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Structural and Thermodynamic Analysis of PDZ–Ligand Interactions

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Abstract

Tiam-family guanine exchange proteins are activators of the Rho GTPase Rac1 and critical for cell morphology, adhesion, migration, and polarity. These modular proteins contain a variety of signaling domains, including a single postsynaptic density-95/discs large/zonula occludens-1 (PDZ) domain. Here, we show how structural and thermodynamic approaches applied to the Tiam1 PDZ domain can be used to gain unique insights into the affinity and specificity of PDZ–ligand interactions with peptides derived from Syndecan1 and Caspr4 proteins. First, we describe a fluorescence anisotropy-based assay that can be used to determine PDZ–ligand interactions, and describe important considerations in designing binding experiments. Second, we used site-specific mutagenesis in combination with double-mutant cycle analysis to probe the binding energetics and cooperativity of residues in two ligand binding pockets (S_0 and S_{-2}) that are involved in Tiam1 PDZ–ligand interactions. Peptide ligand binding results and double-mutant cycle analysis revealed that the S_0 pocket was important for Syndecan1 and Caspr4 peptide interactions and that the S_{-2} pocket provided selectivity for the Syndecan1 ligand. Finally, we devised a “peptide evolution” strategy whereby a Model consensus peptide was “evolved” into either the Syndecan1 or Caspr4 peptide by site-directed mutagenesis. These results corroborated the PDZ mutational analysis of the S_0 pocket and identified the P_{-4} position in the ligand as critical for Syndecan1 affinity and selectivity. Together, these studies show that a combined structural and thermodynamic approach is powerful for obtaining insights into the origin of Tiam1 PDZ–ligand domain affinity and specificity.

1. Introduction

A chief goal in the field of biochemistry is to quantitatively describe protein–protein and protein–ligand interactions, and to determine how the physical parameters that describe these interactions relate to biological function. A common method for obtaining this information is by using a reductionist approach, whereby a large protein is divided into its individual domains, and the function of each is investigated separately. Subsequent studies in the context of the full-length protein can then be used to infer regulatory mechanisms and domain–domain interactions. Eukaryotic signaling proteins are often composed of multiple domains and hence are amenable to this approach. Here, we describe the application of this approach to the guanine nucleotide exchange factor (GEF) protein, T-cell lymphoma invasion and metastasis-1 (Tiam1), to gain insight into the affinity and specificity of its

postsynaptic density-95/discs large/zonula occludens-1 (PDZ) domain (Iden and Collard, 2008; Mertens *et al.*, 2006).

Tiam1 and its homolog Tiam2 are GEF proteins that specifically activate the Rho-family GTPase Rac1 (Mertens *et al.*, 2003). Because Rac1 activation is tightly regulated, GEF proteins themselves must be spatially and temporally controlled. This control occurs primarily through chemical signals such as phosphorylation and protein–protein interactions. Tiam1 and Tiam2 possess similar domain architecture and cellular function (Matsuo *et al.*, 2002, 2003). Both contain a Pleckstrin homology-coiled coil-extra (PH_n-CC-Ex) region, followed by a Ras-binding domain (RBD), a PDZ domain, and a Dbl homology–Pleckstrin homology (DH–PH_c) catalytic domain cassette. While most interaction domains in Tiam1 and Tiam2 have high sequence identity, the PDZ domains share only ~28% identity, suggesting that there might be functionally important differences in their specificities (Hoshino *et al.*, 1999).

PDZ domains are small protein–protein interaction domains of ~90 amino acids in length. They fold into a compact β -barrel structure formed by six β -strands and two α -helices and typically bind the 5–10 extreme C-terminal residues of their interaction partner by forming a β -sheet contiguous with the β 2 strand of the PDZ domain (Fig. 4.1; Shepherd *et al.*, 2010). Individual pockets within the PDZ domain are used to accommodate the side chains of ligand residues and are the source of the exquisite specificity of PDZ–ligand interactions. This specificity lends itself to the scaffolding and signaling function of PDZ domains, which are abundant in eukaryotes and prokaryotes (Ponting *et al.*, 1997). For example, the human genome encodes an estimated ~214 PDZ-containing proteins, which collectively harbor ~440 PDZ domains (Schultz *et al.*, 2000; SMART database, June 2010). To date, the structures of nearly 200 PDZ domains have been solved, either alone or in complex with a ligand (reviewed by Lee and Zheng, 2010). In addition, several studies investigating the specificity of PDZ–ligand interactions have uncovered the general rules that define PDZ–ligand specificity (Chen *et al.*, 2008; Songyang *et al.*, 1997; Stiffler *et al.*, 2007; Tonikian *et al.*, 2008). Despite this knowledge, gaps in our understanding of the thermodynamics of PDZ–ligand interactions remain. Here, we highlight structural and thermodynamic methods for studying PDZ–ligand affinity and specificity using the Tiam1 PDZ domain as a model system.

