

Role of Electrostatic Interactions in PDZ Domain Ligand Recognition<sup>†</sup>Baruch Z. Harris,<sup>‡,§</sup> Francis W. Lau,<sup>§</sup> Naoaki Fujii,<sup>||</sup> R. Kiplin Guy,<sup>||</sup> and Wendell A. Lim<sup>\*§</sup>*Program in Biological Sciences, Department of Cellular and Molecular Pharmacology, and Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143**Received October 24, 2002; Revised Manuscript Received January 15, 2003*

**ABSTRACT:** PDZ domains are protein–protein interaction modules that normally recognize short C-terminal peptides. The apparent requirement for a ligand with a free terminal carboxylate group has led to the proposal that electrostatic interactions with the terminus play a significant role in recognition. However, this model has been called into question by the more recent finding that PDZ domains can recognize some internal peptide motifs that occur within a specific secondary structure context. Although these motifs bind at the same interface, they lack a terminal charge. Here we have investigated the role of electrostatics in PDZ-mediated recognition in the mouse  $\alpha$ 1-syntrophin PDZ domain by examining the salt dependence of binding to both terminal and internal ligands and the effects of mutating a conserved basic residue previously proposed to play a role in electrostatic recognition. These studies indicate that direct electrostatic interactions with the peptide terminus do not play a significant energetic role in binding. Additional chemical modification studies of the peptide terminus support a model in which steric and hydrogen bonding complementarity play a primary role in recognition specificity. Peptides with a free carboxy terminus, or presented within a specific structural context, can satisfy these requirements.

Cells rely on modular protein–protein recognition domains to assemble multiprotein signaling complexes. PDZ<sup>1</sup> (PSD-95, Dlg, ZO-1 homology) domains form a large family of such modules. PDZ domain-containing proteins play an important role in organizing signaling structures at cell–cell signaling junctions such as synapses (1, 2).

PDZ domains were first characterized for their ability to specifically recognize C-terminal peptide ligands, including the C-termini of receptors and channels (3, 4). Peptide library studies indicate that most of the recognition determinants are contained within six amino acids, and that termination with a specific C-terminal residue is required for recognition (5). Moreover, in the structure of PDZ peptide complexes, the terminal carboxylate was found to lie adjacent to a highly conserved basic residue that occurs in what is termed the

carboxylate binding loop (6). Thus, electrostatic interaction with the peptide terminus was long thought to be a critical part of recognition. However, this model was challenged by the more recent finding that some PDZ domains could recognize specific internal peptide motifs, if the motif occurred in a particular secondary structure context. Specifically, the PDZ domains from syntrophin and PSD-95 (domain 2) were found to recognize a  $\beta$ -hairpin motif in the PDZ domain from nNOS (a specific PDZ heterodimerization interaction) (7–9), or related sequences that are cyclized by an intramolecular disulfide linkage (10) (Figure 1). Structural and biochemical studies show that these internal peptides are related in sequence to canonical terminal recognition motifs, and that they bind at the same binding groove with comparable binding affinities:  $\sim 2$ – $7 \mu\text{M}$  for C-terminal peptides,  $\sim 0.6$ – $5 \mu\text{M}$  for nNOS residues 1–130 (nNOS 1–130), and  $\sim 1.5 \mu\text{M}$  for the cyclic peptide (7). These findings are surprising given that these internal ligands lack the canonical terminal negatively charged carboxylate.

Here we describe experiments aimed at more precisely evaluating the role of electrostatic interactions in PDZ-mediated peptide recognition using the *Mus musculus*  $\alpha$ 1-syntrophin PDZ domain as a model system. First, we have determined the sensitivity of various PDZ-mediated interactions to ionic strength, and second, we have determined the effects of mutating a conserved basic residue in the PDZ binding groove that has been proposed to play a role in electrostatic recognition. These results indicate that electrostatics do not play a significant role in recognition of the terminal carboxylate. In addition, we have examined a library of ligand variants and found that PDZ domains discriminate very tightly between ligands with minor alterations in the precise chemical structure of the C-terminus. Together, these results are consistent with a model in which steric and

