

Single-Amino Acid Substitutions Alter the Specificity and Affinity of PDZ Domains for Their Ligands[†]

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ABSTRACT: PDZ domains are modular protein–protein interaction domains that bind to specific C-terminal sequences of membrane proteins and/or to other PDZ domains. Certain PDZ domains in PSD-95 and syntrophins interact with C-terminal peptide ligands and heterodimerize with the extended nNOS PDZ domain. The capacity to interact with nNOS correlates with the presence of a Lys residue in the carboxylate-binding loop of these PDZ domains. Here, we report that substitution of an Arg for Lys-165 in PSD-95 PDZ2 disrupted its interaction with nNOS, but not with the C terminus of the Shaker-type K⁺ channel Kv1.4. The same mutation affected nNOS binding to α 1- and β 1-syntrophin PDZ domains to a lesser extent, due in part to the stabilizing effect of tertiary interactions with the canonical nNOS PDZ domain. PDZ domains with an Arg in the carboxylate-binding loop do not bind nNOS; however, substitution with Lys or Ala was able to confer nNOS binding. Our results indicate that the carboxylate-binding loop Lys or Arg is a critical determinant of nNOS binding and that the identity of this residue can profoundly alter one mode of PDZ recognition without affecting another. We also analyzed the effects of mutating Asp-143, a residue in the α B helix of α 1-syntrophin that forms a tertiary contact with the nNOS PDZ domain. This residue is important for both nNOS and C-terminal peptide binding and confers a preference for peptides with a positively charged residue at position –4. On this basis, we have identified the C terminus of the Kir2.1 channel as a possible binding partner for syntrophin PDZ domains. Together, our results demonstrate that single-amino acid substitutions alter the specificity and affinity of PDZ domains for their ligands.

PDZ¹ domains are modular protein–protein interaction domains that are specialized for binding to specific amino acid sequences located at the C terminus of membrane proteins and/or to other PDZ domains (1, 2). PDZ domains were originally identified as conserved sequence elements in PSD-95, the *Drosophila* tumor suppressor protein Dlg and the mammalian tight junction protein ZO-1 (3). The overwhelming majority of proteins containing PDZ domains are associated with the plasma membrane (4). Indeed, PDZ domain interactions appear to be a general mechanism for localizing membrane proteins to specific subcellular domains and for coupling them to proteins in signal transduction pathways. In many cases, when proteins containing PDZ

domains are coexpressed with their ligands in heterologous cells, they become coclustered in the plasma membrane (4–6).

PDZ domains recognize their target proteins with remarkable selectivity. Most known PDZ domain-mediated interactions occur by the recognition of short C-terminal peptide motifs and require that the peptide ligands have a free carboxylate group (7). The molecular basis for this type of recognition by PDZ domains has been clarified by the X-ray crystallographic structure of PDZ domains in complex with their cognate peptide ligands (8, 9). An important conclusion from the three-dimensional structures is that the specificity of binding is conferred by at least four to five residues at the C terminus of the peptide, although other upstream residues may contribute additional specificity (10). Another important aspect of peptide recognition by PDZ domains is the ability to stabilize the terminal carboxylate group of the peptide. Not surprisingly, the amino acid sequence of the carboxylate-binding loop, the region that cradles the C-terminal carboxylate group of the peptide, is conserved among many different PDZ domains (8). Highly conserved residues in the carboxylate-binding loop include the G-L-G-F signature sequence and an almost invariant Arg or Lys residue. The ϵ -amide nitrogen of this residue coordinates the terminal carboxylate group of the peptide ligand via a highly ordered water molecule, an interaction that appears to be critical for peptide binding (8).

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¹ Abbreviations: PDZ, PSD-95/Dlg/ZO-1; PSD-95, 95 kDa postsynaptic density protein; nNOS, neuronal nitric oxide synthase; Dlg, discs-large; ZO-1, Zona Occludens-1; PH, pleckstrin homology; SU, syntrophin unique; GST, glutathione S-transferase; PCR, polymerase chain reaction; SPR, surface plasmon resonance; CD, circular dichroism.

