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Interactions outside the boundaries of the canonical binding groove of a PDZ domain influence ligand binding

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Abbreviations

ITC, isothermal titration calorimetry; PDZ, Postsynaptic density protein-95/Discs large/Zonula Occludens-1; PSD-95, Postsynaptic density protein-95

ABSTRACT

The PDZ domain is a protein-protein interaction module with a shallow binding groove where protein ligands bind. However, interactions that are not part of this canonical binding groove are likely to modulate peptide binding. We have investigated such interactions beyond the binding groove for PDZ3 from PSD-95 and a peptide derived from the C-terminus of the natural ligand CRIPT. We found by NMR experiments that up to eight residues of the peptide ligand interact with the PDZ domain, showing that the interaction surface extends far outside of the binding groove as defined by the crystal structure. PDZ3 contains an extra structural element, a C-terminal helix ($\alpha 3$), which is known to affect affinity. Deletion of this helix resulted in loss of several inter-molecular NOEs from peptide residues outside of the binding pocket, suggesting that $\alpha 3$ forms part of the extra binding surface in wild type PDZ3. Site-directed mutagenesis, isothermal titration calorimetry and fluorescence intensity experiments confirmed the importance of both $\alpha 3$ and the N-terminal part of the peptide for the affinity. Our data suggest a general mechanism where different binding surfaces outside of the PDZ binding groove could provide sites for specific interactions.

Postsynaptic density protein-95/Discs large/Zonula Occludens-1 (PDZ) domains are usually part of multi-domain proteins and coordinate signaling and scaffolding by binding and organizing various cellular proteins.¹⁻⁵ They generally bind to short C-terminal sequences in a well-defined binding groove.⁶ There are multiple PDZ domains within the same cell and most likely several competing protein ligands present within the vicinity of each PDZ domain. In crystal or NMR structures of PDZ domains and peptide ligands, usually only four-five ligand residues are visible. Thus, to achieve selectivity towards ligands in a cellular context, other factors than the interactions in the binding groove are likely to play a role. Compartmentalization, local concentration^{7, 8} and allosteric effects^{9, 10} have been proposed as means by which PDZ domains modulate selectivity towards their binding partners.

However, structural elements located outside of the canonical PDZ fold may also influence affinity and specificity. For example, the src homology 3 (SH3) domain modulates the affinity of the adjacent MAGI1 PDZ3.^{11, 12} Another example is the third PDZ domain of PSD-95 (PDZ3), which contains an α helix located at the C-terminus outside of the canonical PDZ fold¹³ (Fig. 1). This helix is referred to as α 3 and it curls towards the ligand-binding groove between α 2 and β 2 of PSD-95 PDZ3. The α 3 helix is however located more than 5 Å from the last residue that is visible in the crystal structure (Gln₃) of the complex between PDZ3 and peptide ligand.¹³ The α 3 thus appears not to interact with the ligand. Nevertheless, deletion of the α 3 helix has a profound effect on the affinity between PDZ3 and peptide ligand and this helix was suggested to regulate a hidden allostery.¹⁴ Although the peptide ligand residue Tyr₅ is not visible in the crystal structure of PDZ3, it contributes to the affinity since its

deletion results in a 24-fold decrease in affinity as measured by isothermal titration calorimetry (ITC).¹⁵ It is possible that Tyr₅ interacts transiently with PDZ3, via the α 3 helix or other parts of the PDZ domain. The presence of such very short-lived interactions may have implications for the whole family of PDZ domains and their interactions with protein ligands.

To test this hypothesis and learn more about interactions outside of the binding groove of PDZ domains, we investigated the role of peptide residues outside of the binding pocket as well as the α 3 helix in PDZ3 by protein engineering in conjunction with NMR, ITC and kinetic experiments based on fluorescence. We demonstrate that binding is indeed modulated by interactions beyond the canonical binding groove.

MATERIALS AND METHODS

Expression and purification of PDZ variants. The PDZ domain used in this study was PDZ3 from human PSD-95 and it contains the mutation F337W (this construct is hereafter referred to as PDZ3). The numbering of residues refers to full length PSD-95 α without exon b and is the same as used in Doyle et al.¹³ The F337W mutation serves as a fluorescence probe in ligand binding experiments and does not affect the affinity of the PDZ3-peptide interaction.¹⁶⁻¹⁸ It also does not affect the energetic coupling pattern between side-chain residues in the PDZ domain and in the peptide ligand⁹, since the coupling energy for F337W is zero with regard to a second mutation in the peptide ligand, either at the C-terminal Val₀ or at Ser₂ (not shown). The sequence corresponding to the α 3-helix (residues 396-401) was deleted from the

cDNA of PDZ3 to generate the α 3-helix-deleted mutant PDZ3 $\Delta\alpha$ 3. The point mutants Y397E, R399A and F400A in the α 3-helix were created by inverted PCR on the cDNA encoding PDZ3, using Pfu Turbo polymerase (Stratagene).