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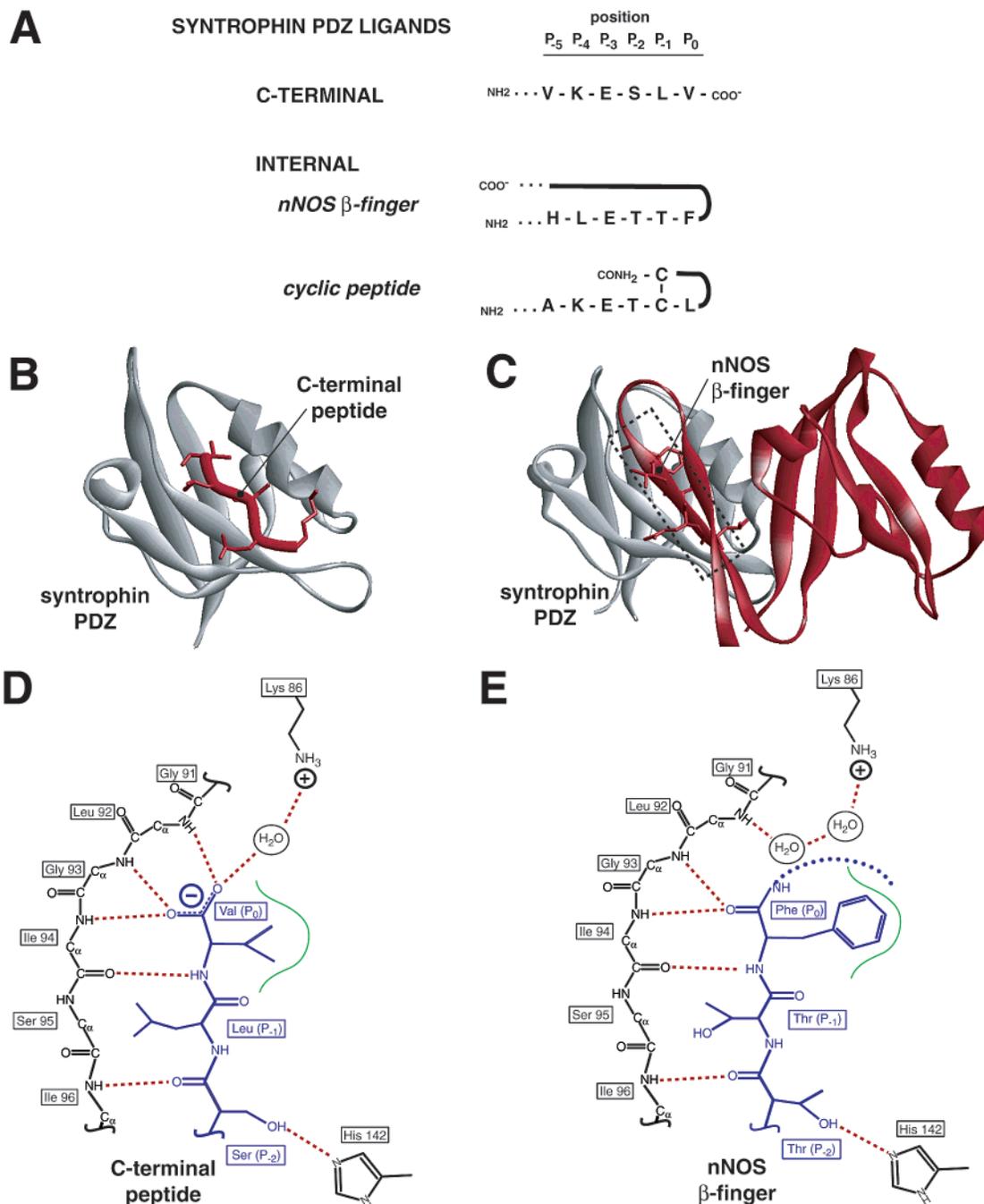
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<sup>1</sup> Abbreviations: DCM, dichloromethane or methylene chloride; DIPEA, diisopropylethanolamine; DMF, dimethylformamide; DTT, dithiothreitol; EDT, ethanedithiol; EDTA, ethylenediaminetetraacetic acid; EtOH, ethanol or ethyl alcohol; Fmoc, 9-fluorenylmethoxycarbonyl; FPLC, fast performance liquid chromatography; HATU, 2-(1*H*-9-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; IPTG, isopropyl  $\beta$ -D-thiogalactopyranoside; MeOH, methanol or methyl alcohol; Ni-NTA, nickel nitriloacetic acid; nNOS, neuronal nitric oxide synthase; PCR, polymerase chain reaction; PDZ, PSD-95, Dlg, ZO-1 homology; TEV, tobacco etch virus; TFA, trifluoroacetic acid; TIS, triisopropylsilane.



**FIGURE 1:** Interaction of the  $\alpha$ 1-syntrophin PDZ domain with different ligand types. (A) Three ligand types recognized by the syntrophin PDZ domain. Panels B and C show the overall structure of the PDZ domain bound to (B) the KESLV peptide [PDB entry 2PDZ (12)] and (B) nNOS 1–130 [PDB entry 1QAV (8)]. The PDZ domain is shown in gray and the ligand in red. Side chains of the ligand are shown in stick form. In the syntrophin–nNOS 1–130 bound structure, the “pseudopeptide” region that is responsible for most contacts is highlighted by a dashed box. Panels B and C were generated using WebLab Viewer Lite (Molecular Simulations, Inc.). Panels D and E show schematic diagrams of interactions made in complexes with peptide (D) and nNOS 1–130 (E) ligands. PDZ domain residues in the binding pocket are depicted in black, and ligand residues are drawn in blue. Likely hydrogen bonds observed as contacts in the complex structures are shown as red dashed lines. The C-terminal amino acid side chain is packed against a hydrophobic pocket in the PDZ domain composed of several hydrophobic amino acid side chains (symbolized by a green line).

hydrogen bonding complementarity are the central determinants for PDZ domain recognition of C-termini.

## EXPERIMENTAL PROCEDURES

**Plasmid Construction and Mutagenesis.** Mutagenesis of the  $\alpha$ 1-syntrophin PDZ domain was performed by two-step PCR as described previously (7). Briefly, overlapping primers were designed by incorporating the desired mutation in their

sequence. Two rounds of PCR were performed, and the resulting product was ligated into the pBH4 vector for inducible expression with an N-terminal His<sub>6</sub> tag (7). Resulting plasmids were transformed in *Escherichia coli* strain BL21(DE3)pLysS for expression.

**Protein Expression and Purification.** The recombinant *M. musculus* N-terminally His<sub>6</sub>-tagged  $\alpha$ 1-syntrophin PDZ domain (wild-type and mutant) was expressed in *E. coli* strain

BL21(DE3)pLysS as described previously (7). Purified proteins were cleaved with ~0.1 mg/mL TEV protease overnight during dialysis against room-temperature 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM EDTA, and 2 mM DTT. After removal of the His<sub>6</sub> tag, the protein was dialyzed overnight against cold 25 mM HEPES, 1 mM EDTA, and 10 mM NaCl (pH 7.0) and further purified by ion exchange chromatography on an HR10/10 Source S Sepharose column in a 10 to 500 mM NaCl gradient using an ÄKTA Explorer FPLC system (Amersham Pharmacia). Final protein stocks were >99% pure and stored in 20 mM HEPES (pH 7.0) at -80 °C.

