. Therefore, to understand whether the conserved mode of interaction, especially of the P0 hydrophobic residues, is affected due to altered beta sheet arrangement, we undertook an extensive analysis of the peptide-PDZ complex. In the simulations, peptides GRRL, GRRI and GRRC, all with a hydrophobic residue at the C-terminus, remain stably bound to PDZ with RMSD $< 4\text{"A}$ (Figure 7A) and the C-terminal leucine, isoleucine and cysteine at the P0 position bury into the hydrophobic pocket. As with GRRF, the bound peptides show reduced flexibility, with the C-terminal region showing decreased flexibility as compared to the N-terminal region (Figure 7C).

Peptide-protein backbone interactions ($I_{218}$-$G_3$, $C_{216}$-$I_0/C_0$, $I_{218}$-$R_2$) stabilize the bound state of the peptide and the PDZ domain (preserved for $\sim 75\%$ of the simulation time). If the sidechain at the C-terminus is removed as in GRRG, the peptide unbinds from the canonical binding mode within $\sim 5$-$10$ ns of the simulation (Figure S6). This unstable binding of the GRRG peptide is in agreement with experimental observations which show that when P0 phenylalanine is mutated to glycine, the peptide no longer inhibits the PSMD9-hnRNPA1 interactions\textsuperscript{7}.

However, in sharp contrast to the unstable GRRG-PDZ complex, the SCGG peptide bound stably in the peptide binding groove with RMSD of $\sim 3.0\text{"A}$ during the simulations (Figure 7A). The bound SCGG peptide is involved in backbone-backbone interactions ($N_{217}$-$C_2$, $C_{216}$-$G_0$, $G_{215}$-$G_0$, $I_{218}$-$S_3$, $Q_{181}$-$G_0$) with the $\beta_5$-strand of the PDZ domain; however, now the C-terminus of the peptide shows increased flexibility compared to the N-terminus probably because of two glycine residues located at the C-terminus. The stable binding of the SCGG peptide is in agreement with experimental observations where the affinity of SCGG peptide is $\sim 44\mu$M (Table 1).
Figure 7. RMSD (RMSD was calculated by superimposing all the residues of sampled structures onto the starting structure of the simulation) of the bound peptide (A) RMSF (C) of the conformations of GRRL (black) GRRI (red) GRRC (green) and SCGG (blue) peptides in complex with PDZ, from its corresponding complex simulations. (B) Calculated MMPBSA binding free energies (ΔG_{bind}) for PDZ-peptide complexes (D) Decomposition of binding free energy on per residue basis for all the four residues in the GRRL (black) GRRI (red) GRRC (green) and SCGG (blue) peptides from the corresponding complex simulations.

Importance of the Cys at position P-2 for the binding of tetra peptides (MD simulations):
From the detailed analysis of the contributions of residues to the interactions of the peptides, it is obvious that C-terminal hydrophobicity is critical for the binding of at least the GRRX series of peptides, but less so for the SCGX series. The residue at position (P-2), i.e., arginine, in fact
disfavors binding in the case of the GRRX series but the residue at this position in the SCGX series, i.e., cysteine, contributes significantly to the binding energy (Figure 6E). Therefore, we next investigated the importance of cysteine at P-2 for binding. Concomitant with our experimental data (Table 1) the SGGF peptide was less stable in our simulations, with the RMSD of the bound conformation reaching values as high as 7Å (Figure S7). Although no complete unbinding of the peptide was observed during the MD simulation, (P0 phenylalanine remains buried in the pocket), the rest of peptide moves away from the binding site, with no protein–peptide hydrogen bonds observed during the simulations.

Detailed MD simulations revealed that P-2 cysteine contributes favorably to binding, while arginine at this position has a negative influence on the occupancy of P0 phenylalanine. Together these explain how the replacement of arginine in GRRF with cysteine to yield GCRF results in increases in affinity (from 439 µM to 5.6 µM). Arginine has an inhibitory effect on the affinity of GRRF; the introduction of cysteine at this position mimics the effect seen in SCGF wherein cysteine at P-2 was found to contribute significantly (ΔG < -3.0 kcal/mol; Figure 4) to the binding energy. MD simulations revealed that the GCRF peptide remained stably bound with RMSD ~3Å (Figure 8A) with both the phenylalanine at P0 and cysteine at P-2 now buried into the binding pocket and the bound peptide stabilized by backbone – backbone hydrogen bond interactions (C216-F0, I218-C2, I218-S3; stable for ~90% of the simulation time) with the β5 strand from the binding groove (Figure 9A). The tighter binding of the GCRF peptide is also mirrored in our energetic calculations, the ΔG of GCRF is -26.8 kcal/mol (Figure 8B) which is as good as that of SCGF with ΔG of -27.2 kcal/mol (Figure 6D) while the ΔG of GRRF is only -17.5 kcal/mol (Figure 6D). As seen with SCGF, the cysteine at P-2 makes significant contributions to the binding free energy (Figure 8D). P-2 cysteine with a shorter side chain along with the side chain of P0
phenylalanine are buried deep into the binding groove, enabling the peptide to be involved in stable hydrogen bond interactions with residues from the β5 strand (Figure 9).