Transformed *E. coli* BL21 DE3 pLysS cells were selected by growing in a 2TY medium containing ampicillin (50 μ g/ml) and chloramphenicol (35 μ g/ml) at 37°C until the OD₆₀₀ was 0.4-0.6. Protein expression was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside and the cells were allowed to express protein overnight at 30°C. The cells were collected by centrifugation (7000 g for 10 min) and re-suspended in buffer (50 mM Tris/HCl, pH 8.5, 400 mM NaCl). The cells were then disrupted by ultra-sonication, followed by centrifugation (35000 g) for 1 hour. The supernatant thus obtained was filtered successively through 0.45 and 0.2 μ m filters (Sarstedt). The filtered supernatant was loaded onto a Nickel (II)-charged chelating Sepharose FF column (GE healthcare), equilibrated with 50 mM Tris/HCl pH 8.5, 400 mM NaCl and washed with 300 ml of the same buffer. The bound protein was eluted with 250 mM imidazole pH 7.9. The fractions containing the PDZ were pooled, concentrated and dialyzed overnight against 50 mM Tris/HCl, pH 8.5. The dialyzed protein samples were filtered through 0.2 μ m filters and loaded onto a Q-column (GE Healthcare), equilibrated with 50 mM Tris/HCl, pH 8.5. The protein did not bind to the column and appears in the un-bound fraction. The fraction containing the protein was run on an S-column (GE Healthcare), equilibrated with 50 mM Tris/HCl, pH 8.5. The bound protein was eluted with a gradient of 0-1 M NaCl in 50 mM Tris/HCl, pH 8.5. The fractions containing the PDZ were pooled and the purity of the PDZ variants were checked by SDS-PAGE stained with coomassie brilliant blue and the identity of the mutants were confirmed by MALDI-TOF mass spectrometry. Purified mutants

were analyzed by urea denaturation experiments. We observed the typical sigmoidal denaturation curve for the point mutants (not shown), which, together with the fact that they bound peptide ligand, confirmed that they were properly folded.

Peptide ligands. For ITC binding experiments the peptides KQTSV and YKQTSV (GL Biochem, Shanghai) corresponding to the five or six last residues of CRIPT was used. A dansylated version of the longer peptide, **D**-YKQTSV, was used in stopped flow experiments. Numbering of the peptide ligand is as in Figure 1, with the C-terminal residue numbered 0 and subsequent residues -1, -2 etc towards the N-terminus. For the NMR measurements a longer version of the CRIPT C-terminus¹⁹ was expressed as a double-labeled ¹⁵N ¹³C lipoyl fusion protein in *E. coli*. The lipoyl-peptide fusion protein was loaded onto a nickel column, washed with 50 mM Tris/HCl, pH 8.5, 400 mM NaCl and subsequently eluted with 250 mM imidazole, pH 7.9. Fractions containing lipoyl-peptide were then pooled, filtered with a 0.2 µm filter and loaded onto a Q-column equilibrated with 50 mM Tris/HCl, pH 8.5. Pure lipoyl-peptide was eluted by a gradient of 0-500 mM NaCl in 50 mM Tris/HCl, pH 8.5. The peptide was cleaved off from the fusion protein with thrombin and then purified by reversed phase HPLC on a C18 column, lyophilized and stored at -20°C. The peptide contained two extra residues at the N-terminus, GS, from the thrombin cleavage site, thus resulting in the following peptide: GSKNYKQTSV. The identity was confirmed by MALDI-TOF mass spectrometry. Prior to NMR experiments, the peptide was dissolved in the appropriate buffer and its concentration was determined by UV absorbance using a calculated extinction coefficient of 1490 cm⁻¹M⁻¹.