**Synthesis and Modification of Peptides.** Peptides were synthesized using conventional solid-phase Fmoc amino acid chemistry on an ABI 381 synthesizer. For peptides with free carboxyl termini, Wang resin with the C-terminal amino acid pre-conjugated was used (Novabiochem). Amidated peptides and the dansylated cyclic peptide were synthesized using Rink-Amide resin (Novabiochem). Deprotection of the N-terminal Fmoc group was performed on the machine. Dansylation reactions were accomplished using the following procedure. A solution of 70 mg of dansyl chloride (Molecular Probes), 16  $\mu$ L of DIPEA, and 1.9 mL of DMF was added to 50  $\mu$ mol of peptide equivalents on resin and the mixture incubated for 2 h at room temperature. Resin was then washed with DMF and DCM and dried under vacuum. The reaction was repeated once before cleavage from the Rink-Amide resin. For cleavage, the resin was incubated for 3 h at room temperature in a 10 mL solution containing 9.25 mL of TFA and 0.25 mL each of 1,2-EDT, TIS, and water. Afterward, the resin was filtered and the effluent collected. The resin was washed further with two additional 1 mL aliquots of TFA. Crude peptide solutions were precipitated with cold ethyl ether and lyophilized overnight, and then resuspended in 0.1% TFA with an amount of acetonitrile that was as small as necessary to solubilize the crude peptide. Peptides were purified on a Rainin Dynamax HPLC system using a Vydac 25 cm  $\times$  2.2 cm, 10 cm C18 column with a gradient of 0 to 90% acetonitrile in 0.1% TFA. Fractions corresponding to major peaks detected at 215 nm were lyophilized and redissolved in 0.1% TFA. The purity of fractions was assessed by HPLC on a Vydac 15 cm  $\times$  0.46 cm analytical C18 column; pure fractions were subsequently lyophilized, and a small amount was dissolved in buffer for electrospray mass spectrometry. The molecular masses of all peptides were verified to be within 0.5 Da by electrospray mass spectrometry, and final stocks were made in filtered deionized water at a concentration that was appropriate for binding experiments.

Peptides with modified C-termini were synthesized as follows (listed by C-terminal chemical group).

(1) *Hydroxamide.* The protected peptide was synthesized by a standard solid-phase peptide synthesis protocol using 2-chlorotrityl chloride resin (0.065 mmol) and Fmoc chemistry, and reacted with *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxyamine (76 mg, 0.65 mmol), HATU (246 mg, 0.65 mmol), and DIPEA (0.22 mL, 1.3 mmol) in dry DMF (2 mL) at 50 °C for 12 h. Additional *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxyamine (38 mg, 0.32 mmol), HATU (123 mg, 0.32 mmol), and DIPEA (0.11 mL, 0.63 mmol) were added to the reaction mixture, and the mixture was heated for an additional 10 h. The reaction mixture was diluted with ethyl

acetate (30 mL), washed with 1 N hydrochloric acid (5 mL) followed by a saturated aqueous sodium bicarbonate solution (10 mL) and a saturated aqueous sodium chloride solution (10 mL), dried over anhydrous sodium sulfate, and evaporated. The residue was dissolved with 1.2 mL of a peptide deprotection cocktail (96% TFA, 2% water, and 2% TIS), kept at ambient temperature for 0.5 h, and evaporated. The crude residue was triturated in dry diethyl ether (1 mL) for purification.

(2) *Amide.* Peptides were synthesized on Rink-Amide resin (Novabiochem) using conventional Fmoc chemistry and prepared as described above.

(3) *Alcohol, Hydrazide, and Methyl Ester.* Synthesis was performed on HMB-AM resin (Novabiochem) and a C-terminal valine attached using the symmetrical anhydride method. Remaining residues on the peptide were added using conventional Fmoc chemistry. Deprotection was first performed with 95% TFA, and specific cleavage reactions were performed as follows. For peptide alcohol, resin was treated with 126 mg of NaBH<sub>4</sub> in 50% aqueous EtOH for 4 h, and collected by filtration. For hydrazide, resin was treated with 5% hydrazine hydrate in DMF for 1 h (washed with DMF and TFA). Peptide was collected by filtration and rotary evaporation. For methyl ester, resin was treated overnight with a DIPEA/MeOH/DMF (1:5:5) mixture. Peptide was collected by filtration (washed with a 1:1 MeOH/DMF mixture) and rotary evaporation.

(4) *C-Terminal Isobutylamine.* The protected peptide was synthesized by standard solid-phase peptide synthesis using 2-chlorotrityl chloride resin (0.065 mmol) and Fmoc chemistry, and reacted with isobutylamine (0.065 mL, 0.65 mmol), HBTU (123 mg, 0.32 mmol), and DIPEA (0.11 mL, 0.63 mmol) in dry DMF (1 mL) at ambient temperature overnight. The reaction mixture was diluted with ethyl acetate (30 mL), washed with 1 N hydrochloric acid (5 mL) followed by a saturated aqueous sodium bicarbonate solution (10 mL) and a saturated aqueous sodium chloride solution (10 mL), dried over anhydrous sodium sulfate, and evaporated. The residue was dissolved with 1.2 mL of a peptide deprotection cocktail (96% TFA, 2% water, and 2% TIS), kept at ambient temperature for 0.5 h, and evaporated. The crude residue was triturated in dry diethyl ether (1 mL) for purification. Following cleavage reactions, all modified peptides were dissolved in aqueous buffer and acetonitrile, lyophilized, and purified by HPLC as described above. Molecular masses of modified peptides were verified to within 0.5 Da by electrospray (carboxylate, amide, alcohol, hydrazide, or methyl ester) or MALDI-TOF (hydroxamide or C-terminal isobutylamine) mass spectrometry.

**Fluorescence Perturbation Binding Assays.** Binding assays were performed by monitoring dansyl fluorescence perturbation as described previously (7) and using either direct (i.e., two-component) binding or competition binding to measure binding constants. Briefly, changes in dansyl fluorescence were monitored with 335 nm excitation and 545 nm emission wavelengths in a Photon Technology Inc. Quantum Master Fluorometer using a 1 cm  $\times$  1 cm quartz stirred-cell cuvette. Data were fit to the appropriate equation using nonlinear least-squares analysis using the program ProFit 5.1.0 (Quantum Soft).

**Circular Dichroism Studies.** Circular dichroism spectroscopy was performed using a JASCO 715 spectropolarimeter.

Scans and melts were performed with untagged, purified proteins in 20 mM HEPES (pH 7.0) with either no salt or 500 mM NaF as indicated in the text; a 1 cm × 1 cm quartz stirred-cell cuvette was used. Denaturant melts were performed by manual addition of buffered, fresh 8 M urea with correction for volume changes.  $\Delta G_{\text{folding}}$  at 0 M denaturant was estimated by linear extrapolation as previously described (11).