A second type of PDZ domain interaction is not dependent upon recognition of a carboxy-terminal motif (2). A well-characterized example is the binding of the PDZ domain of neuronal nitric oxide synthase (nNOS) to the second PDZ domain of PSD-95 or to the PDZ domain of α 1-syntrophin (11). The former couples nNOS to NMDA receptor activation at synaptic sites in the brain, while the latter localizes nNOS to the dystrophin complex in skeletal muscle (11). These interactions may regulate nNOS activity and, consequently, the action of NO by targeting the enzyme to specific intracellular membrane domains. The structure of the nNOS—syntrophin complex has revealed that an internal peptide motif following the consensus nNOS PDZ domain (amino acids 1–99) mediates the so-called PDZ–PDZ heterodimerization (12). These additional amino acids (amino acids 100–130) form a “pseudopeptide” extension, which adopts a β -hairpin finger structure that interacts with the binding pocket of the PDZ domain of α 1-syntrophin (and PSD-95). Cyclic peptides containing an internal PDZ recognition motif that bind to syntrophin PDZ domains may adopt a similar conformation, since a constrained structure induced by disulfide-bonded cysteine residues is necessary for their interaction (13).

In this report, we have generated recombinant PDZ domains of PSD-95 and syntrophins with single-amino acid substitutions that alter the binding specificity and affinity for the extended nNOS PDZ domain and for C-terminal peptide ligands. Specific mutations were identified that disrupted one mode of PDZ recognition without affecting another. Collectively, our results indicate that the Lys or Arg residue in the carboxylate-binding loop plays a critical role in the selection of ligands and that subtle changes at key residues can affect the specificity and affinity of PDZ domains for their ligands.

EXPERIMENTAL PROCEDURES

Wild-Type and Mutant PDZ Domain cDNA Constructs. Constructs encoding individual PDZ domains of α 1-, β 1-, and β 2-syntrophin have been described previously (14). The γ 1-syntrophin PDZ domain (amino acids 56–136) was generated using the polymerase chain reaction (PCR) with the following primers using a human cDNA as a template: forward (5'-ATATGAATTCTTCTATTCTGGTGAAGAA-CGGTG) and reverse (5'-ATATCTCGAGTGGGAGTTT-GAGGAAAGCAGGTG). The *Eco*RI and *Xho*I restriction sites are underlined. The γ 1-syntrophin cDNA was a generous gift from L. Kunkel (Harvard University, Cambridge, MA). The R62K mutant was amplified using the reverse primer (above) and 5'-ATATGAATTCGAAA-GAACGGTGACCATCAGAAAACAAACAGTAGGAGG. To generate mutants of PSD-95 PDZ domains, a cDNA encoding full-length rat PSD-95 (15) (accession number M96853) was used as a template for PCR (a gift from M. Sheng, Harvard University). The following primers were used: K165R, 5'-AGAGGAATTCGAAAAGGTCATG-GAGATCAAACATCAGAGGGCCTAAAGGACTTGGC; R70K, 5'-AGAGGAATTCATGGAGTATGAGGAGAT-CACATTGGAAAAGGGTAACTCAGG; and R70A, 5'-AGAGGAATTCATGGAGTATGAGGAGATCACATTGG-AAAAGGGTAACTCAGG.

The following wild-type PDZ domain constructs of PSD-95 were also provided by M. Sheng and have been

described previously (5): PDZ1(41–161), PDZ2(154–267), and PDZ3(302–401). The numbers in parentheses indicate the amino acids encoded by each construct. A construct encoding Chapsyn-110 PDZ3(418–503) has also been described (10). A cDNA encoding the N-terminal domain of nNOS (amino acids 1–150) was a gift from K. Christopherson and D. Bredt (University of California, San Francisco, CA) (16).