Figure 8. (A) RMSD (RMSD was calculated by superimposing all the residues of sampled structures onto the starting structure of the simulation) of bound peptide, RMSF (C) of the conformations of GCRF (black) GCRG (red) GCGF (green) peptides in complex with PDZ, from the corresponding complex simulations. (B) Calculated MMPBSA binding free energies (ΔGbind) for PDZ-peptide complexes (D) Decomposition of binding free energy on per residue basis for all the four residues in the GCRF (black) GCRG (red) GCGF (green) peptides from the corresponding complex simulations.
Figure 9. Snapshots from MD simulations of various peptides GCRF (A), GCGF (B) GCRG (C) SCGG (D) bound with PDZ from the corresponding complex simulations.

In the majority of PDZ-peptide complex structures tested here, glycine residue at P-3 position of the peptide makes unfavorable contribution ($\Delta G \sim +1.5 \text{ kcal/mol}$; Figure 8D) while serine makes favorable contribution ($\Delta G \sim -1.5 \text{ kcal/mol}$; Figure 6E, Figure 7D) to the binding energy of PDZ-peptide interaction. We, therefore evaluated the role of serine at the P-3 position in SCGF for binding to the PDZ domain by testing a variant of the peptide GCGF (with glycine at P-3 instead of serine) peptide with the PDZ domain. This peptide had the same affinity for the PDZ domain.
(K\textsubscript{D}: 8.89±0.82\mu M) as that of SCGF peptide (K\textsubscript{D}: 11.06±\mu M) (Table 1). These results indicate that cysteine is the primary mediator for high affinity interactions in these peptides, and serine has little or no effect. The positional occupancy of cysteine is also important in dictating maximal binding affinity since moving the cysteine from P-2 to P0 as in GRRC improved the affinity only by 5-fold (72\mu M for GRRC vs 439\mu M for GRRF) (Table1).

**Contribution of residues in the binding pocket towards peptide binding:**

The docking studies pinpointed crucial residues P147, A148, F162, I176, V180, L201, L203, G215, C216, N217, I218) in the PDZ domain that contribute to the bulk of peptide binding energy. We investigated the contribution of three primary residues, F162 (β2), Q181 (α2) and I218 (β5) to peptide binding by mutating each of them to a glycine residue. All the three mutations led to modest reduction in affinity (Table 1), indicating the importance of these residues in peptide binding. These results are similar to single amino acid substitutions made in PDZ3 domain of PSD-95. Besides modifying cysteine with DTNB (Table1), we also modified the PDZ domain by incubating it with glutathione. The unreacted glutathione was removed by desalting and PDZ domain was tested for interaction with peptide. The peptide binding was unaffected (K\textsubscript{D}: 9.94±0.64) (Table 1). These results indicate that I218 in β5 has a better contribution to the binding energy than C216.

**Discussion and conclusion**

More than 250 non-redundant PDZ domains have been identified from 150 PDZ containing proteins. Despite low sequence identity (<30%), the overall fold is conserved across the family with the variations localized largely to the loop regions. The PDZ domain, like other protein interacting domains such as the SH3, WW, SH2, mediates biological processes often through transient interactions involving small regions of the interacting partner. In accordance, these
isolated domains often recognize short peptides with specific sequence motifs. The small nature
of the binding surface combined with the specific and conserved nature of PDZ-peptide
interactions makes the PDZ domains attractive ‘druggable’ targets and have guided the rational
design of several small molecule and peptide-based inhibitors. In addition peptide analogs or
blockers that disrupt PDZ domain interactions have also been useful for understanding how these
domains regulate functions of their interacting proteins. While these results have been
extremely encouraging, the low sequence conservation and lack of extensive structural data on apo
and peptide or protein bound structures of the PDZ complexes remains a major deterrent in
exploiting these interactions exhaustively for designing novel inhibitors for blocking associated
signaling cascades. This lacuna is considerably overcome by extremely detailed biochemical and
biophysical investigations on the sequence determinants of binding which in some cases is
accompanied by mutagenesis studies to identify per residue contributions to binding affinity. Some
of the major outcomes of these studies have been a) the identification of the common determining
factor for peptide recognition by the PDZ domains which is a hydrophobic residue at the C-
terminus of the peptide; b) the interaction is typically governed by the binding of the C-terminus
of an interacting peptide into a groove formed by a strand (β2) and a helix (α2) of the PDZ domain
leading to the interacting peptide assuming an antiparallel β-strand (relative to β2 of most
PDZ domains); c) P0 hydrophobic residue and the amino acid at P-2 position drive the bulk of
binding energy in most of the known PDZ domains.