## RESULTS

### *Ionic Strength Dependence of PDZ Domain Interactions.*

As a first step toward understanding the role of electrostatics in PDZ domain recognition, we performed binding experiments using buffer conditions with an increasing ionic strength. In all experiments, we used the *M. musculus*  $\alpha 1$ -syntrophin PDZ domain, which is a well-characterized model system (7, 8, 10, 12). A problem with these types of studies is that ionic strength perturbation may alter various aspects of PDZ binding. In addition to altering the putative electrostatic interaction between the ligand terminal carboxylate and the positively charged PDZ binding pocket, ionic strength would also be expected to effect other (nonterminal) electrostatic interactions between the peptide and domain. For example, all ligands of the syntrophin PDZ domain require a glutamate residue at peptide position P<sub>-3</sub>, which appears to participate in a salt bridge interaction with syntrophin residue lysine 95. Moreover, ionic strength might also effect electrostatic interactions involved in domain folding, and therefore might indirectly effect recognition through destabilization of the domain.

We therefore took two steps to control for these other ionic strength effects. First, we assayed the salt dependence of domain stability to urea denaturation using circular dichroism spectroscopy (Figure 2A). NaF at 500 mM (fluoride was used to avoid interference due to far-UV absorption) was found to have no significant effect on PDZ domain stability (in fact, a slight stabilization of  $\Delta\Delta G$  of  $-0.5$  kcal/mol was observed), indicating that increasing ionic strength would not indirectly reduce the level of binding of PDZ ligands through domain destabilization. Second, to control for perturbation of nonterminal electrostatic interactions, we performed ionic strength perturbation assays not only on the C-terminal peptide ligands but also on internal ligands lacking a terminal charge. These internal ligands, which include nNOS 1–130 (7, 10, 12) and the cyclic binding peptide (Figure 1A), all share similar nonterminal electrostatic properties with the C-terminal ligand (they retain glutamate at ligand position P<sub>-3</sub>). Thus, if recognition of the C-terminal carboxylate occurs via an electrostatic mechanism, we would expect to see a dependence of binding affinity on salt concentration that is far greater for C-terminal ligands than for internal ligands.

As can be seen in Figure 2B, all classes of PDZ ligands, including internal motifs, exhibit a linear dependence of  $\Delta G_{\text{binding}}$  on ionic strength over the range of 0–500 mM sodium chloride. Two C-terminal peptide ligands, Ndan-SIESDV-COOH and Ndan-VKESLV-COOH, exhibited similar salt-dependent slopes of 2.7 and 2.3 cal mol<sup>-1</sup> (mM NaCl)<sup>-1</sup>. Salt dependence for the dansylated cyclic peptide is somewhat lower at 0.9 cal mol<sup>-1</sup> (mM NaCl)<sup>-1</sup>. Most notably, however, similar salt effects are seen for ligand types

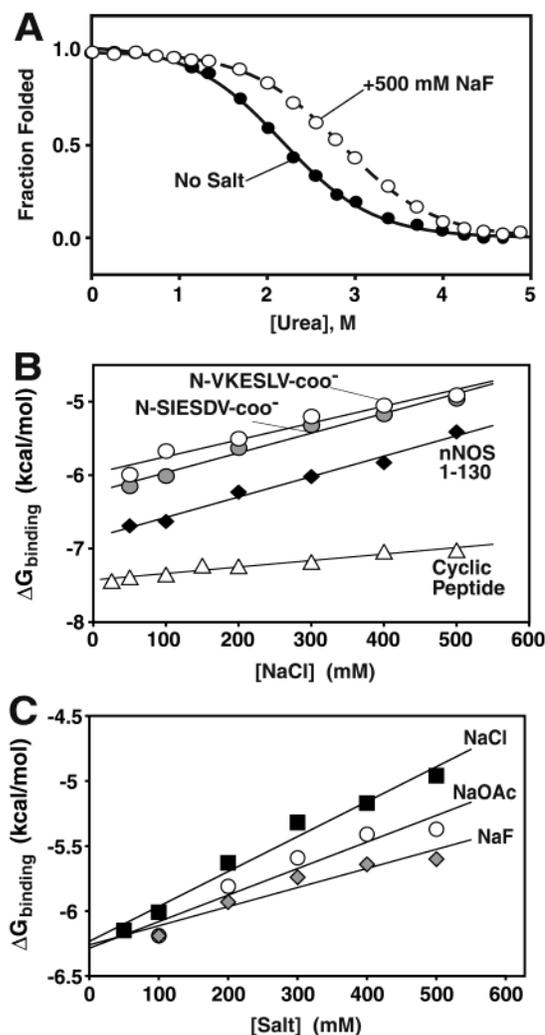


FIGURE 2: Salt dependence of PDZ domain–ligand interactions. (A) The effect of salt on domain stability is minimal. Urea denaturation curves determined by CD spectroscopy in the presence (○) and absence (●) of 500 mM NaF are shown. (B) Plot of  $\Delta G_{\text{binding}}$  for binding to the  $\alpha 1$ -syntrophin PDZ domain with respect to the salt buffer concentration. (B) Dependence of binding ( $\Delta G_{\text{binding}}$ ) for different PDZ domain ligands on NaCl concentration. N-Terminally dansylated peptides with free C-terminal carboxylate: Ndan-SIESDV-COO<sup>-</sup> (gray circles), slope = 0.0027,  $R^2 = 0.977$ ; Ndan-VKESLV-COO<sup>-</sup> (○), slope = 0.0023,  $R^2 = 0.956$ ; nNOS 1–130 (◆), slope = 0.0028,  $R^2 = 0.984$ ; and N-terminally dansylated cyclic peptide (Ndan-AKETCLAGYYC-CONH<sub>2</sub>) (△), slope = 0.0009,  $R^2 = 0.950$ . A clear salt dependence exists for interaction of all three ligand types with the PDZ domain. In addition, there is no obvious relationship between the degree of salt dependence and the presence of a C-terminal carboxylate. (C) Effect of different anions on the salt dependence of PDZ–peptide interactions. The salt dependence of binding is shown for the Ndan-SIESDV-COO<sup>-</sup> peptide with sodium chloride (■, slope = 0.0027,  $R^2 = 0.977$ ), sodium acetate (○, slope = 0.0020,  $R^2 = 0.917$ ), and sodium fluoride (gray diamonds, slope = 0.0015,  $R^2 = 0.915$ ) at identical pH.