Expression and Purification of Recombinant Proteins. PCR products were subcloned into the *Eco*RI–*Xho*I sites of the pET-32a expression vector (Novagen, Inc., Madison, WI) which contains sequences encoding thioredoxin, an N-terminal 15-amino acid S tag, and a hexahistidine nickel binding motif. Clones with the correct sequence were electroporated into BL21(λ DE3)pLysS competent cells and grown overnight in 2 mL of LB medium containing 200 μ g/mL ampicillin. Following centrifugation, the cells were resuspended in 1 mL of fresh LB medium. One liter of LB medium (containing ampicillin) was inoculated with the cells, then grown at 37 °C for ~3 h, and induced overnight at 28 °C with 1 mM isopropyl β -D-thiogalactopyranoside (IPTG). The expressed fusion proteins were all recognized by the S-protein HRP conjugate (Novagen) and were purified from the soluble fraction on nickel–Sephacrose columns according to the manufacturer's instructions. The fusion protein purity was determined by Coomassie blue staining of SDS–polyacrylamide gels. The protein concentration was determined by the Bradford method (17).

Peptides. Synthetic peptides corresponding to the 10 C-terminal amino acids of the adult skeletal muscle Na⁺ channel SkM1 (VRPGVKESLV), the embryonic skeletal muscle Na⁺ channel SkM2 (SPDRDRESIV), and the Shaker-type K⁺ channels Kv1.4 (SNAKAVETDV) and Kv1.5 (CLDTSREDTL) were synthesized by Macromolecular Resources (Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO). Peptides corresponding to the 10 C-terminal amino acids of Kir2.1 (PRPLRRESEI) and Kv2.1 (AHGSTRDQSI) were synthesized by the University of Waterloo Department of Chemistry Peptide Synthesis Facility (Waterloo, ON). All peptides contained an additional four-amino acid linker (SGSG) at the N terminus and an N-terminal biotin.

Blot Overlay Assays. Blot overlay assays were carried out as described previously (11, 14) with the following modifications. Signals generated by enhanced chemiluminescence were captured with a Digital Image Station (Kodak). The net intensity of each band was determined using the software provided by the manufacturer. The results were normalized to the amount of fusion protein loaded in each lane as determined by densitometric analysis of Ponceau S-stained blots captured digitally prior to blocking. Results are the average of three separate experiments \pm the standard deviation. The Student's unpaired *t* test was used to test for differences in nNOS binding to the wild-type and K86R for four independent experiments.

Surface Plasmon Resonance. All experiments were performed on a BIAcore 3000 instrument (BIAcore Inc., Piscataway, NJ). Biotinylated peptides were immobilized on Neutravidin-coated sensor chips as described previously (21). Increasing concentrations of syntrophin PDZ domains were injected onto the peptide surfaces at a flow rate of 20 μ L/min for 2 min in 20 mM HEPES (pH 7.5), 150 mM NaCl,

3 mM EDTA, and 0.005% surfactant P-20. Between successive measurements, the surfaces were regenerated with 10 mM HCl (2 min contact time). Binding to Neutravidin (Pierce, Rockford, IL) alone was also assessed and was used to subtract nonspecific interactions. Blank sensorgrams recorded for injection of running buffer were also subtracted. Background-corrected sensorgrams were fitted to a single-site binding model using the numerical integration functions of the BIAevaluation 3.0 software (BIAcore AB, Uppsala, Sweden).

Circular Dichroism (CD). CD measurements were performed on an AVIV 62ADS circular dichroism spectrophotometer equipped with a temperature controller. Cells with a path length of 1 mm were used. A 5 s time constant with a 1 nm bandwidth was used during acquisition over a wavelength range of 190–260 nm. Buffer conditions were 10 mM potassium phosphate at pH 8.0. Three spectra were recorded and averaged for each protein. Spectra for buffer were collected in a similar manner and subtracted. Protein concentrations for CD analysis were determined spectrophotometrically using extinction coefficients calculated from amino acid composition. All measurements were performed at 20 °C.