NMR titrations. Titration experiments were performed on a Varian INOVA 600 MHz spectrometer equipped with a cryogenically cooled probe, at 283 K and 277 K. Peptide samples were dissolved in 50 mM potassium phosphate, pH 7.5, 10% D₂O and ¹H-¹⁵N HSQC spectra for peptide (290 μM or 150 μM) were recorded at an increasing concentration of unlabeled PDZ3 (0, 260, 270, 310, 370 μM, respectively) or unlabeled PDZ3Δα3 (0, 520, 820, 940 μM, respectively). For backbone resonance assignments, ¹⁵N NOESY-HSQC, ¹⁵N TOCSY-HSQC, HNCACB, CBCA(CO)NH, HN(CA)CO, and HNCO spectra were recorded for the bound/unbound peptide. Aliphatic side-chain resonance assignments were obtained using HCCH-TOCSY and ¹³C NOESY-HSQC experiments. The aromatic side-chain of Tyr₅ was assigned using ¹³C edited NOESY spectra optimized for the aromatic resonances. ¹³C edited NOESY experiments were used to detect inter/intra molecular NOEs. Data processing and analysis were done with NMRpipe²⁰ and Sparky (Goddard and Kneller, SPARKY 3, University of California, San Francisco), respectively.

Stopped-flow experiments. Pre-equilibrium ligand binding experiments were performed on an SX-20 MV stopped-flow spectrometer (Applied photophysics, Leatherhead, UK). For *k_{on}* measurements, the concentration of protein (PDZ3, PDZ3Δα3 and point mutants Y397E, R399A and F400A) was kept constant at 3 μM and the concentration of **D**-YKQTSV peptide was varied. The experiments were performed at 10°C in 50 mM potassium phosphate pH 7.5. Excitation of the Trp₃₃₇ was carried out at 280 nm while the emission was recorded using a 330 nm band-pass filter. Traces obtained from pre-equilibrium ligand binding were fitted to a single exponential function (Eq. 1).

$$A = \Delta A_{EQ} (1 - e^{-k_{obs} \cdot t}) + C \quad (\text{Eq. 1})$$

A is the change in florescence over time t and k_{obs} is the observed rate constant. The k_{obs} values were plotted against the peptide concentration and fitted to Eq. 2.

$$k_{obs} = (k_{on}^2 (n - [A]_0)^2 + k_{off}^2 + 2 k_{on} k_{off} (n + [A]_0))^{0.5} \quad (\text{Eq. 2})$$

where k_{on} is the association or on-rate constant and k_{off} is the dissociation or off-rate constant. $[A]_0$ and n are the total concentrations of peptide and protein (held constant at 3 μM), respectively.²¹

The off-rate constant was measured separately for PDZ3, PDZ3 $\Delta\alpha 3$ and point mutants Y397E, R399A and F400A. For these experiments protein (3 μM) was pre-incubated with the **D**-YKQTSV peptide (3 μM). The complex was dissociated with the addition of unlabeled YKQTSV peptide and the resulting traces were fitted to Eq. 1. k_{obs} values thus obtained were plotted against the concentration of unlabeled peptide and fitted to Eq. 3.

$$k_{obs} = k_{off} + k_{on}' (K_D / (K_D + n))$$

(Eq. 3)

where n is the concentration of unlabeled peptide, K_D is the equilibrium dissociation constant for the unlabeled peptide and the PDZ, and k_{on}' is the apparent (first order) on-rate constant for the PDZ and the dansylated peptide at the particular concentration of peptide in the experiment. At high n , the observed rate constant k_{obs} approaches the dissociation rate constant k_{off} , which is well determined in the curve fitting.

Eq. 4 was used to determine the change in binding free energy of PDZ-peptide interaction upon mutation, $\Delta\Delta G$, in Table 1.

$$\Delta\Delta G = RT \times \ln ((k_{off}^{WT} k_{on}^{mut}) / (k_{off}^{mut} k_{on}^{WT})) \quad (\text{Eq. 4})$$

R is the gas constant and T is the temperature in Kelvin.

Competition ligand binding experiments. To estimate K_D values of YKQTSV and KQTSV, competition ligand binding experiments were performed in the SX-20 MV stopped-flow spectrometer in 50 mM potassium phosphate pH 7.5 at 10°C. For the measurements, a complex formed by pre-incubating 2 or 3 μM protein (either PDZ3 or PDZ3 $\Delta\alpha 3$) with 4 or 6 μM **D**-YKQTSV peptide, respectively, was dissociated with different concentrations of the non-dansylated peptide (either YKQTSV or KQTSV). The resulting change in the dansyl fluorescence was monitored and plotted versus peptide concentration. Excitation was done at 280 nm (excitation of the Trp₃₃₇) while the emission was recorded using a 330 nm band-pass filter in the stopped flow. Data from the competition ligand binding were fitted to Eq. 5.¹⁸

$$F = [(K_{D1} + K_{D1}[L_2] / K_{D2} + [P]_t + [L_1]_t) / 2 - ((K_{D1} + K_{D1}[L_2] / K_{D2} + [P]_t + [L_1]_t)^2 / 4 - [P]_t[L_1]_t)^{0.5}] \times B + C \quad (\text{Eq. 5})$$