that do not contain a free carboxylate, and in the case of nNOS 1–130, the salt dependence of binding shows a slope of 2.8 cal mol<sup>-1</sup> (mM NaCl)<sup>-1</sup>. Thus, the dependence is nearly identical for ligands with and without a free carboxylate. These data are consistent with a model in which the free terminal carboxylate of PDZ peptide ligands does not participate in ionic interactions with the PDZ binding pocket.

We also tested several different types of salts for differential effects on PDZ–peptide binding (Figure 2C). The

salt dependence of binding for different anions (using  $\text{Na}^+$  as a cation) using the Ndan-SIESDV-COOH peptide showed slightly different effects for different salts, as might be expected from predicted known solution properties for these different ions (13, 14) (all solutions were titrated to pH 7.5). The fluoride anion, for example, has a high energy of hydration and would be expected to penetrate a protein–protein interface more weakly than chloride (13, 15). If anions affect PDZ binding by interacting with the surface of either the PDZ domain or the ligand, the slope of chloride dependence would be shallower than that of fluoride dependence. Slopes of salt dependence were  $2.7 \text{ cal mol}^{-1} (\text{mM NaCl})^{-1}$  for sodium chloride,  $2.0 \text{ cal mol}^{-1} (\text{mM NaCl})^{-1}$  for sodium acetate, and  $1.5 \text{ cal mol}^{-1} (\text{mM NaCl})^{-1}$  for sodium fluoride ( $\text{Cl}^- > \text{OAc}^- > \text{F}^-$ ).

**Mutagenesis of the Positive Charge of the Conserved Binding Pocket.** The structures of PDZ–peptide complexes have shown that a conserved arginine/lysine residue in the carboxylate-recognition loop (sometimes termed the “GLGF” loop) is in an excellent position to participate in electrostatic interactions with the terminal peptide ligand carboxylate (Figure 1D); in the structure of the syntrophin PDZ domain complexed with the KESLV peptide, it is the only basic residue within  $10 \text{ \AA}$  of the peptide carboxylate (12). Thus, to further probe the role of electrostatic interactions in terminus recognition, we mutated this residue in the syntrophin PDZ domain, lysine 86, to a nearly isosteric methionine (K86M). This mutation was designed to maintain the stability of the PDZ domain hydrophobic core and binding pocket, while eliminating the conserved positive charge [in contrast to previous mutations of this conserved residue which have often been more drastic (16)]. The K86M mutant was assayed by circular dichroism during urea denaturation and found to have a stability ( $\Delta\Delta G = -1.2 \text{ kcal/mol}$ ) greater than that of the wild-type domain (Figure 3A); observed losses in binding affinity of this mutant for PDZ ligands are therefore not due to a defect in stability. It is important to note that the K86 residue may contribute to binding in ways beyond electrostatics. Thus, we not only assayed the effect of this mutation on the binding affinity but also measured its effect on the ionic strength dependence of binding. If K86 plays a crucial electrostatic role in recognition, we would expect not only a decrease in C-terminal binding affinity but also, more importantly, a decrease in the salt dependence of binding.

The K86M mutation decreases the affinity for all ligands, but has the most significant effect on PDZ domain binding to peptide ligands (Figure 3B); a  $\Delta\Delta G_{\text{binding}}$  of approximately  $1.5 \text{ kcal/mol}$  was observed at low salt (50 mM). The effect of the mutation on binding of the cyclic peptide and nNOS 1–130 was smaller in magnitude ( $\Delta\Delta G_{\text{binding}} \sim 0.5 \text{ kcal/mol}$ ). These results indicate that the wild-type K86 residue plays an important role in recognition of the C-terminal ligands.

However, we also measured the effect of the K86M mutation on the slope of the salt dependence for all three ligand types (Figure 4). Strikingly, the salt dependence of binding changes only minimally, and does not decrease significantly in any case. The K86M mutation actually increases the slope of salt dependence from  $3.4$  to  $6.1 \text{ cal mol}^{-1} (\text{mM NaCl})^{-1}$  for the C-terminal peptide Ndan-SIESDV (Figure 4A). Effects on slope of the salt dependence plot for the other ligands are minimal: from  $2.6$  to  $2.5 \text{ cal}$

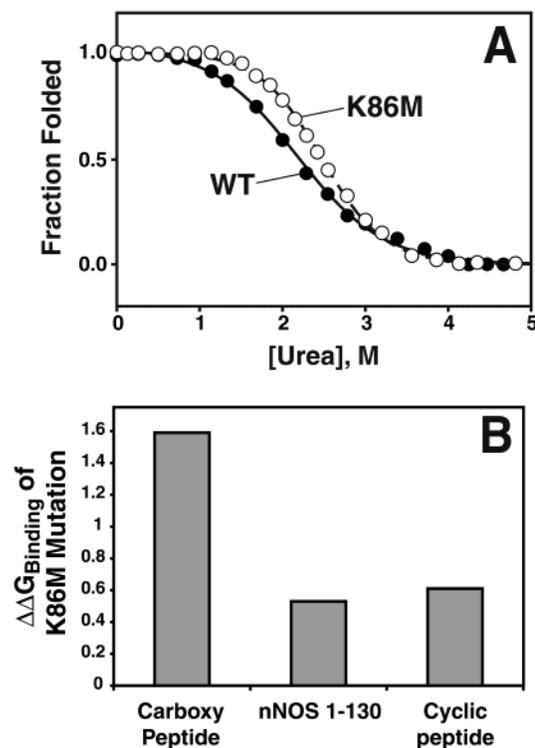


FIGURE 3: Effect of the K86M mutation on PDZ–ligand interactions. (A) The K86M mutation has minimal effects on the stability of the syntrophin PDZ domain. Urea denaturation curves determined by CD spectroscopy for the wild-type (●) or K86M mutant (○)  $\alpha$ 1-syntrophin PDZ domain are shown. (B) Effect of the K86M mutation on binding to different ligands: carboxy peptide, nNOS 1–130, and cyclic peptide. The  $\Delta\Delta G$  of the K86M binding pocket mutation was assayed at low salt (50 mM NaCl). The K86M mutation destabilizes all of the interactions, although its effect is greatest on the carboxy–peptide interaction.