2. Structural Studies of the Tiam1 PDZ Domain

Several studies have identified peptides that bind the Tiam1 PDZ domain. Early studies by Songyang *et al.* (1997) identified a synthetic peptide capable of binding the Tiam1 PDZ domain. This peptide ligand (SSRKEYYA_{COOH}, henceforth referred to as the Model peptide) was shown to bind the Tiam1 PDZ domain with low affinity ($K_d \sim 112 \mu\text{M}$). More recently, the cell–cell and cell–matrix adhesion receptors Syndecan1 and CADM1 were identified as physiological Tiam1 PDZ domain binding proteins (Masuda *et al.*, 2010; Shepherd *et al.*, 2010). In addition, bioinformatic analysis identified several putative Tiam-family PDZ-binding proteins, including the neuronal cell–cell receptor proteins Contactin-associated protein-like4 (Caspr4) and Neurexin1 (Shepherd, Hard, Murray, Pei and Fuentes, unpublished data). The C-terminal sequences of these proteins are similar but contain

differences at key positions that result in distinct PDZ-binding properties. For instance, the Syndecan1 (TKQEEFYA_{COOH}) and Caspr4 (ENQKEYFF_{COOH}) peptides bound the Tiam1 PDZ domain, but the Neurexin1 (NKDKEYYV_{COOH}) peptide did not. Subsequent studies with the Tiam2 PDZ domain indicated a different pattern of specificity, where the Caspr4 and Neurexin1 peptides bound the PDZ domain tightly, but the Syndecan1 peptide did not (Shepherd, Hard, Murray, Pei and Fuentes, unpublished data).

We determined the crystal structure of the Tiam1 PDZ domain bound to the Model peptide ligand to gain insight into the molecular details of Tiam-family PDZ/ligand specificity. In particular, two specificity pockets, S_0 and S_{-2} , were identified (Fig. 4.1A). The side chains of residues Y858, F860, L915, and L920 form the S_0 pocket that accommodates the C-terminal residue of the ligand. This pocket is relatively shallow, partly explaining the preference for an Ala residue at the P_0 position of the ligand (where P_0 is the most C-terminal residue and P_{-n} denotes the residue position n amino acids from the C-terminus). The structure also showed how the S_{-2} pocket can accommodate a large hydrophobic side chain in the ligand at P_{-2} and implicated residues L911 and K912 as determinants of ligand specificity (Fig. 4.1).

To understand how the Tiam1 PDZ domain achieves specificity for several distinct peptide ligands, we performed NMR-based titration experiments (Shepherd *et al.*, 2010). NMR spectroscopy is a powerful technique because it can provide site-specific information regarding the structure, dynamics, and energetics of protein–ligand interactions. In the titration experiments, we added either the Caspr4 or Syndecan1 peptide to a sample containing the U-¹⁵N-labeled PDZ domain of Tiam1 and then acquired a ¹H-¹⁵N HSQC spectrum at each ligand concentration. Because the position, or chemical shift, of each peak within the HSQC spectrum reflects the chemical environment surrounding that amide group, it is possible to determine which residues participate in the binding reaction by monitoring the changes in the chemical shift of individual cross-peaks as a function of peptide ligand. Figure 4.2 summarizes the titration data for Caspr4 and Syndecan1 peptides, highlighting differences in chemical shifts between these two PDZ–ligand complexes (Shepherd *et al.*, 2010). Figure 4.2A shows the chemical shift changes of each complex mapped onto the surface of the Tiam1 PDZ/Model structure. It is clear that both ligands recognize the same binding cleft. However, the data also identify significant differences in $\alpha 2$ and $\beta 6$ that likely reflect interactions responsible for binding specificity. In particular, L911, K912, L915, and L920 in the S_0 and S_{-2} pockets were identified as candidate residues that might determine the specificity of the Tiam1 PDZ–ligand interaction (Figs. 4.1 and 4.2). Interestingly, none of these four residues are conserved between the Tiam1 and Tiam2 PDZ domains (Fig. 4.2C), consistent with the notion that these two homologous PDZ domains may have distinct specificities. Here, we focus on the Tiam1 PDZ domain and show how thermodynamic analyses can be used to assess the role of PDZ and ligand residues hypothesized to be important in PDZ–ligand interactions.

3. Fluorescence Anisotropy Methods for Measuring the Energetics of PDZ–Ligand Interactions

Several techniques are available for determining the energetics of protein–protein and protein–ligand interactions, including isothermal titration calorimetry, surface plasmon resonance, NMR, and fluorescence-based methods. Each technique has its relative strengths and weaknesses and can potentially contribute unique information. Fluorescence-based methods rely on measuring the change in fluorescence intensity or anisotropy that occurs upon ligand binding. This method is sensitive, can be carried out relatively quickly, requires only modest amounts of protein, and is highly reproducible. In this section, we describe a fluorescence anisotropy binding assay for studying PDZ–ligand interactions.