F is the observed fluorescence signal at equilibrium, K_{D1} and K_{D2} are the dissociation constants of L_1 and L_2 respectively. $[L_1]_t$ is the total concentration of the **D**-YKQTSV peptide that was held constant at 4 or 6 μM while L_2 is either YKQTSV or KQTSV peptide whose concentration was varied between 0-100 μM and 0-470 μM ,

respectively. $[P]_t$ is the total concentration of either PDZ3 or PDZ3 $\Delta\alpha 3$ held constant at 2 or 3 μM . B is the total fluorescence change and C is the end point. The K_{D1} value for the dansylated peptide for PDZ3 and PDZ3 $\Delta\alpha 3$, respectively, was calculated from the ratio of k_{off} and k_{on} (Table 1) and used in Eq. 5. The K_{D2} values for YKQTSV and KQTSV are reported in the legend to Fig. 6.

Isothermal titration calorimetry (ITC) experiments. ITC experiments were carried out in an ITC200 microcalorimeter with a 200 μl cell volume (GE healthcare). PDZ solution dialyzed in 50 mM potassium phosphate pH 7.5 was placed in the sample cell. Peptide stock was prepared in 50 mM potassium phosphate, pH 7.5 and aliquots of 1.5 μl (0.12-0.24 mM for YKQTSV and 0.24-1 mM for KQTSV) were injected into the cell containing either PDZ3 (8-13 μM) or PDZ3 $\Delta\alpha 3$ (15 μM) protein solution at 25°C. Once the titration was completed, the raw data were then fitted using the “one-binding-site model” of the MicroCal version of ORIGIN. Experiments were performed in duplicate or in triplicate (for the wild type and mutant proteins, respectively). As a control, the heat exchange of peptide dilution into buffer has been measured and taken into account in data analysis. In our experiments, although the baseline is a little noisy, as expected for a highly sensitive instrument, the reproducibility is very good as shown by the low experimental errors given in Table 2. Therefore, although the c value is slightly low,²² in particular for the mutant, the curve is not too shallow and is completed in a single experiment. The heat of binding (ΔH), the stoichiometry (n), and the dissociation constant K_D were then calculated from plots of the heat evolved per mole of ligand injected *versus* the molar ratio of ligand to protein using the software provided by the manufacturer.

RESULTS

First we performed NMR experiments to see which peptide ligand residues that interact with the protein. Next, in order to determine the contribution to the binding free energy of the $\alpha 3$ helix to PDZ3-peptide complex formation, we performed equilibrium and pre-equilibrium binding experiments with peptide ligand and wild type or mutant PDZ3.

At least eight peptide residues interact with PDZ3. In order to determine which residues in the peptide that likely interacts with PDZ3, we monitored chemical shift changes in ^1H - ^{15}N HSQC and ^1H - ^{13}C HSQC experiments of a fully labeled peptide (290 μM or 150 μM) with saturating amounts of PDZ3 (520 μM) and a variant where the $\alpha 3$ helix was removed, PDZ3 $\Delta\alpha 3$ (940 μM), respectively. The chemical shifts were assigned for free and bound peptide. In the free peptide eight of the resonances were observed at 277 K and six of the resonances were observed at 283 K (Fig. 2). The discrepancy is likely due to higher amide proton exchange rate with solvent at the higher temperature. In the bound form the backbone ^1H - ^{15}N resonances of eight residues (Fig. 2A) and the ^1H - ^{13}C resonances of the side chains of all residues (data not shown) were observed and assigned with the exception of CB of Thr₂ for peptide bound to PDZ3 $\Delta\alpha 3$. ^1H - ^{13}C and ^1H - ^{15}N combined chemical shift perturbations upon binding (CSP) were observed throughout the peptide except for Gly₉ both for the backbone and side chain residues showing that most of the peptide experiences significant changes in the environment on interaction with the protein (Fig. 3). However, our data cannot be used to conclusively state that all residues of the peptide

are interacting with the PDZ domain since CSPs for residues that do not interact can be the result of conformational changes of the peptide itself due to interaction with PDZ3 in another part of the peptide. It is however noteworthy that the CSPs, for residues Lys₋₇ to Gln₋₃, differ significantly for binding to PDZ3 and PDZ3 $\Delta\alpha$ 3. It is therefore tempting to speculate that these residues interact differently with the two forms of the PDZ domain and that the α 3 helix is involved in the binding to wild type PDZ3.