$\text{mol}^{-1} (\text{mM NaCl})^{-1}$  for nNOS 1–130 (Figure 4B) and from  $0.9$  to  $0.8 \text{ cal mol}^{-1} (\text{mM NaCl})^{-1}$  for the dansylated cyclic peptide (Figure 4C).

The fact that removal of a conserved positive charge from the binding pocket does not reduce the salt dependence of binding is counterintuitive, and suggests that this residue, although it plays an important energetic role, does not play a significant electrostatic role. Since it is the only binding pocket residue which could conceivably participate in an ionic interaction with the C-terminal carboxylate, these results lend credence to the notion that the peptide free C-terminal carboxylate is recognized by a nonelectrostatic mechanism.

**Testing Alternate Termini Ligand Variants.** Our findings that the ligand terminal carboxylate is not recognized through electrostatic interactions support an alternative, previously suggested model in which the PDZ domain selects ligands by steric occlusion (7). All of the internal binding peptides to date share a common structural feature: the core recognition motif occurs immediately before a sharp turn in the peptide chain. Thus, the PDZ binding groove may simply be selecting for ligands that contain a specific sequence motif followed by a sudden termination or change in direction of the peptide chain. In support of this model is the observation that the conserved “carboxylate” binding loop sterically blocks forward continuation of the peptide chain (8).

If this steric exclusion model were true, however, one would expect that ligand variants that had chemically diverse termination chemistries would still be recognized by the PDZ

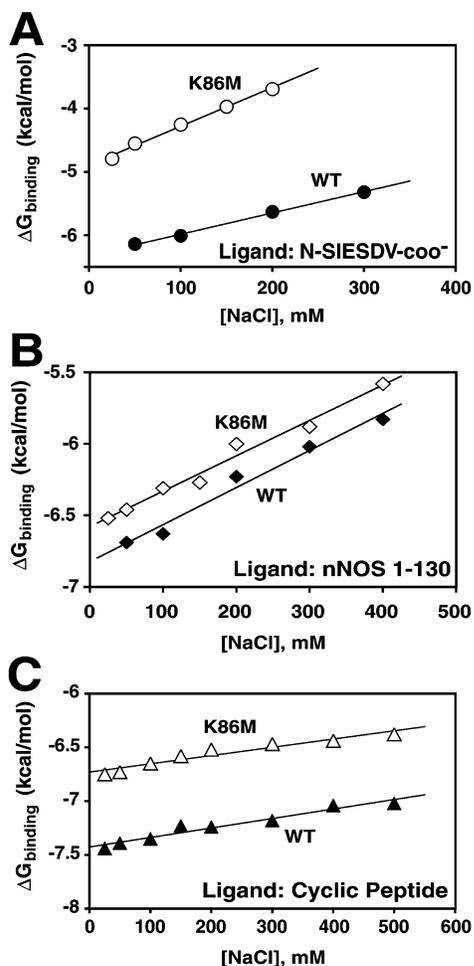


FIGURE 4: Effect of the K86M mutation on the salt dependence of PDZ–ligand interactions. Binding of a given ligand to the wild-type syntrophin PDZ domain (black symbols) or the K86M mutant syntrophin PDZ domain (white symbols) is shown. (A) N-Terminally dansylated peptide with a free C-terminal carboxylate (Ndan-SIESDV-COOH, circles): wild-type (WT) slope = 0.0034 and  $R^2 = 0.997$ , K86M mutant slope = 0.0061 and  $R^2 = 0.993$ . (B) nNOS 1–130 (diamonds): WT slope = 0.0026 and  $R^2 = 0.998$ , K86M mutant slope = 0.0025 and  $R^2 = 0.981$ . (C) N-Terminally dansylated cyclic peptide (triangles): WT slope = 0.0009 and  $R^2 = 0.950$ , K86M mutant slope = 0.0008 and  $R^2 = 0.925$ . In no case does the K86M mutant show a smaller salt dependence slope than the wild-type PDZ domain, indicating that the contributions of K86M to binding are unlikely to be electrostatic in nature.

domain. Therefore, to test this hypothesis, we synthesized a variety of peptides with identical amino acid sequences (VKESLV) but with chemically modified C-termini (Figure 5). These include termini structures that are approximately equal in size to the carboxylate group (both slightly smaller and larger) but that differ in their precise chemical nature. We assayed the binding affinity of modified peptides for the syntrophin PDZ domain using a competition binding assay (7). We and others had previously found that peptides with an amide C-terminus bind very poorly to PDZ domains (17, 18), but we also found that many other C-termini with a variety of chemical structures are also poor binders. The only C-terminal group with reasonable binding affinity is the hydroxamide (18). These results indicate that the simple steric occlusion model is not sufficient to explain PDZ terminus recognition. Although the carboxylate can be eliminated in PDZ ligands with internal sequence motifs,

C-terminal Group	Structure	Affinity
Carboxylate		2–8 $\mu\text{M}$
nNOS 1–130		0.5 $\mu\text{M}$
Cyclic Peptide		0.1–1.0 $\mu\text{M}$
Hydroxamide		105 $\mu\text{M}$
Amide		>500 $\mu\text{M}$
Alcohol		>500 $\mu\text{M}$
Hydrazide		>500 $\mu\text{M}$
Methyl Ester		>500 $\mu\text{M}$
C-terminal isobutylamine		>500 $\mu\text{M}$

FIGURE 5: Binding to alternative terminal ligands indicates that the arrangement of hydrogen bonding interactions is essential to high-affinity PDZ domain binding. A variety of modified peptides were synthesized, all with the sequence N-acetyl-VKESLV, but with alternative terminal moieties. These were tested for binding to the  $\alpha$ 1-syntrophin PDZ domain in a competition binding assay. For comparison, the affinities of strongly binding internal ligands (nNOS 1–130 and cyclic peptide) are also shown. No binding is detectable for derivatives with peptide amide, alcohol, hydrazide, methyl ester, or C-terminally modified peptide that can bind to the  $\alpha$ 1-syntrophin PDZ domain with measurable affinity is the peptide hydroxamide, which may recapitulate some of the favorable hydrogen bonding interactions with the carboxylate recognition loop.

recognition of the carboxylate chemical structure in peptide ligands is very specific.

