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Investigation of the PDZ Domain Ligand Binding Site Using Chemically Modified Peptides

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Abstract—Several chemically modified analogues to a tightly binding ligand for the second PDZ domain of MAGI-3 were synthesized and evaluated for their ability to compete with native peptide ligands. *N*-methyl scanning of the ligand backbone amides revealed the energetically important hydrogen bonds between the ligand backbone and the PDZ domain. Analogues to the ligand's conserved threonine/serine(−2) residue, involved in a side chain to side chain hydrogen bond with a conserved histidine in the PDZ domain, revealed that the interaction is highly sensitive to the steric structure around the hydroxyl group of this residue. Analogues of the ligand carboxy terminus revealed that the full hydrogen bond network of the GLGF loop is important in ligand binding.

The identification of adaptor proteins has led to the idea that organization of protein complexes at the cellular membrane is a crucial aspect of signal transduction.[1] Adaptors normally utilize clusters of modular protein interaction domains to control protein complex assembly. One important class of interaction domain in adaptors is the PDZ domain, a 100 amino acid module that normally binds a short carboxy terminal motif on its cognate ligands.[2] PDZ domains segregate into three major classes: Class I, which binds the S/T-X-V motif; Class II, which binds F/L-X-V/L; and Class III, which binds D/E-X-V. Structural[3] and biochemical[4] studies of PDZ domains have suggested some requirements for interactions between PDZ domains and their ligands. The most commonly observed contacts for class I PDZ domains (Fig. 1) are: (1) a hydrogen bond network between the ligand carboxylate anion and both amide protons from the backbone of the PDZ domain's GLGF motif and the side chain of a conserved basic residue on the PDZ domain, (2) specific hydrogen bonds between the backbone amides of the PDZ domain and the ligand, (3) hydrophobic interactions between the ligand's terminal V(0) side chain and a PDZ domain pocket lined with hydrophobic side chains, and (4) a hydrogen bond between the ligand S/T(−2) side chain and a H in α helix 2 of the PDZ domain. Other points of interaction that might allow for discrimination between related ligands by the PDZ domain include (1) specific interactions between the (−1) residue side chain of the ligand and those of β strand C of the PDZ domain, (2) the ligand's (−3) residue interacting with β strand B, and (3) ligand residues past (−3) interacting with a variable loop region between β strands B and C. As part of our program aimed at specifically blocking function of individual PDZ domains, we desired to carefully evaluate the role of these elements.

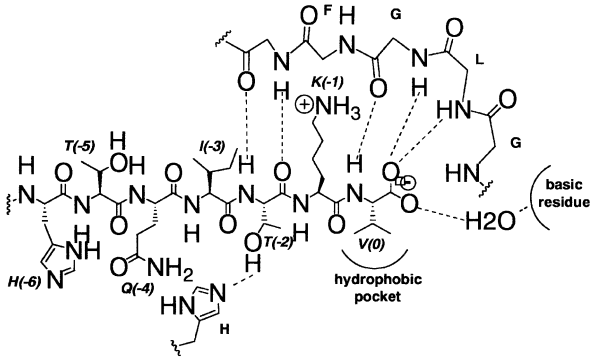

Figure 1. Recognized features of the ligand–PDZ domain interaction and proposed interactions under investigation as adapted from Doyle et al.[3a] The peptide sequence shown is that of the native PTEN carboxy terminus.

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To date, there have been no reports of detailed investigation of the hydrogen bond network between domain/ligand backbones or the size and shape of the PDZ domain's hydrophobic pockets. To address these issues, we prepared a library of peptides and peptidomimetics designed from the homology model of PDZ2 of MAGI-3 bound to a peptide ligand representing the carboxy terminus of tumor suppressor PTEN.[5] We chose the following peptide modifications (Fig. 2): (1) serial truncation of the peptide to identify the minimum length for the efficient binding (Panel A), (2) serial substitution of peptide-bond amide hydrogens with methyl groups (*N*-methyl scanning) to identify important hydrogen bond donors (Panel B), (3) replacement of the carboxy terminus with other functionalities (Panel C), and (4) modification of the hydrophobic regions of the X(−1) and S/T(−2) side chains (Panel C). We compared the binding affinity of these modified ligands toward PDZ2 of MAGI-3 with that of a native ligand using a fluorescence polarization competition assay.[4d,6]