3.1. Theoretical background

Fluorescence anisotropy has been extensively reviewed elsewhere (Eccleston *et al.*, 2005; Eftink, 1997; Lakowicz, 1999; Royer and Scarlata, 2008); here we provide a basic overview to introduce key aspects that are important for experimental design. Fluorescence anisotropy relies on selectively exciting a subpopulation of fluorophores with polarized light and monitoring polarized emission. Because a fluorophore has a defined excited-state lifetime that is on a timescale (ns) similar to that which molecules tumble (i.e., experience rotational diffusion), the polarity of the emitted light is sensitive to molecular size. This property allows one to monitor a protein/ligand binding reaction based on accompanying changes in polarization (or anisotropy).

The fundamental equation describing fluorescence anisotropy (r) is

$$r = \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + 2I_{\perp})} = \frac{I_{\parallel}/I_{\perp} - 1}{I_{\parallel}/I_{\perp} + 2} \quad (4.1)$$

where $I_{\parallel}$ is the intensity of the detected light when the excitation and emission polarization is parallel and $I_{\perp}$ is the intensity of detected light when the excitation and emission polarization is perpendicular. Thus, fluorescence anisotropy is the ratio of the difference in intensity between the emitted parallel and perpendicular polarized light ($I_{\parallel} - I_{\perp}$) to the total intensity of polarized light emitted by the sample ($I_{\parallel} + 2I_{\perp}$) (Lakowicz, 1999), and is consequently dimensionless and independent of the concentration of the fluorophore. In the experimental setting, we use a laboratory reference frame, where I_{VV} is defined as the intensity of light when both the excitation and emission polarizers are mounted vertically, and I_{VH} is the intensity of light when the excitation polarizer is mounted vertically and the emission polarizer is mounted horizontally. Because the experimental sensitivity in these channels may not be identical, a correction factor G (or G -factor) is introduced

$$\frac{I_{VV}}{I_{VH}} = G \frac{I_{\parallel}}{I_{\perp}} \quad (4.2)$$

Equation (4.2) alone does not uniquely determine the G -factor because of its dependence on the intensity of $I_{\parallel}$ and $I_{\perp}$; therefore, we must rely on an alternate experimental configuration. With horizontally polarized light, both the excitation and emission components of the excited-state distribution are equal and proportional to $I_{\perp}$ (Lakowicz, 1999). Thus, measured changes between horizontally (I_{HH}) and vertically (I_{HV}) polarized light in the emission path can be used to experimentally determine the G -factor as defined by

$$\frac{I_{HV}}{I_{HH}} = G \frac{I_{\perp}}{I_{\parallel}} = G \quad (4.3)$$

Combining Eqs. (4.1) and (4.2) yields the fluorescence anisotropy in measurable quantities

$$r = \frac{(I_{VV}/I_{VH})G - 1}{(I_{VV}/I_{VH})G + 2} = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (4.4)$$

Furthermore, the Perrin equation (Eq. 4.5) (Perrin, 1926) relates fluorescence anisotropy to the rotational correlation time (τ_c) of the labeled molecule and the fluorescence lifetime of the fluorophore (τ)

$$r = \frac{r_0}{\left(1 + \frac{\tau}{\tau_c}\right)} \quad (4.5)$$

where r_0 is the upper limit of anisotropy when the fluorophore is “frozen in” (i.e., no motion during the excited-state lifetime). In the approximation of a spherical molecule, the rotational correlation time (τ_c) is related to the molecular weight (M) of the molecule of interest according to

$$\tau_c = \frac{\eta M \bar{v}_h}{RT} \quad (4.6)$$

where η is the solution viscosity, $\bar{v}_h$ the specific volume of a hydrated protein, R the gas constant, and T is the absolute temperature. By combining and rearranging Eqs. (4.5) and (4.6), we have

$$r = \frac{r_0 \eta M \bar{v}_h}{\eta M \bar{v}_h + \tau RT} \quad (4.7)$$

This equation shows that anisotropy increases hyperbolically with molecular mass of the fluorophore-bound molecule (Fig. 4.3; Lakowicz, 1999). Hence, a binding reaction that has a reasonable change in mass (or τ_c) between the free and bound states can be detected by changes in anisotropy. Equation (4.7) is dependent on viscosity and temperature; therefore, these parameters should be controlled during the titration experiment. Figure 4.3 also shows simulated curves for fluorescence lifetimes of 4 and 13 ns that indicate that relatively small changes in the fluorophore lifetime can significantly affect the absolute anisotropy up to a

value of ~0.1 (Fig. 4.3, inset). Moreover, these simulations indicate that analyzing PDZ–ligand interactions (changes in molecular weight from ~1 to 12 kDa upon peptide binding) requires a fluorophore with a fluorescence lifetime (τ) on the order of ~1–4 ns to obtain a significant change in anisotropy between the free and bound state. A few assumptions are implicit in Eq. (4.7) as applied to protein–ligand interactions. First, it is assumed that the fluorescence lifetime or quantum yield does not appreciably change upon binding ligand. Second, it is assumed that the fluorophore does not tumble independently of the ligand to which it is bound. If these conditions are met, it is possible to use changes in anisotropy to monitor the fraction bound of a protein–ligand complex.