Together, these results are most consistent with a model in which, in addition to steric features, the specific pattern of hydrogen bonding groups presented by the ligand terminus is critical for recognition. Both the C-terminal peptides and the specific internal ligands (and, to a limited degree, the hydroxamide ligand) appear to be capable of satisfying these specific requirements.

## DISCUSSION

PDZ domains are widely distributed protein binding domains that are best known for recognizing short peptide motifs with a required free C-terminal carboxylate. To dissect the mechanism of PDZ domain ligand recognition, we have performed extensive analysis on the role of electrostatics in recognition of three known ligand types: peptide ligands, cyclic peptides constrained with an intramolecular disulfide bond, and  $\beta$ -hairpin ligand nNOS 1–130. Our experiments focused on the mouse  $\alpha$ 1-syntrophin PDZ domain, which is the PDZ domain model system best characterized in terms

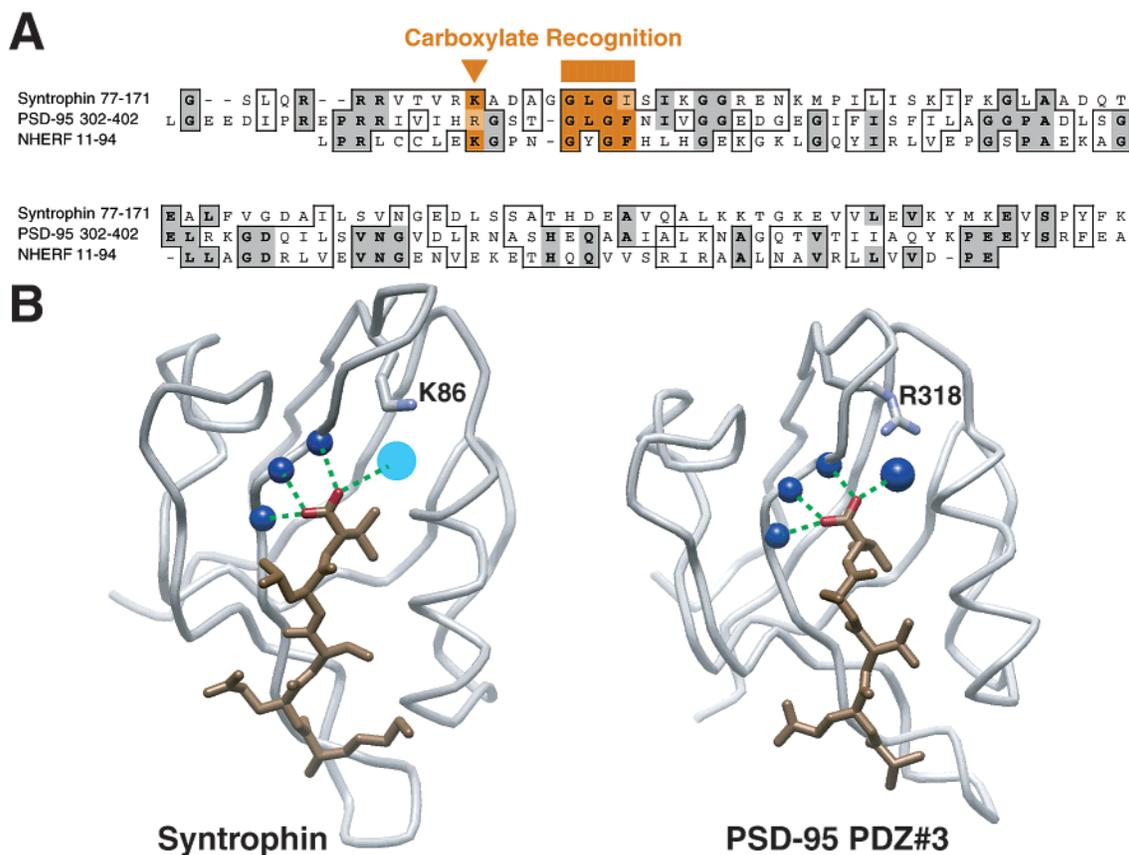


FIGURE 6: Similarity of the mouse  $\alpha$ 1-syntrophin PDZ domain to other class I PDZ domains. (A) Primary sequence alignment of the *M. musculus*  $\alpha$ 1-syntrophin PDZ domain (8) with other PDZ domains for which ligand-bound crystal structures are available: that of *Rattus norvegicus* PSD-95/SAP90 PDZ#3 (6) and *Homo sapiens* NHERF/EBP50 PDZ#1 (20). Dark gray shading indicates identical sequence (40% overall), and light gray indicates similar sequence (72% overall). For this alignment, we have used the borders of sequence regions that were used in structural studies. The alignment was generated using Macvector version 6.5.3 (Oxford Molecular). Residues that participate in carboxylate recognition are indicated in orange; the conserved arginine/lysine is marked with an orange triangle, and the conserved GLGF loop is indicated with an orange bar above the alignment. (B) Comparison of peptide-bound structures for the syntrophin PDZ domain (left, PDB entry 2PDZ) and the PSD-95/SAP90 PDZ#3 domain (right, PDB entry 1BE9). Peptides are drawn in brown with terminal carboxylate oxygens in red, and PDZ domains are drawn as gray  $\alpha$ -carbon traces. Atoms that participate in interactions with the peptide carboxylate are explicitly shown as dark blue spheres (main chain amide nitrogens and water in the PSD-95 structure); the side chains of conserved arginine/lysine residues are shown in stick form with side chain nitrogens indicated in light blue (syntrophin lysine 86 and PSD-95 arginine 318, labeled). The syntrophin diagram structure is drawn from the most representative chain (1) in the ensemble of 15 NMR-derived structures; because it is an NMR structure, the ordered binding pocket water is not included. This ordered water is observed in the nNOS-syntrophin complex crystal structure (PDB entry 1QAV), and we have drawn a light blue disk to indicate its approximate location. This ordered water is observed in all crystal structures of related PDZ domains. Illustrations were generated using the program WebLab Viewer Lite (Molecular Simulations, Inc.). The two PDZ domain structures are highly similar, especially within the regions that are important for peptide recognition.