We produced OregonGreen™-tagged fluorescent peptides **1a** (the native sequence of PTEN,[7] a tumor suppressor that binds to MAGI-2/3 PDZ2 domain[8]) and **1b** (a high affinity peptide for the MAGI-3 PDZ2 domain[5]) by standard Fmoc peptide synthesis protocols. Consistent with prior ELISA results, peptide **1b** showed almost a 10-fold tighter affinity for MAGI-2/3 PDZ2 than **1a**.[5] Anticipating relatively weak binding from our library of modified peptides, we chose the natural sequence **1a** as our probe for the competition binding assay. We synthesized several modified versions of **1b** in order to test hypotheses about the role in ligand binding of the various moieties.

Few reports have addressed the significance of residues further amino terminal than S/T(−2) in the ligand consensus motif.[4c] To investigate the contribution of other residues in the extended motif we truncated the AcHTQITWV-OH peptide (**3d**) sequentially (Fig. 2A) by one amino acid. All of the truncated peptides **3a**–**3d** showed weaker affinity than longer peptide **2b**. Presumably, the contribution by residues more amino terminal than the H(−6) imparts tighter affinity as seen with **2b**. This is also evident in the weak binding shown by the negative control peptide **2a**, which has an incomplete pharmacophore. However, peptides **3b**, **3c** and **3d** of five, six, and seven amino acids, respectively, showed similar affinity, while **3a**, a peptide of only four amino acids, showed even weaker binding. While these data are consistent with reported observations that five residues confer PDZ domain recognition, they do indicate a role for the more extended amino acids as has been previously proposed.[4a,c]

Based on these results, we carried out *N*-methyl scanning of **3c** at the five amide nitrogens in the peptide backbone (Fig. 2B) by a reported methodology.[10] The steric effects of the valine isopropyl precluded **4a** from being synthesized through this method. A liquid-phase version of the solid-phase method was applied to synthesize *N*-methyl valine derivatives as described in Figure 2. All of the methylated peptides, **4a**–**4e**, showed weaker binding than **3c**. *N*-Methylation may change amide bond geometry from *E*-form to *Z*-form[11] thus inducing the overall reduction in binding ability. Disparity in the affinity of these peptides may reveal the importance of the amide hydrogens or may reflect a greater propensity for β branched residues to allow a conformational change. The order of affinity of the peptides: **4e**/**4b** > **4d** > **4c** >> **4a** may indicate that amide hydrogens on V(0) and T(−2) are important and the I(−3) amide hydrogen is also contributing to efficient binding. Meanwhile, amide hydrogens on W(−1) and Q(−5) have significantly less contribution. These results are consistent with the hydrogen bond networks inferred from the co-crystal structure of KQTSV peptide binding to the PSD-95 PDZ3 domain.[3a]