RESULTS

A Conserved Lys or Arg Residue in PDZ Domains of PSD-95 Is a Key Determinant for Interaction with nNOS. PSD-95 contains three tandem PDZ domains that have distinct binding specificities. The second PDZ domain, which has been shown to interact with the PDZ domain of nNOS (11, 12), has a Lys residue in the carboxylate-binding loop (at the position corresponding to Arg-318 in the structure of the third PDZ domain of PSD-95; residues shaded in Figure 1A). The first and third PDZ domains, which do not bind nNOS, each have an Arg residue at this position. On the basis of these observations, we hypothesized that a Lys residue may be required for interaction with nNOS and, conversely, that an Arg residue may be incompatible with nNOS binding. To test this idea, we substituted Lys-165 in the second PDZ domain of PSD-95 with Arg and expressed both the wild-type (PDZ2) and mutant (K165R) PDZ domains as thioredoxin fusion proteins in bacteria. Both were expressed at high levels and were highly soluble. Their ability to interact with nNOS was assayed by overlaying blots with a fusion protein containing the canonical nNOS PDZ domain (amino acids 1–99) as well as an additional ~30 amino acids downstream that are required for high-affinity binding to PSD-95 (11, 12, 16). As shown in Figure 1B, the wild-type PDZ2 domain bound strongly to nNOS. In contrast, the K165R mutant failed to give a detectable signal, even at longer exposure times (Figure 1B and data not shown).

Previous studies have shown that PDZ2 of PSD-95 also interacts with the C terminus of NMDA receptor NR2B subunits (18) and with Shaker-type Kv1.4 K⁺ channel α -subunits (5). To determine if the Lys to Arg mutation also affects C-terminal peptide binding, we tested PDZ2 and K165R for the ability to bind a biotinylated peptide corresponding to the last 10 amino acids of Kv1.4 in overlay assays. A previous study has shown that the last 11 amino acids of Kv1.4 are sufficient, and the last four amino acids are required, to interact with PDZ2 (5). As expected, PDZ2

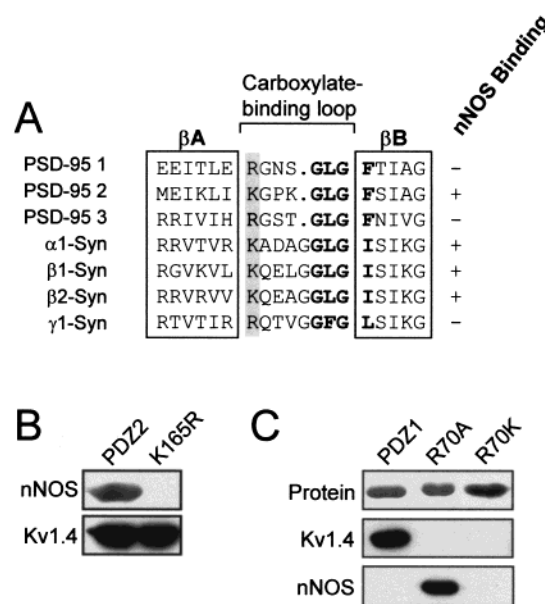


FIGURE 1: Mutations in the carboxylate binding loop sequences of PSD-95 PDZ domains selectively disrupt binding to nNOS or Kv1.4. (A) Comparison of carboxylate binding loop sequences from PSD-95 PDZ domains 1–3 and syntrophin PDZ domains. Shown are the amino acid sequences of the β A and β B strands and the intervening carboxylate binding loop. The conserved Arg or Lys residues in the carboxylate binding loops are shaded. The GLGF (GLGI in the case of syntrophins) motifs are in bold. The ability to interact with nNOS is indicated with a + or – to the right of each sequence. (B and C) Blot overlay analysis of wild-type and mutant PSD-95 PDZ domains. Approximately equal amounts of the indicated PDZ domain fusion proteins were analyzed by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were either stained for total protein with Ponceau S (Protein) or overlaid with a peptide corresponding to the 10 C-terminal amino acids of Kv1.4 (Kv1.4) or a fusion protein (amino acids 1–150) containing the PDZ domain of nNOS (nNOS).

bound the Kv1.4 peptide (Figure 1B). Interestingly, K165R bound Kv1.4 to the same extent as PDZ2. Thus, the Lys to Arg substitution disrupts the ability of PDZ2 to bind nNOS without affecting its ability to bind Kv1.4. This suggests that the lack of nNOS binding was not due to nonspecific structural alterations in the protein caused by the introduced mutation. Thus, an Arg residue in the carboxylate-binding loop of PSD-95 PDZ domains appears to be incompatible with nNOS binding.