In a typical experiment, the solution of fluorophore-labeled molecule is contained within a quartz cuvette. The G -factor is determined using Eq. (4.3), by exciting the sample with horizontally polarized light and detecting the intensity of the polarized light that is emitted horizontally (I_{HH}) and vertically (I_{HV}). The nonfluorescing titrant (the PDZ domain in the present case) is then added to the analyte (peptide) solution, and the entire sample is exposed to vertically polarized excitation light, while horizontally and vertically emitted light is detected at each titration step. The anisotropy at each titration step is then calculated using Eq. (4.4). Plotting the data as the concentration of the titrant versus the change in fluorescence anisotropy provides a binding curve that can be fit to a hyperbolic binding model (Eq. 4.8)

$$r - r_i = \frac{(r_{\max} - r_i)[\text{PDZ}]}{K_d + [\text{PDZ}]} \quad (4.8)$$

where r is the anisotropy at each titration step, r_i the initial anisotropy of analyte alone, $r_{\max}$ the maximum anisotropy when the binding is saturated, K_d the dissociation constant, and $[\text{PDZ}]$ is the total concentration of the PDZ domain protein in the analyte solution.

In the studies described here, nonlinear regression analysis was used to fit the fluorescence anisotropy data. Using the known quantities $(r - r_i)$ and $[\text{PDZ}]$, $(r_{\max} - r_i)$ and K_d were fit to Eq. (4.8) (Sigma Plot; SPPS Inc.). Equation (4.8) is valid only if several conditions are met. First, a 1:1 stoichiometry is assumed, and in this case consistent with the crystal structure of the Tiam1 PDZ/Model complex (Shepherd *et al.*, 2010). Second, we assume that the concentration of free PDZ domain is on the order of the concentration of the total PDZ domain. Finally, significant changes in the lifetime or intensity of the fluorophore in the bound state require that a correction factor be applied (Eftink, 2000; Mocz *et al.*, 1998). In our studies, we did not see a significant change in fluorescence intensity (<10%) upon PDZ binding and no corrections were applied.

3.2. Experimental design considerations

To maximize the change in mass and hence the anisotropy upon binding, we chose to fluorescently label the peptide and titrate this species with the larger PDZ domain. Additional considerations for the design of fluorescence anisotropy-based binding assays are also required. First, one must select the fluorophore to be used. While measuring intrinsic fluorescence using tryptophan or tyrosine may be an option, their relatively low quantum

yield and scarcity within typical PDZ ligands may be limiting. Several extrinsic fluorophores—such as dansyl, rhodamine, and fluorescein—are useful because of their short lifetimes (1–4 ns). Other fluorophores can be considered, but their quantum yield and fluorescence lifetime should be used to guide the choice for particular applications (Owicki, 2000). The characteristic excitation and emission wavelengths of each fluorophore may also influence this decision, because the individual capability of fluorimeters is variable. Other parameters of the fluorophore, such as solubility, as well as sensitivity to changes in pH, salt concentration, and photo bleaching, should also be considered. Another consideration is the position of the fluorophore linkage to the peptide. In PDZ–ligand studies, peptide labeling is restricted to the N-terminus because of the necessity for an unmodified carboxyl terminus in PDZ–ligand interactions.

The second major experimental consideration is the source of the peptide ligand. One possibility is to use recombinant methods to express the peptide using any of the myriad of available biological overexpression systems. However, this can be laborious and inefficient. The convenience and availability of custom peptide synthesis makes it a highly cost-effective alternative for obtaining small peptides, and provides the opportunity to chemically link an extrinsic fluorescent chromophore.

A third experimental design consideration is the length of the peptide. With recent reports of PDZ–ligand interactions involving up to P_{-10} in the ligand (Feng *et al.*, 2008; Tyler *et al.*, 2010), it may be necessary to test several peptides of differing length to optimize ligand design. Too short of a peptide may result in strong fluorophore–PDZ interactions and too long of a peptide can result in a fluorophore that tumbles independently of the peptide, artificially lowering the experimental anisotropy.

For our studies, we chose to have all peptides commercially synthesized as 8-residue peptides modified at their N-termini with the dansyl fluorophore. The choice of an 8-residue peptide was guided by the crystal structure of the Tiam1 PDZ/Model peptide, which showed no interactions beyond position P_{-5} (Shepherd *et al.*, 2010). The choice of the dansyl fluorophore was made in part because this moiety could be readily attached to the amino-termini of the synthetic peptides. Moreover, this fluorophore is pH-independent from pH 5–8 and has a reasonably short excited-state fluorescence lifetime (~4 ns) in aqueous solutions (Hoenes *et al.*, 1986). Finally, the relatively small size of the dansyl fluorophore minimizes the potential that it will interact with the PDZ domain.