In the crystal structure (pdb code: 1BE9) of PDZ3 in complex with the peptide TKNYKQTSV the motif closest to the N-terminus of the peptide is the α 3, which is 5 Å away from the peptide residue Gln₋₃. No electron density was observed for the residues TKNYK in the N-terminus of the peptide.¹³ However, in the crystal structure of PDZ3 in complex with another peptide, KKETPV (pdb code: 1TP3) Lys₋₄ is about 4 Å from the side-chain His₃₇₂. In order to see if the combined chemical shifts observed were due to direct interaction with the protein or if they were merely due to titration perturbation (upon addition of PDZ3), we performed NOESY-HSQC and HCCH-TOCSY experiments of the peptide in complex with PDZ3 and PDZ3 $\Delta\alpha$ 3, respectively. By identifying peaks present in the NOESY-HSQC but absent in the HCCH-TOCSY spectra, we observed several NOEs (Fig. 4) between the respective protein and the peptide backbone and side chain residues from position 0 to -6.

To further rule out the possibility that NOEs identified as inter-molecular were in fact not intra-molecular, we carefully checked the NOESY spectra for candidates with matching chemical shifts and confirmed that corresponding peaks were absent (Fig.4, panel V). Importantly, the inter-molecular NOEs obtained with the wild-type PDZ3

were different from those with PDZ3 $\Delta\alpha$ 3, demonstrating that the peptide form distinct transient interactions in the two cases and suggesting that the α 3-helix may interact directly with the peptide ligand. To prove the latter hypothesis we would however need to perform the reverse experiment, i.e. to use isotopically labeled PDZ domain and determine which of its nuclei that interact with the peptide in NOESY experiments. Admittedly, the sensitivity is poor in all NOESY experiments due to low concentration and low temperature, especially experiments involving peptide bound to PDZ3 $\Delta\alpha$ 3. Thus, we likely observed too few rather than too many intermolecular contacts.

In a previous study where the influence of the α 3-helix of PDZ3 binding was investigated, the authors concluded that α 3 modulates PDZ3 binding through entropy driven dynamic allostery rather than direct interactions.¹⁴ They did not detect any significant chemical shift changes for peptide residues other than 0 to -3 using natural abundance ^1H - ^{13}C HSQC spectra recorded at 298 K (Fig. 6 of ref. ¹⁴.) Furthermore, the concentration dependence of the chemical shift changes of these nuclei in these residues suggests exchange in the fast regime. However, in the present work we used a fully ^{13}C - ^{15}N labeled peptide and saturating concentration of protein and recorded our data at 277 and 283 K using a slightly different form of the peptide. As noted during our NMR titrations, at 89 % saturation, most residues were in slow exchange and appeared as double peaks (14 in total) in the HSQC (Fig. 2B). Complete perturbation was observed only at 99 % saturation, which could explain the discrepancy regarding the number of peptide residues making direct contact to the protein (the previous data was for 15% molar ratio) (Fig. 2B). In addition, Petit et al. used a peptide with slightly different sequence in the N-terminus and including N-

acetylation, which was previously shown to reduce the affinity for peptide-PDZ3 interactions.¹⁵

Deletion of the $\alpha 3$ helix and Tyr.₅ both induce a favorable entropy of binding. To analyze the thermodynamics of the PDZ-peptide interaction and in particular the role of Tyr.₅, since this peptide residue may interact with $\alpha 3$, we performed ITC experiments for both PDZ3 and PDZ3 $\Delta\alpha 3$ with two peptides, KQTSV and YKQTSV (Fig. 5). The fitted parameters from three of these four separate experiments are summarized in Table 2. PDZ3 interacts with the peptide YKQTSV with a highly favorable enthalpy ($\Delta H = -10$ kcal/mol) and slightly unfavorable entropy ($-T\Delta S = 1.9$ kcal/mol). On deletion of the Tyr.₅ in the peptide, the enthalpy of binding was reduced to -4 kcal/mol and this was associated with a favorable entropic term ($-T\Delta S = -3.8$ kcal/mol). This result is similar to that when the $\alpha 3$ helix was deleted from PDZ3 ($\Delta H = -3.4$ kcal/mol and $-T\Delta S = -3.7$ kcal/mol). Unfortunately, we did not see any heat change when both the $\alpha 3$ helix and Tyr.₅ were deleted (data not shown). The likely reason for this is a low affinity between PDZ3 $\Delta\alpha 3$ and KQTSV, rather than a low enthalpic term, or possibly a combination of the two. Nonetheless, the short peptide was found to bind PDZ3 $\Delta\alpha 3$ in a competition assay (Fig. 6). We attempted to directly demonstrate an interaction between the $\alpha 3$ -helix and Tyr.₅ through a double mutant cycle (Fig. 6) but the error in K_D for the interaction between PDZ3 $\Delta\alpha 3$ and KQTSV was too large, due to the low affinity, which precluded a reliable quantitative analysis of the coupling energy.