Given the results described above, we reasoned that it might be possible to induce nNOS binding in a PDZ domain that does not normally have this activity. To this end, we substituted Arg-70 in PDZ1 of PSD-95 with Lys (R70K) and expressed the mutant as a bacterial fusion protein. PSD-95 PDZ1, like PDZ2, binds the C terminus of Kv1.4, albeit less strongly (5). In agreement with these results, we found that the recombinant PDZ1 domain was able to bind the C terminus of Kv1.4 (Figure 1C, PDZ1). In addition, PDZ1 did not bind to nNOS as expected. Interestingly, the R70K mutation did not induce nNOS binding (Figure 1C, R70K). However, the R70K mutant also failed to bind the C terminus of Kv1.4, suggesting that the mutation disrupted the PDZ domain binding site. Consistent with this idea, R70K was expressed at low levels compared with the wild-type PDZ1 domain.

We constructed an additional mutant in which we replaced Arg-70 of PDZ1 with Ala (R70A). Remarkably, and in

contrast to the wild-type PDZ1 domain, R70A strongly bound nNOS (Figure 1C, R70A). However, R70A failed to bind Kv1.4. Thus, a single-amino acid substitution (Arg to Ala) confers nNOS binding on PDZ1 of PSD-95, but disrupts C-terminal peptide binding. These results suggest that an Arg or Lys residue in the carboxylate-binding loop of PDZ domains is required for binding to C-terminal peptides and are consistent with this residue being highly conserved. In addition, these data demonstrate that an Arg or Lys residue is not absolutely required for nNOS binding.

Effect of Lys to Arg Substitution on Syntrophin PDZ Domain Interactions. Besides the second PDZ domain of the closely related PSD-95 family member PSD-93/Chapsyn-110, the only other PDZ domains that bind nNOS are found in a family of proteins called syntrophins, intracellular peripheral membrane proteins of the dystrophin-associated protein complex (11, 14, 19). The three well-characterized syntrophin isoforms (α 1, β 1, and β 2) share a common domain organization which includes two tandem PH domains, a C-terminal domain unique to syntrophins (SU), and a single PDZ domain (19–22). α - and β -syntrophin PDZ domains all bind nNOS and have a Lys residue in the carboxylate-binding loop (Figure 1A) (11, 14). To determine if this Lys is critical for nNOS binding, we substituted Lys-86 in the α 1-syntrophin PDZ domain with Arg (K86R) and compared the ability of K86R and wild-type α 1-PDZ to bind nNOS using overlay assays. We quantified the amount of nNOS bound using enhanced chemiluminescence detection and a digital gel documentation system. The results were expressed as the amount of each ligand bound to K86R as a percentage of binding to the wild-type (see Experimental Procedures). When assayed by this method, the level of binding of nNOS to K86R was decreased by $\sim 15\%$ in comparison with that of the wild-type α 1-PDZ domain (Figure 2A, nNOS). Thus, in comparison with PSD-95 PDZ2, the substitution of Arg for Lys in α 1-PDZ has a measurable but much less dramatic effect on nNOS binding.

We have previously shown that syntrophin PDZ domains interact with the C terminus of the skeletal muscle Na⁺ channel α -subunits, SkM1 and SkM2 (14). Syntrophin PDZ domains also bind to the C terminus of the K⁺ channel Kv1.5.² We evaluated the ability of K86R to bind to C-terminal peptides of these channels and saw little or no difference compared to the wild-type using overlay assays (Figure 2A).