3.3. Experimental procedures

3.3.1. Synthetic peptides—To minimize any contaminating source of background fluorescence, we used reagents with the highest optical purity available. Peptides were commercially synthesized, labeled with an N-terminal dansyl moiety, and judged 95% pure by analytical HPLC (GenScript USA, Inc.). The identity of each peptide was confirmed by mass spectrometry (GenScript USA, Inc.). Stock peptide solutions were made by resuspending lyophilized peptides in binding buffer (20 mM PO_4 , 50 mM NaCl, and 0.5 mM EDTA at pH 6.8) at a concentration of 1 mM. Special attention was paid to adjusting the pH of the peptide solution because trace acid may remain from the original HPLC purification. In our studies, the buffer was chosen to match the buffer system used in the NMR studies.

However, in general, care should be taken in the choice of buffer to ensure that precipitation of both the protein and peptide is minimized and that the fluorophore is not quenched by buffer components. Finally, all buffers should be filtered and thoroughly degassed to minimize light scattering from aggregates and/or gas (bubbles), which interfere with anisotropy measurements. The concentration of each dansylated peptide was calculated based on A_{280} measurements and the molar extinction coefficient of the peptide, which was estimated by summing the contributions of the dansyl ($1596 \text{ M}^{-1} \text{ cm}^{-1}$) moiety and peptide at this wavelength. Concentrated stock peptides and working dilutions were stored in light-resistant tubes to avoid photo bleaching and were frozen at -20 or -80 °C for long-term storage.

3.3.2. Protein expression and purification—The Tiam1 PDZ domain expression plasmid was a modified pET21a (Novagen) vector that contained an N-terminal 6× histidine tag and tobacco etch virus (rTEV) protease cleavage site (Shepherd *et al.*, 2010). All protein expression was conducted in BL21(DE3) (Invitrogen) *Escherichia coli* cells. *E. coli* cells were grown at 37 °C in Luria–Bertani medium supplemented with ampicillin (100 µg/mL) under vigorous agitation until an A_{600} of ~0.6–1.0 was reached. Cultures were cooled to 25 °C and protein expression was induced by the addition of isopropyl 1-thio-D-galactopyranoside to a final concentration of 1 mM. Induced cells were incubated for an additional 6–8 h at 25 °C and harvested by centrifugation.

The histidine-tagged Tiam1 PDZ domain was purified by nickel-chelate (GE-Healthcare) and size-exclusion chromatography (G-50 or S-75). The rTEV protease was used to remove the N-terminal His₆ affinity tag. The digested PDZ domain was isolated from undigested fusion protein, cleaved His₆ tag, and histidine-tagged rTEV by nickel-chelate chromatography by collecting the flow through fractions. The concentration of all PDZ proteins was calculated based on the predicted extinction coefficient determined using the program SEDNTERP (v1.09). The final yield of pure PDZ protein was ~20 mg/L of culture. The protein purity was ~95% as judged by SDS-PAGE. Samples were used immediately or stored at -20 °C.

3.3.3. Equilibrium fluorescence binding assays—All binding experiments were conducted in 1.3 mL of binding buffer containing peptide at a concentration of either 2 or 5 µM. Measurements were made in a 2-mL quartz cuvette that was stirred and maintained at constant temperature (25 °C). The anisotropy measurements were recorded on a Fluorolog-3 (Horiba Jobin Yvon, NJ) spectrofluorimeter with polarizers in excitation and emission channels. The maximum excitation and emission wavelength for each dansylated peptide was determined by obtaining a preliminary fluorescence spectrum. The individual peptides had nearly identical excitation and emission wavelengths ($\lambda_{\text{ex}} = 340$ and $\lambda_{\text{em}} = 555$ nm) and these were used for all fluorescence measurements. The slit widths for the control of excitation and emission intensity were adjusted to optimize the signal-to-noise ratio and maximum intensity and set to 3 and 8 nm, respectively. To allow convenient and reliable delivery of at least 3 µL of solution per titration step, 1:100 and 1:10 dilutions of the stock PDZ protein solution (~1 mM) were prepared in binding buffer. For each experiment, 20–30 individual titration steps were performed until the sample had little or no change in

anisotropy. The change in fluorescence anisotropy was plotted against protein concentration and fit to a standard hyperbolic ligand-binding curve (Eq. 4.8). Each titration was carried out in triplicate, and the reported values in Table 4.1 are the average K_d and standard deviation of the mean. The change in free energy (ΔG_b) of the PDZ–ligand binding was calculated from the dissociation constant and the error in free energy was obtained by error propagation.

4. Double-Mutant Cycle Analysis of PDZ-Binding Pockets

The use of double-mutant cycles was pioneered by Fersht and colleagues (Carter *et al.*, 1984; Horovitz and Fersht, 1990) and has been applied to many proteins, including PDZ domains (Saro *et al.*, 2007). This technique measures the coupling between two distinct perturbations in a system by comparing the thermodynamics of each perturbation individually and then together. In proteins, the most common perturbation is a site-specific mutation and the monitored thermodynamic process is generally protein folding or ligand binding (Fersht, 1998). In studying PDZ–ligand binding events, we begin by measuring the free energy of binding for the wild-type protein (G_{WT}), two single mutants (G_{M1} , G_{M2}), and a double mutant (G_{DM}) that combines the mutations present in the two single mutants. Cooperativity or coupling is assessed by comparing the sum of the binding free energies of the single mutants (G_{M1} and G_{M2}) to those of the wild-type and double mutant (G_{WT} and G_{DM}), or equivalently by evaluating two legs of a thermodynamic box (Eq. (4.9)).