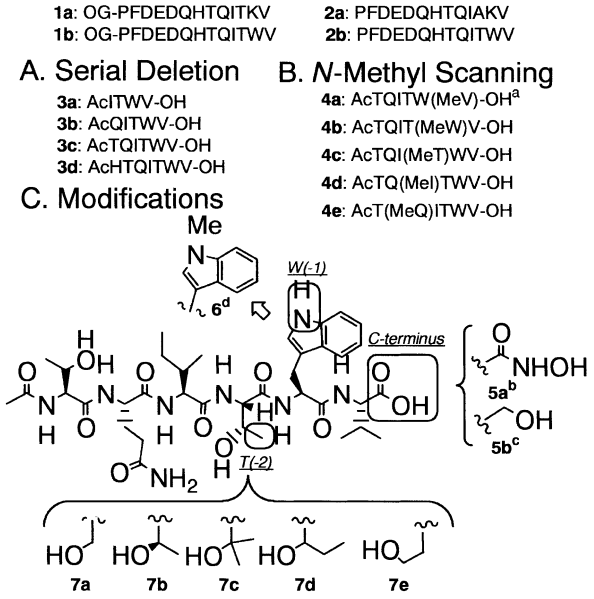

Figure 2. Designed peptides from the carboxy terminus sequence of PTEN. Unless otherwise specified, peptides were synthesized from commercially available amino acids using standard Fmoc protocols.[9] All were purified to homogeneity by RP-HPLC. Structures were confirmed by HRMS. [a]Conditions for generating **4a**: (a) HCl·H-V-OMe (1 equiv), 2-$(O_2N)PhSO_2Cl$ (1.1 equiv), DIPEA (3 equiv), DMF, rt, 3 days: then added 4-$(O_2N)PhSO_3Me$ (2.6 equiv), MTBD (6 equiv), rt, 4 h, 97% overall; (b) MeOH, NaOH aq (10%), 50 °C, 4 h; (c) PMB-Cl (1.2 equiv), K_2CO_3 (2.2 equiv), DMF, rt, 12 h; (d) $HOCH_2CH_2SH$ (5.0 equiv), DBU (2.5 equiv), DMF, rt, 10 min, 3 steps 85% overall yield; (e) Fmoc-W(Boc)-OH (1 equiv), HATU (1.3 equiv), DIPEA (3 equiv), DMF, rt, 1 h; (f) 96% TFA, 2% TIS, 2% H_2O, rt; (g) 2-chlorotrityl chloride resin, DIPEA , CH_2Cl_2, rt, 3 days; (h) repeat the following protocols: Fmoc amino acid (2.5 equiv), HBTU (2.4 equiv), DIPEA (5 equiv), DMF, rt, 0.5 h then 20% piperidine in DMF, rt, 10 min, three times; (i) Ac_2O (4 equiv), DIPEA (5 equiv), DMF, rt, 0.5 h; (j) 96% TFA, 2% TIS, 2% H_2O, rt; [b]Preparation of **5a**: (a) H_2NOTHP (10 equiv), AcT(*t*Bu)Q(Tr)IT(*t*Bu)W(Boc)V-OH (1 equiv), HATU (9 equiv), DIPEA (20 equiv), DMF, 50 °C, 12 h; (b) 96% TFA, 2% TIS, 2% H_2O, rt. [c]Preparation of **5b**: (a) *l*-valinol (3 equiv), AcT(*t*Bu)Q-(Tr)IT(*t*Bu)W-(Boc)-OH (1 equiv), HBTU (2.4 equiv), DIPEA (8 equiv), DMF, 50 °C, 1 h; (b) 96% TFA, 2% TIS, 2% H_2O, rt. [d]Preparation of **6**: (a) H-W-OH (1 equiv), $PhCH_2Br$ (3.2 equiv), K_2CO_3 (4 equiv), DMF, 50 °C, 12 h; (b) MeI (large excess), NaH (large excess), DMF, rt, 0.5 h, two steps quantitative; (c) HCO_2NH_4 (10 equiv), 10% Pd/C, MeOH, THF, H_2O, reflux, 12 h; (d) Fmoc-OSu (2.2 equiv), THF, H_2O, pH 9, rt, 12 h, two steps 57% overall; (e) H-V-OWang resin, HBTU (2.4 equiv), DIPEA (5 equiv), CH_2Cl_2, rt, 1 h then 20% piperidine in DMF, rt, 10 min, three times; (f) repeat the following protocols: Fmoc amino acid (2.5 equiv), HBTU (2.4 equiv), DIPEA (5 equiv), DMF, rt, 0.5 h then 20% piperidine in DMF, rt, 10 min, 3 times; (g) Ac_2O (4 equiv), DIPEA (5 equiv), DMF, rt, 0.5 h; (h) 96% TFA, 2% TIS, 2% H_2O, rt.