It is likely that small differences in the affinity of the PDZ domains for the peptide ligands would not be detected using overlay assays. Therefore, to more accurately measure the differences, we analyzed the binding of wild-type α 1-PDZ and K86R to peptide ligands using surface plasmon resonance (SPR or BIAcore). Biotinylated SkM1, SkM2, and Kv1.5 peptides were immobilized on a Neutravidin-coated sensor chip, and the level of binding of fusion proteins was measured versus time. At a fusion protein concentration of 2.5 μ M, there was a $\sim 50\%$ reduction in the level of binding of K86R to SkM2 and Kv1.5 peptides in comparison to that of wild-type α 1-PDZ (panels A and B of Figure 3, respectively). There was a similar reduction in the level of binding to SkM1 (not shown). Using SPR data measured

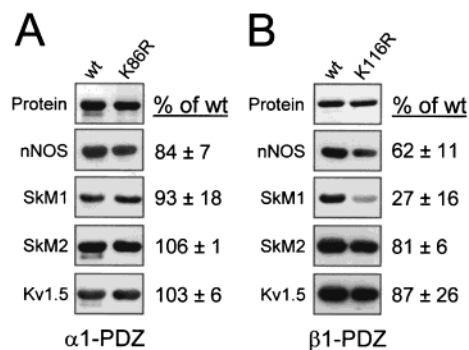


FIGURE 2: Analysis of the binding of nNOS and C-terminal peptides to syntrophin PDZ domain mutants by blot overlay assays. Approximately equal amounts of purified syntrophin PDZ domain fusion proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes. The amount of each protein that was loaded was determined by staining with Ponceau S. Replicate blots were overlaid with an nNOS fusion protein (amino acids 1–150) or with biotinylated peptides corresponding to the C terminus of the Na⁺ channels SkM1 and SkM2 or the K⁺ channel Kv1.5. Bound peptides were detected with streptavidin-labeled horseradish peroxidase followed by enhanced chemiluminescence. Bound nNOS PDZ was detected with a GST antibody followed by goat anti-mouse HRP and chemiluminescence. The amount of bound ligand was quantified using a digital image analysis system and normalized to the amount of protein loaded. The values are expressed as a percentage of ligand bound to the wild-type domain (% of wt) and are the average of three separate experiments \pm the standard deviation. The difference in nNOS binding to wild-type α 1-PDZ and K86R was statistically significant (paired *t* test, *P* < 0.05, *n* = 4).

from a range of concentrations, we calculated dissociation constants for the binding of SkM1, SkM2, and Kv1.5 peptides to α 1-PDZ and K86R (Table 1). For each peptide ligand, the affinity for K86R was about 2-fold lower than for the wild-type.

We analyzed the effect of the same mutation on binding to the PDZ domain of β 1-syntrophin. In this case, the mutation (K116R) had a more pronounced effect on nNOS binding (Figure 2B, nNOS). Quantification of overlay assays revealed a $\sim 40\%$ decrease in the level of binding compared to that of the wild-type domain. Interestingly, the mutation also dramatically decreased the level of binding to SkM1, but did not appreciably alter binding to Kv1.5 or SkM2. The latter result was confirmed by the BIAcore analysis, which showed no decrease in the affinity of the mutant for the SkM2 or Kv1.5 peptides (not shown). Although we were able to detect binding of SkM1 to β 1-PDZ and β 1-PDZK116R in overlay assays, we were unable to obtain sufficient binding of either fusion protein to the immobilized SkM1 peptide to calculate dissociation constants, perhaps due to the lower affinity of SkM1 for syntrophin PDZs. This difference may be explained by the fact that in overlay assays the biotinylated peptide is coupled to streptavidin-labeled horseradish peroxidase prior to binding. This allows formation of a tetrameric complex, which may increase the apparent affinity of the interaction by an avidity effect. Nevertheless, these data indicate that the Lys to Arg mutation has a more dramatic effect on the binding of ligands to β 1-PDZ than to α 1-PDZ and are consistent with the higher affinity of these ligands for α 1-PDZ than for β 1-PDZ.

The PDZ Domain of γ 1-Syntrophin Does Not Bind nNOS. γ 1-Syntrophin is a newly identified syntrophin isoform expressed specifically in the brain and is the most divergent

² S. Gee and S. Froehner, unpublished observations.