$$\begin{aligned}\Delta\Delta\Delta G_{\text{int}} &= \Delta\Delta G_{WT-M1} - \Delta\Delta G_{M2-DM} \\ &= (\Delta G_{WT} + \Delta G_{DM}) - (\Delta G_{M1} + \Delta G_{M2})\end{aligned}\quad (4.9)$$

Thus, if the difference in the binding free energies between the first mutant and the wild-type ($G_{WT-M1} = G_{WT} - G_{M1}$) is equivalent to the difference between the second mutant and the double mutant ($G_{M2-DM} = G_{M2} - G_{DM}$), then the energy of interaction (G_{int}) is additive and no coupling is observed. However, if G_{int} is nonzero, then the two mutations are energetically coupled (or cooperative), and together they influence ligand binding to a greater extent than either one alone. The sign of the coupling is important and indicates whether the cooperativity enhances ($G_{\text{int}} < 0$) or reduces ($G_{\text{int}} > 0$) binding compared to that of the single mutants.

Double-mutant cycle analysis was applied to the Tiam1 PDZ domain to gain insight into the energetics of ligand binding and PDZ specificity. Specifically, we were interested in determining whether residues within the S_0 (L915 and L920) and S_{-2} (L911 and K912) binding pockets act cooperatively in recognizing ligands. We probed the importance of these residues by using site-directed mutagenesis to change each residue to the corresponding amino acid in the Tiam2 PDZ domain (see Fig. 4.1C) and subjecting the purified protein to a fluorescence anisotropy ligand-binding assay, as described in Section 3.3. Double-mutant cycle analysis indicates that residues L915 and L920 in the S_0 pocket are coupled in the context of binding to both Syndecan1 and Caspr4 (Shepherd, Hard, Murray, Pei and Fuentes, unpublished data). In contrast, residues L911 and K912 were variably coupled depending on the sequence of the ligand: they were not coupled in binding to Syndecan1, as the total change in energy was determined by the single mutant K912E. However, residues

L911 and K912 were clearly coupled when binding to the Caspr4 peptide (Shepherd, Hard, Murray, Pei and Fuentes, unpublished data). In this case, the L911M mutation counteracted the negative effect on affinity of the K912E mutation. These data indicate that residues within each pocket work coordinately to modulate and fine-tune the affinity and exquisite selectivity of the PDZ domains.

5. Peptide Evolution as a Tool for Probing PDZ Specificity

5.1. The peptide evolution strategy

The Model peptide (SSRKEYYA_{COOH}) used in our structural analysis of the Tiam1 PDZ served as a valuable tool for understanding PDZ–ligand interactions. Interestingly, this peptide has high sequence identity to both the Syndecan1 and Caspr4 peptides. We took advantage of this fact and devised a “peptide evolution” strategy to convert the Model peptide into the Syndecan1 or Caspr4 ligand while assessing the energetic consequences for PDZ binding at each step in the evolution. As depicted in Fig. 4.4, all three peptides have the identical core sequence from P_0 to P_{-4} , while the Model ligand contains an Arg at P_{-5} . By mutating the -5 position from Arg to Gln ($R_{P_{-5}}Q$), we created a peptide with the sequence **QKEYYA**-COOH, which corresponds to the “Core” sequence of the evolved peptides. This change had only a small effect on the dissociation constant measured for the Tiam1 PDZ domain compared to that previously reported for the Model peptide (Table 4.1). Conveniently, the Core peptide can be changed to either the Caspr4 or Syndecan1 peptide (Fig. 4.4) by two distinct mutations. Mutating the Core peptide at P_{-2} from Tyr to Phe ($Y_{P_{-2}}F$ -Sdc1) and at P_{-4} from Lys to Glu ($K_{P_{-4}}E$ -Sdc1) yields a peptide whose final six C-terminal residues are identical to those in the Syndecan1 peptide (**QEEFYA**-COOH), whereas mutating both P_0 and P_{-1} to Phe ($A_{P_0}F$ -Caspr4 and $Y_{P_{-1}}F$ -Caspr4) yields a peptide with a sequence whose last six amino acids are identical to those of the Caspr4 peptide (**QKEYFF**-COOH) (Fig. 4.4). The residues at positions P_{-6} and P_{-7} were not considered further because no electron density was evident for these residues in the Tiam1 PDZ/Model peptide crystal structure, suggesting that they do not interact with the PDZ domain.

5.2. Double-mutant cycle analysis of evolved peptides

The Core peptide and each peptide in the evolution path were synthesized with an N-terminal dansyl adduct to facilitate fluorescence anisotropy measurements. The free energy of binding for each peptide was determined, as outlined in Section 3.3. Figure 4.5A and B shows representative binding curves for each peptide. Because the peptide evolution strategy incorporates single and double mutants, we constructed double-mutant thermodynamic cycles to assess whether the individual residue changes lead to cooperativity in binding the Tiam1 PDZ domain.