of structure and function. Although our results formally apply only to this PDZ domain, the syntrophin PDZ domain is highly homologous to other PDZ domains, particularly so-called “class I” PDZ domains (Figure 6). Thus, the behavior reported here is likely to be applicable to other PDZ domains as well.

*C-Terminal Recognition Appears To Be Dependent on Hydrogen Bonding, and Not on Electrostatic Interactions.* We have found that binding affinities of all three types of ligands for the syntrophin PDZ domain decrease in a linear fashion with increasing ionic strengths. We therefore believe that there is an electrostatic contribution to ligand recognition in this system. However, because this ionic strength dependence is observed for ligands both with and without a free carboxylate, we postulate that electrostatic interactions are not a significant factor in recognition of the terminal carboxylate anion in the peptide ligand. In addition, mutation of a highly conserved positively charged residue (the only

such residue in the binding pocket which could contact the peptide carboxylate) does not affect the salt dependence of binding, though it does specifically affect carboxy-peptide binding relative to the other two ligand types. We therefore believe that, although this basic residue plays a key role in recognition of the C-terminal carboxylate, it does so by a nonelectrostatic mechanism.

If recognition of the C-terminus is not via ionic interactions, what is the mechanism of specificity for carboxylate recognition? In the case of the syntrophin PDZ domain, our evidence argues against an electrostatic mechanism of recognition. Our data, combined with structural information from a variety of other studies (6, 12, 19–23), support a model in which PDZ domains specifically recognize a C-terminal amino acid sequence through a precise arrangement of hydrogen bonding partners and hydrophobic groups. Three main chain amides and a water molecule coordinated by the conserved K86 side chain are spatially arranged within

the binding pocket (see Figure 1) to recognize the C-terminal carboxylate by its shape and positioning of hydrogen bond acceptor groups. The position of the coordinated water is well-conserved in all known PDZ structures.

How then is a PDZ domain able to recognize structurally distinct types of ligands such as the nNOS  $\beta$ -finger? We postulate that this network of hydrogen bonding groups is capable of some degree of flexibility. In the structure of the syntrophin PDZ domain in complex with the nNOS internal ligand, the ligand carbonyl group that corresponds to the terminal carboxylate in peptide ligands only makes hydrogen bonding contacts with two of the three main chain amides normally used for carboxylate recognition (Figure 1E). However, it is striking that in this structure a second ordered water molecule is observed that hydrogen bonds to both the remaining domain amide and the first ordered water molecule. Thus, the same number of hydrogen bonds observed in the terminal peptide complex is reconstituted. We therefore postulate that the structure of these particular internal ligands not only allows maintenance of a subset of the original interactions but also allows insertion of an additional water that can functionally mimic the missing second oxygen of the carboxylate. Other chemical termini are unlikely to function because either they cannot recapitulate the original hydrogen bonding network or they sterically do not allow insertion of an additional water to replace the interactions of the second carboxylate oxygen. The hydroxamide, which is partially functional, may be capable of positioning its hydroxyl group appropriately to partially reconstitute the network.

The sensitivity of PDZ domains to the exact nature of the ligand C-terminus suggests that cells could make use of C-terminal modifications to modulate PDZ domain interactions *in vivo*. For example, the C-terminal leucine in bovine protein phosphatase 2A has been shown to be reversibly modified by methyl esterification (24), and such a modification to a PDZ domain peptide ligand would abrogate binding. There is a precedent for covalent modifications regulating PDZ domain interactions, specifically phosphorylation of serine at the P<sub>-2</sub> position in the peptide ligand (25).

*Energetic Implications of the PDZ Recognition Mechanism.* Nonelectrostatic mechanisms for recognition of ionic ligands have been observed before. For example, in phosphate and sulfate transport receptors, anionic ligands appear to be held in place by extensive, specific hydrogen bond interactions (26, 27), and it is thought that this network of interactions accounts for exquisite specificity (28). Such a mechanism is thought to contribute to interactions of high specificity, while preventing significant competition by other free anions or amino acids. Although a recognition mechanism more dependent on Coulombic interactions might increase the overall interaction affinity, it would likely decrease the overall ligand specificity.

It is intriguing that PDZ domains also appear to use a nonelectrostatic mechanism to recognize the critical carboxylate group in its ligands. There may be similar reasons why it would be functionally disadvantageous to utilize the full interaction potential of the carboxylate. Although direct electrostatic interaction might yield a higher-affinity interaction, this would yield several potential problems. First, PDZ domains might recognize other competing ionic groups, or other carboxylate-containing side chains or small molecules.

Second, such a mechanism might lead to reduced sequence specificity; a disproportionate dependence on the carboxylate for binding energy would reduce the level of discrimination based on more N-terminally located residues (individual PDZ domains each recognize unique signature C-terminal sequences). Third, full optimization of the binding potential might also yield extremely high-affinity interactions. Such high-affinity interactions might be inappropriate for PDZ domain function in assembling diverse and often dynamically regulated signaling complexes. Thus, this type of recognition mechanism helps to explain how PDZ domains can achieve the proper balance of binding specificity and energy for their unique function.

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