Single point mutants in $\alpha 3$ destabilize the PDZ3-peptide complex. The results thus far suggested that residues Val₀ to Lys.₇ in the peptide are involved in complex

formation and that $\alpha 3$ in PDZ3 increases the affinity. We therefore tested which individual side-chain residues in the $\alpha 3$ helix that contribute to binary complex formation, by making the following point mutants in the $\alpha 3$ helix: Y397E (a phosphorylation mimic²³), and the deletion mutations R399A and F400A. The side-chains of these residues point towards the peptide binding groove and may interact with a bound peptide. The point mutants as well as the wild-type and PDZ3 $\Delta\alpha 3$ were subjected to presteady-state binding experiments (Fig. 7, Table 1). The k_{off} for the interaction between the mutants, Y397E, R399A and F400A, with dansylated peptide, **D**-YKQTSV increased by factors of 26, 4 and 30, respectively, showing that these residues contribute significantly to the binding of the dansylated peptide ligand (Table 1). It is therefore likely that these side-chains will contribute to binding of natural ligands, either through direct and transient interactions or indirectly by stabilizing the $\alpha 3$ helix.²⁴

DISCUSSION

The binding energy in the interaction between PDZ domains and their ligands comes mainly from hydrophobic interactions and hydrogen bonds in the canonical binding groove.^{13, 15} But, since the PDZ domain ligand is often very flexible, it has the possibility to wrap around the surface of the PDZ to make additional favorable interactions outside of the canonical binding groove. This binding groove is typically occupied by only four ligand residues, as defined by hydrogen bonds between backbones of the peptide and the second β -strand of the PDZ domain (see Fig. 1).

The contribution to the binding free energy of the PDZ3-peptide interaction by residues in the peptide ligand other than the four at the C-terminus was thoroughly investigated by Saro et al. using ITC.¹⁵ Their results showed that residues 0 to -5 (See Fig. 1) are important for PDZ3-peptide affinity, while further addition had minimal effect on the thermodynamics of binding. For example, the presence of Tyr.₅ was found to increase the affinity 10-fold at pH 6.0. Our data further confirm the importance of Tyr.₅ in the PDZ3-peptide interaction. However, we also showed that residues -4 to -8 show changes in chemical shift upon binding, although the net energetic contribution of residues -6 to -8 to the affinity is low. Nonetheless, it is clear from our data that the N-terminal regions of long C-termini of PDZ ligands may explore a large binding surface outside of the peptide-binding groove.

All residues in the vicinity of the binding groove may afford such an extra binding surface, but the α 3-helix in PDZ3 is particularly interesting in this respect since it is a so-called non-canonical structural element^{25, 26} only found in some PDZ domains and for example not in the other two PDZ domains of PSD-95. Our ITC data suggest that the α 3-helix in PDZ3 contributes a binding free energy for the CRIPT ligand of approximately 1.1 kcal mol⁻¹, and the fluorescence based binding data give free energies of 2.4 kcal/mol (dansylated peptide) and 2.2 kcal/mol (Fig. 6, large error), in qualitative agreement with published data on a longer peptide (1.8 kcal/mol).¹⁴ Moreover, we detected inter-molecular NOEs between the PDZ3 and Tyr.₅ of the peptide. The absence of these inter-molecular NOEs in experiments with labeled peptide and PDZ3 $\Delta\alpha$ 3, indicates that the α 3-helix may in fact be involved in direct short-lived interactions with peptide ligand side-chains although it remains to be proved.

The point mutations in $\alpha 3$ further demonstrate the importance of this helix. A recent computational study by Mostarda et al.²⁴ shed light on the destabilizing effects of the mutations. They found by molecular dynamics simulations that Tyr₃₉₇ and Phe₄₀₀ make stabilizing hydrophobic interactions with each other and with residues in PDZ3, Arg₃₉₉ makes a transient salt bridge with Glu₃₃₄, which in turn stabilizes the $\beta 2$ - $\beta 3$ loop, and Glu₄₀₁ interacts with Lys₃₅₅. Thus, mutation of these residues (as well as the complete removal of $\alpha 3$) might influence binding indirectly by destabilization of a pre-organized binding site for the peptide. But, they also observed transient salt bridges between Glu₃₃₁ and Asp₃₃₂ in PDZ3 and Lys₋₄ and Lys₋₇ in the CRIPT peptide, along with unstable hydrophobic contacts between the peptide and $\alpha 3$. The E331A mutation indeed results in both a decreased k_{on} and an increased k_{off} .⁹ Thus, simulation²⁴ and experiment conspire to reveal a protein-ligand interaction for PDZ3 governed by (i) stable interactions ($t_{1/2} \sim 1/k_{off}$) in the canonical binding site^{9, 13, 15}, (ii) transient ($t_{1/2} \ll 1/k_{off}$) but direct interactions outside of this binding site involving for example Glu₃₃₁ and most likely portions of $\alpha 3$, and (iii) long-range interactions.^{9, 14, 17}