The fitted binding parameters for each titration series are summarized in Table 4.1. The $Y_{P_{-2}}F$ -Sdc1 peptide was found to have very little effect on binding to the PDZ domain (Fig. 4.5A and Table 4.1). However, the binding affinity of the $K_{P_{-4}}E$ -Sdc1 peptide was two-fold higher compared to that of the Core peptide (Fig. 4.5A), indicating that a significant amount of the binding energy comes from this residue. We constructed a double-mutant cycle from the binding results of these two mutant peptides and the Syndecan1 peptide (Shepherd *et al.*,

2010; Table 4.1 and Fig. 4.5C). Analysis of these data indicates that P_{-2} and P_{-4} in the Syndecan1 peptide are not cooperative ($G_{\text{int}} = 0.17 \pm 0.13$ kcal/mol).

Our examination of the energetics of binding (Table 4.1) indicates that the affinity of the Syndecan1 peptide is mainly determined by position -4 in the ligand. Consistent with this, examination of the Tiam1 PDZ/Model structure (Fig. 4.1) suggests that the side chain of residue K912 of the S_{-2} pocket may interact with the Glu side chain at P_{-4} of the Syndecan1 ligand. Indeed, the affinity of the K912E (S_{-2} pocket) PDZ mutant for the Syndecan1 peptide was reduced and near the levels of the affinities of the Model and Core peptides for the wild-type PDZ domain (Shepherd, Hard, Murray, Pei and Fuentes, unpublished). Moreover, the difference in binding free energy (G_b) for the K912E PDZ/Syndecan1 compared to wild-type PDZ/Syndecan1 was very similar to that for the wild-type PDZ/ $K_{P_{-4}}E$ -Sdc1 versus the Core peptide. Thus, ~ 1 kcal/mol of binding energy results from this Lys/Glu ion pair, and changes in the Lys/Glu ion pair due to changes in residues in either the ligand or the PDZ domain result in similar effects on the binding energetics. Collectively, these results suggest that K912 interacts with the Glu at P_{-4} of Syndecan1, and this interaction is critical for the PDZ–Syndecan1 interaction.

Use of the peptide evolution strategy also allowed for the investigation of the origin of the Tiam1 PDZ domain specificity for the Caspr4 peptide. The $Y_{P_{-1}}F$ -Caspr4 peptide had an approximately three-fold negative effect on ligand affinity (Table 4.1 and Fig. 4.5B). Interestingly, the K_d determined for this peptide was nearly identical to that found for the Caspr4(F $\rightarrow$ A) C-terminal mutation (ENQKEYFA COOH ; $K_d = 64.8 \pm 5.9$ μM) (Shepherd *et al.*, 2010), verifying that N-terminal residues of the ligand are not critical for binding. The $A_{P_0}F$ -Caspr4 peptide had an approximately two-fold increase in ligand affinity, but did not restore binding to the level of that of the Caspr4 peptide (Table 4.1 and Fig. 4.5B). Together, the $Y_{P_{-1}}F$ -Caspr4 and $A_{P_0}F$ -Caspr4 mutations recreate the Caspr4 peptide, and it was possible to analyze this system by double-mutant cycle analysis (Table 4.1 and Fig. 4.5D). From this analysis, it is clear that the two C-terminal residues (P_0 and P_{-1}) are important for affinity and that they act cooperatively ($G_{\text{int}} = -0.51$ kcal/mol) in binding the Tiam1 PDZ domain (Fig. 4.5D). The molecular origin of this cooperativity is not understood and awaits further structural analysis.

6. Conclusions

In this chapter, we show how a combined approach using structural and thermodynamic methods can provide unique insights into PDZ–ligand specificity. We describe a fluorescence anisotropy-based binding assay and highlight important considerations in designing binding experiments. We apply this fluorescence anisotropy assay to determine the binding energetics for PDZ–ligand interactions. In particular, we probed residues in the S_0 and S_{-2} pockets of the Tiam1 PDZ domain. The binding results and double-mutant cycle analysis revealed that the S_0 pocket was important for PDZ interactions with Syndecan1 and Caspr4 and that the S_{-2} pocket provided selectivity for the Syndecan1 ligand. In addition, we introduced a “peptide evolution” strategy and showed that in combination with double-mutant cycles, it can be used to probe the origin of specificity from the perspective of the ligand. These results corroborated the PDZ mutational analysis of the S_0 pocket and

identified the P_{-4} position in the ligand as critical for Syndecan1 affinity and selectivity. Future application of this combined structural and thermodynamic approach to other PDZ domains should provide additional insights into the origin of affinity and specificity of PDZ domains.

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Abbreviations

CADM1	cell adhesion molecule-1
Caspr4	Contactin-associated protein-like 4
NMR	nuclear magnetic resonance
PDZ	postsynaptic density-95/discs large/zonula occludens-1

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Figure 4.1.