In this report we pursue one such structure-activity relationship of the PDZ domain of PSMD9 and
find that hydrophobicity of the C-termini, which is the hallmark of peptide recognition by other
PDZ domain proteins, does not fully account for the binding preference of the PSMD9 PDZ
domain. The initial set of point mutations in PSMD9 peptide interactions underscore the
requirement of hydrophobicity at the C-terminus of the peptide. Peptide binding is lost if the P0 phenylalanine in GRRF is mutated to glycine, but retained if mutated to leucine (GRRL) or isoleucine (GRRRI)\textsuperscript{7}. These observations are similar to those reported by other groups\textsuperscript{3, 39-45}. However, by investigating other peptide interactions with the PDZ domain, we found that the P0 hydrophobicity is not the primary driving force of the interaction; rather an unusual non-covalent interaction via the cysteine residue at P-2 with the residues in the β5 strand of the circularly permuted PDZ domain is a major affinity determinant (Table 1). Computational studies explain these experimental observations; classical hydrophobic residues such as phenylalanine can add to the affinity when the P-2 is a cysteine, as seen from the measurable loss in ΔG of binding when the phenylalanine at P0 is mutated in the high affinity peptides or super binders\textsuperscript{7}. Based on the modelled structures, one can envisage that in SCGG and GCRG peptides, the absence of phenylalanine generates an unfilled pocket, which is energetically unfavorable. Since the aromatic side chain of phenylalanine is involved in stacking interactions with other aromatic residues, it may play a significant role in recognition of specific client proteins and hydrophobic ligands\textsuperscript{46}. Our simulations and energetic analyses also explain the complexity of the contributions of individual residues from the GRRX and SCGX series. Arginine at the P-2 position in the GRRX series contributed negatively to the binding free energy and is not favored in the richly hydrophobic and positively charged binding pocket whereas in the case of SCGF and SCGG, the shorter and hydrophobic side chain of cysteine, is well tolerated. The side chain of cysteine buries deep into the pocket while arginine is extruded into solvent. The residue at P-1 in either series does not engage in side chain interactions and therefore does not dictate or influence specificity, similar to the observation made for other PDZ domains\textsuperscript{47}. The residue at P-3 may marginally influence binding or not at all in the series tested here.
Thus, in conclusion, we report that C-terminal peptides, the sequence of which were derived from the C-termini of proteins, interact with the isolated PDZ domain of PSMD9. The domain contributed to the binding affinity of the full-length protein in entirety and the magnitude of binding is affected identically (full-length PSMD9 vis a vis the PDZ domain) by mutations in the peptide. The floor of the binding pocket which is occupied by the β5 strand in the circularly permuted structure (as suggested by the model built based on the Nas2 crystal structure) contributes to the interacting residues. A hitherto unidentified motif with cysteine occupying P-2 position introduces a new class of binding preferences of the PDZ domains at large. So far, based on ligand binding specificities, 16 distinct specificity classes of PDZ domains have been identified and yet cysteine was not observed at P-2 position as a strong contributor of affinity.

Collectively, our parallel in depth biochemical, structure guided modelling and robust MD simulations establish a unique signature motif in C-terminal peptides for high-affinity interactions with the PDZ domain of PSMD9 pinpointing position specific positive and negative contributors of binding energy. The results led to the design of a super binding peptide GCRF, which is capable of inhibiting the PSMD9-hnRNPA1 interaction. The unique signature motif can be utilized as a scaffold for the design of peptide-based inhibitors for blocking NF-kB signaling in cancers dependent on NF-kB for survival and chemo/radioresistance.

ASSOCIATED CONTENT

Additional experimental and computational results in the supplementary information.

Supplementary Information.pdf

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS
PDZ, Post-synaptic Discs large, ZO-1 proteins; PPIs, Protein-protein Interactions; hnRNPA1, heterogenous nuclear ribonuclear protein A1; GH, Growth Hormone;

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