The additional C-terminal extension is not unique for PDZ3.^{25, 26} In MAGI PDZ1 for example, an extended C-terminal loop makes direct interactions with Arg₋₄, Arg₋₅ and Thr₋₆ of the peptide ligand and mutation in this C-terminal loop decreases the binding affinity drastically.²⁷ Thus, from previous work^{11, 12, 14, 24, 27} together with the present data we conclude that some PDZ domains contain certain structural elements at their C-terminus, which have a direct effect on binding affinity.

An appealing model is that such binding surfaces in PDZ domains not only increase affinity for the ligand but also the specificity. For example, the peptide residues at positions -6 to -8 of CRIPT appears not have a net positive energetic effect on the binding of PDZ3¹⁵, but they might well have a negative effect on binding to other PDZ domains. In the case of PDZ3 from PSD-95, the α 3-helix is likely to provide part of an extended binding surface and in this way increase the specificity toward its natural ligand, CRIPT.

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Tables

Table 1: Binding constants for the interaction between dansylated peptide, **D**-YKQTSV and wild-type and mutant PDZ3. k_{on} and k_{off} values were obtained from fitting Eqs. 2 and 3, respectively, to the k_{obs} values from pre-equilibrium ligand binding stopped-flow measurements. Errors in k_{on} and k_{off} are fitting errors, while the other errors are propagated fitting errors.

PDZ	k_{off} (s^{-1})	k_{on} (μMs^{-1})	K_{D} ($k_{\text{off}}/k_{\text{on}}$) (μM)	$\Delta\Delta G$ (kcal/mol)
PDZ3	1.6 ± 0.20	11 ± 0.20	0.15 ± 0.02	
PDZ3 $\Delta\alpha 3$	150 ± 40	13 ± 3	11 ± 4	-2.4 ± 0.4
Y397E	40 ± 2	8.0 ± 0.3	5 ± 0.3	-2.0 ± 0.15
R399A	6.0 ± 0.3	10 ± 1	0.6 ± 0.07	-0.8 ± 0.2
F400A	50 ± 1.0	19 ± 3	2.7 ± 0.4	-1.6 ± 0.2

Table 2: ITC binding data for PDZ-peptide interactions. n.d., not detectable.

PDZ	YKQTSV				KQTSV			
	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	K_{D} (μM)	n	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	K_{D} (μM)	n
PDZ3	-10 ± 1	1.9 ± 0.2	1 ± 0.1	0.8 ± 0.1	-4.0 ± 0.1	-3.8 ± 0.4	2.0 ± 0.3	1 ± 0.1
PDZ3 $\Delta \alpha 3$	-3.4 ± 0.6	-3.7 ± 0.6	6 ± 0.2	0.9 ± 0.1	n.d.	n.d.	n.d.	n.d.

Figure legends

Figure 1. Structure of PDZ3 in complex with a peptide ligand, TKNYKQTSV.

PDZ3 with CRIPT peptide adapted from ref.²⁴ The helix $\alpha 3$ and the Tyr₃₉₇ side chain are shown in blue. The peptide residues are colored purple with the amide nitrogens in blue. The numbering of peptide ligand residues is according to the usual convention, starting from 0 at the C-terminus and then -1, -2 etc. This is one of several structures from an MD simulation²⁴ and not a representative average structure. However, it suggests that both Tyr₃₉₇ and Phe₄₀₀ may form transient interactions with Tyr₋₅ of the peptide ligand. The figure was drawn in PyMOL (The PyMOL Molecular Graphics System, DeLano Scientific, LLC).

Figure 2. ¹H-¹⁵N HSQC spectra of the peptide GSKNYKQTSV in complex with

PDZ3 and PDZ3 $\Delta\alpha 3$. A) The apo-peptide is shown in black, the peptide in complex with PDZ3 is shown in blue (>99 % saturation) and the peptide in complex with PDZ3 $\Delta\alpha 3$ is shown in red (>98 % saturation). The nomenclature of the residues is as follows: a_V0 is the C-terminal valine in the apo-peptide, d_V0 is the C-terminal valine in the peptide in complex with PDZ3 $\Delta\alpha 3$, and V0 the C-terminal valine in the peptide in complex with PDZ3. B) HSQC spectrum of peptide at 0 % (black), 89 % (green) and >99% (blue) saturation of PDZ3. Note that apo-peptide spectrum in A) was recorded at 277 K whereas the one in B) was recorded at 283 K, hence the slightly different appearance. As expected more peaks are present at the lower temperature.