We next modified individual residues to investigate the flexibility and character of the PDZ binding pocket. The carboxy terminus was substituted with other functional groups (Fig. 2C). A neutral alcohol **5b** almost completely abolished the peptide's binding while a weakly acidic hydroxamide **5a** retains weak binding ability. These results suggest that the electrostatic interaction of the ligand carboxylate and a conserved cationic lysine in the MAGI-3 PDZ2 domain which has been suggested to mediate ligand binding are more important than preserving size. Terminating the peptide as an amide, a modification that increases bulk and decreases electrostatic potential, has previously been reported to significantly decrease affinity of the ligand.[4d,5] There are several reports about the lack of firm preferences for a particular side chain at the X(−1) position.[4a,4b] To investigate this residue, the W(−1) of was minimally modified by methylation on its indole-1 position (Fig. 2C). Unexpectedly, **6** demonstrated reduced affinity compared with **3c** suggesting that there is a possibility to design selective ligands for PDZ domains by modification of the X(−1) region.[4c] Finally, we modified the T(−2) side chain region by changing the methyl group to other small hydrophobic moieties (Fig. 2C). As expected, serine-substituted peptide **7a** binds with similar extent to **3c**. Otherwise, all other modifications including an inversion of chirality of the threonine hydroxyl group (**7b**), dimethyl substitution (**7c**), ethyl substitution (**7d**), and elongation (**7e**) nearly abolished binding. Therefore, interaction between the S/T(−2) hydroxyl group and histidine side chain in the α helix 2 in class I PDZ domain is strictly regulated by the approximate size of the binding pocket (Table 1).

Table 1. Affinity of modified peptides for MAGI-3 PDZ2

Direct binding Ligand	K_d[a] (μM) ± standard error[b]
1a	1.0 ± 0.05
1b	0.02 ± 0.0005
Competition binding ligand	**IC_{50}[c] (μM) ± standard error[b]**
2a	14.4 ± 0.6[d]
2b	0.74 ± 0.2
3a	14.8 ± 1.5[d]
3b	4.5 ± 0.1[e]
3c	5.2 ± 0.2[e]
3d	4.1 ± 0.6[e]
4a	Not detected[g]
4b	10.6 ± 0.2[f]
4c	29.0 ± 1.8
4d	20.4 ± 2.1
4e	9.8 ± 0.7[f]
5a	42.1 ± 7.9
5b	Not detected
6	34.0 ± 0.6
7a	5.1 ± 0.6[e]
7b	Not detected
7c	Not detected
7d	Not detected
7e	Not detected

[a]Dissociation constants determined by plotting data to $y = \text{bottom} + (\text{top} - \text{bottom}) * x/(K_d + x)$.
[b]Values are means of three experiments.
[c]Competitor concentration that inhibits probe binding by 50% as determined by fitting data to one site competition equation $y = \text{bottom} + (\text{top} - \text{bottom})/(1 + x/IC_{50})$.
[e,f]Results are not statistically different ($p \leq 0.05$) by Mann–Whitney test.
[g]Indicates $IC_{50} > 50$ μM and unable to be determined by our assay.

Our results indicate that the ligand binding pocket of the MAGI-3 PDZ2 domain is highly sensitive to alterations in the carboxylate terminus and the individual residue side chains. In particular, the S/T(−2) residue, which has been shown to interact with a well conserved histidine in class I binding PDZ domains, could not be substituted with electrostatically similar but more bulky alcohol side chains. Additionally, the carboxylate binding pocket cannot accommodate large changes in electrostatic potential or added bulk.[4d] Therefore, it is essential for affinity to a class I PDZ domain that these interactions be fulfilled. Any successful competitor must satisfy these requirements and incorporate features that will discriminate between PDZ domains. The study of the modified (−1) side chain shows that alterations in this position can greatly affect binding affinity. Making use of this region in concert with more extended interactions with β strand C and the variable loop between β strands B and C may be important to gain specificity. The information obtained by this work would be useful in the design of novel inhibitors of the PDZ domain/ligand interaction, especially between MAGI-3 PDZ2 domain and PTEN.

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