The structure of the Tiam1 PDZ domain bound to a model consensus peptide. (A) A cartoon representation of the PDZ/Model structure (chain B, PDB code 3KZE) is shown. The secondary structure of the PDZ domain is shown in gray and the Model peptide is colored in yellow. The specificity pockets of the PDZ domain (S_0 and S_{-2}) are labeled. Residues discussed in the text are colored red. (B) The sequence of the Model peptide used in the crystal structure determination is shown with the ligand position (P_x) shown above each residue (Shepherd *et al.*, 2010).

Figure 4.2.

Structural analysis of the Tiam1 PDZ domain identifies residues important for specificity. (A) The surface representation of the Tiam1 PDZ domain structure is color coded to indicate the extent of chemical shift changes [$\delta^2(\text{H}, ^{15}\text{N}) = (\delta^2(\text{H}) + (0.4 \delta(^{15}\text{N}))^2)^{1/2}$] upon titration with Syndecan1 or Caspr4 peptides. Residues shown in black could not be assigned and those in gray had a change in chemical shift that was less than the global average. Residues that had a significant change in chemical shift (greater than the global average) are colored continuously from yellow to red, where red indicates maximal changes. (B) An expanded view of ^{15}N -HSQC spectra obtained during a titration series for the Tiam1 PDZ domain with Syndecan1 and Caspr4 peptides. Labeled residues are implicated in Tiam1 PDZ specificity. (C) A histogram plot summarizing the chemical shift changes per residue in the Tiam1 PDZ domain upon titration with Syndecan1 (lower bars) and Caspr4 (upper bars). The value of each bar represents the absolute difference in chemical shift between the two complexes [$\delta = |\delta(\text{Caspr4})| - |\delta(\text{Syndecan1})|$]. A value of zero indicates that changes in chemical shift in the two complexes were identical in magnitude. Differences indicate unique chemical shifts changes in either complex. Residues in red underwent changes greater than 1σ from the average, while those in yellow underwent changes of less than 1σ from the average. Data taken from (Shepherd *et al.*, 2010).

Figure 4.3.

Dependence of anisotropy on fluorescence lifetime and molecular mass. The fluorescence anisotropy was simulated using Eq. (4.7) for two fluorescence lifetimes (4 and 13 ns). The parameters used in the simulation where $r_0 = 0.4$, $v_h = 0.96 \text{ cm}^3/\text{g}$, $R = 8.314 \text{ cm}^3 \text{ MPa K}^{-1} \text{ mol}^{-1}$, $T = 298.15 \text{ K}$, and $\eta = 0.94 \times 10^{-6} \text{ MPa s}$ (Lakowicz, 1999). Data were simulated using molecular weights ranging from 0.1 to 200 kDa, and the inset expands the region from 0.1 to 16 kDa. For reference, a dotted line is placed at 10 kDa, the approximate molecular weight of an individual PDZ domain.

Figure 4.4.

A schematic of the peptide evolution strategy. The path for each evolved peptide is shown with the mutated residue(s) underlined and highlighted in bold.

Figure 4.5.

Fluorescence titration curves and thermodynamic cycles for binding of the Tiam1 PDZ domain to evolved peptides. Binding curves for peptides that had been converted to the (A) Syndecan1 or (B) Caspr4 sequences via the evolution approach. (C) A summary of the binding energetics for peptides evolved into Syndecan1 (P_{-2} and P_{-4} ligand positions). (D) A summary of the binding energetics for peptides evolved into the Caspr4 peptide (P_{-0} and P_{-1} ligand positions).

Table 4.1
Wild-type Tiam1 PDZ domain binding and double-mutant cycles with evolved peptides

Peptide	Sequence	K_d (μ M)	G_b^a	G_b^b	G_{int}	Σ Singles
Model peptide	SSRKEYYA	112 ± 5^c	-5.38 ± 0.05			
Core peptide	SSQKEYYA	119 ± 6	-5.35 ± 0.05			
K _{P-4} E-Sdc1	SSQEEYYA	40 ± 2	-5.99 ± 0.06	-0.65 ± 0.07		
Y _{P-2} F-Sdc1	SSQKEFYA	106 ± 7	-5.42 ± 0.07	-0.07 ± 0.08		
Syndecan1	TKQEEFYA	26.9 ± 0.9^2	-6.23 ± 0.03	-0.88 ± 0.06	-0.17 ± 0.10	-0.71 ± 0.11
Y _{P-1} F-Caspr4	SSQKEYFA	60.1 ± 0.6	-5.75 ± 0.01	-0.40 ± 0.05		
A ₇₀ F-Caspr4	SSQKEYYF	89 ± 5	-5.52 ± 0.06	-0.17 ± 0.08		
Caspr4	ENQKEYFF	19.0 ± 0.4	-6.43 ± 0.02	-1.09 ± 0.05	-0.51 ± 0.08	-0.57 ± 0.09

b, binding; int, interaction.

^a units of kcal/mol.

^b $G_b = G_b(\text{Core}) - G_b(\text{peptide})$.

^c Data taken from Shepherd *et al.* (2010).