Figure 3. Chemical shift differences upon peptide binding. $^{13}\text{C}/^1\text{H}$ chemical shift perturbation for peptide side-chains upon binding to either PDZ3 (blue) or with PDZ3 $\Delta\alpha 3$ (red), respectively. The inset shows the $^{15}\text{N}/^1\text{H}$ chemical shift difference of the backbone amides upon binding to PDZ3 (blue) or PDZ3 $\Delta\alpha 3$ (red), respectively. The chemical shift perturbations were calculated as $(\Delta\varpi_{\text{H}}^2 + (\Delta\varpi_{\text{C}} \times 0.25)^2)^{0.5}$ and $(\Delta\varpi_{\text{H}}^2 + (\Delta\varpi_{\text{N}} \times 0.1)^2)^{0.5}$, respectively. Assignments of the type HB1 and HB2 do not indicate stereospecific assignments but is merely a way of labeling protons with distinct chemical shifts that are bound to the same carbon.

Fig. 4. NOESY-HSQC spectra of Lys₄ and Tyr₅. Intra and inter-molecular NOEs involving HE of Tyr₅(I-II) and HG2 of Val₀ (III-IV) of the peptide (290 μM or 150 μM) saturated with PDZ3 (I, III) or PDZ3 $\Delta\alpha 3$ (II, IV), respectively, as well as HA of Lys₄ of peptide in complex with PDZ3 (V). For clarity, the peaks in panels showing peptide in complex with PDZ3 are colored blue and those for PDZ3 $\Delta\alpha 3$ are colored red. Assignments of intra-molecular NOEs are shown and unassigned inter-molecular NOEs to the PDZ domain are highlighted with an arrow. Absence of putative intra-molecular NOEs are indicated with a cross. Note the disappearance of certain NOEs when helix $\alpha 3$ is deleted (I vs. II and III vs. IV, respectively).

Figure 5. Thermodynamics of the PDZ-peptide interaction. Isothermal titration calorimetric experiments of A) PDZ3 with YKQTSV and B) PDZ3 with KQTSV, respectively. Upper panels: Raw ITC data. Lower panels: Integrated peak areas (black squares) and fit with the one-binding-site model of ORIGIN provided by MicroCal (continuous line). The concentrations used in these experiments were: A) 8 μM PDZ3 with 0.12 mM YKQTSV and B) 13 μM PDZ3 with 0.18 mM KQTSV. C) Plot of the

ΔG (black bars), ΔH (white bars) and $-T\Delta S$ (grey bars) derived from the data and also reported in Table 2.

Fig. 6. Equilibrium displacement experiments. A complex of **D**-YKQTSV (3 μM) and either A) PDZ3 or B) PDZ3 $\Delta\alpha 3$ was dissociated with YKQTSV or KQTSV in separate experiments. If the affinity for **D**-YKQTSV (K_{D1}) is known the affinity for the unlabeled peptides (K_{D2}) can be estimated using Eq. 5 as described in Materials. K_{D2} values were 1.0 ± 0.3 μM and 4.3 ± 0.3 μM for PDZ3 with YKQTSV and KQTSV, respectively. However, the low affinity of PDZ3 $\Delta\alpha 3$ for both YKQTSV (43 ± 36 μM) and in particular KQTSV (360 ± 320 μM) yielded an error in the analysis that is on the same order as the value, precluding a quantitative analysis of the interaction and the calculation of a reliable coupling free energy between $\alpha 3$ and Tyr₅. These experiments were performed in the stopped-flow fluorimeter and normalized kinetic endpoints are plotted on the y-axes.

Figure 7. Pre-equilibrium binding data for PDZ3 and mutants. A) Observed rate constants for the interaction of PDZ and peptide plotted against increasing concentration of the dansylated (**D**) peptide **D**-YKQTSV. B) Observed rate constants for the displacement of the PDZ3/**D**-YKQTSV complex plotted against increasing concentration YKQTSV. The rate constant at the high concentration of unlabeled peptide is equal to the off-rate constant in the interaction of PDZ3 and **D**-YKQTSV.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

A **Fig. 6****B**

Fig. 7

TOC graphics