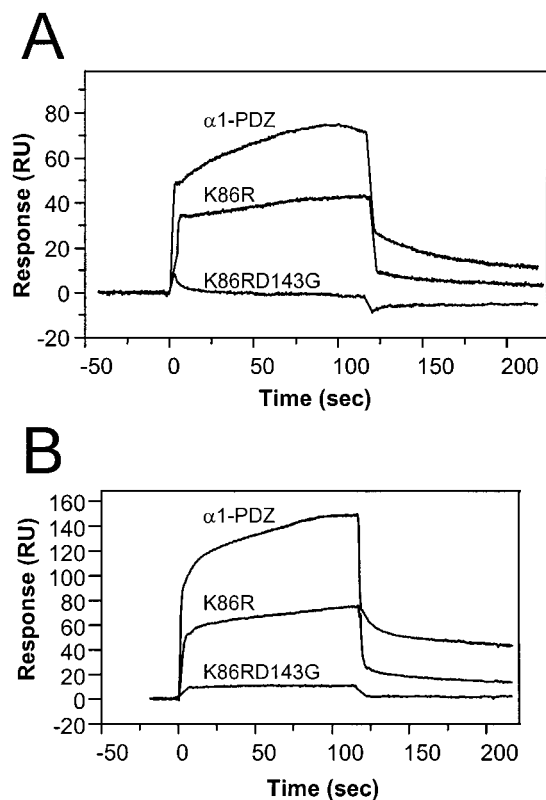


FIGURE 3: Surface plasmon resonance analysis of peptide binding to wild-type and mutant syntrophin PDZ domains. The binding of wild-type and mutant syntrophin PDZ domains to biotinylated peptides immobilized on the surface of a sensor chip was detected by changes in resonance units (RU) over time. (A) Superimposed sensorgrams of the binding of the indicated recombinant PDZ domains to the SkM2 C-terminal peptide at a concentration of 2.5 μ M. (B) Binding of the same fusion proteins to the Kv1.5 C-terminal peptide at 2.5 μ M. In both instances, the K86R mutation results in a \sim 50% decrease in the level of binding. Together, the K86R and D143G mutations decrease the level of binding to less than 5%. Binding constants derived from these and other sensorgrams are summarized in Table 1.

Table 1: Dissociation Constants (μ M) for Normal and Mutant Syntrophin PDZ Domain Binding to Peptide Ligands^a

syntrophin PDZ domain	peptide ligand		
	Kv1.5	SkM1	SkM2
α 1-PDZ	6.5	24	2.6
α 1-PDZ K86R	12	41	5.7
α 1-PDZ K86R/D143G	nb	nb	35

^a The binding of various concentrations of the indicated peptide ligands to each of the indicated syntrophin PDZ domains was assessed by surface plasmon resonance.

of the known syntrophins (23). In particular, the PDZ domain differs from that of the other syntrophin isoforms at several specificity-determining residues, including the presence of an Arg (amino acid 62) in place of the conserved Lys in the carboxylate-binding loop (Figure 1A). On the basis of the identity of this and other amino acid residues, we predicted that the γ 1-syntrophin PDZ domain would not bind to nNOS. We evaluated nNOS binding to a γ 1-syntrophin PDZ domain fusion protein in overlay assays and found that, as predicted, γ 1-PDZ did not bind (Figure 4A). Thus, γ 1-syntrophin is the only syntrophin isoform identified to date that does not interact with nNOS. This result adds support to the idea that an Arg residue in the carboxylate-binding loop of PDZ

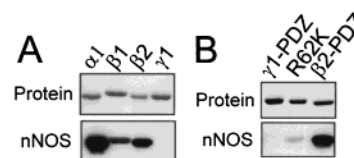


FIGURE 4: The PDZ domain of γ 1-syntrophin does not bind nNOS. (A) Approximately equal amounts of fusion proteins of the PDZ domains of α 1-, β 1-, β 2-, and γ 1-syntrophin were overlaid with the GST-nNOS fusion protein (amino acids 1–150). nNOS bound to α 1-, β 1-, and β 2-PDZs but not to the PDZ domain of γ 1-syntrophin. (B) Partial restoration of nNOS binding to the γ 1-syntrophin PDZ domain via the Arg to Lys substitution. Substitution of a Lys residue for Arg-62 (R62K) in the γ 1-syntrophin PDZ domain induced weak binding of nNOS. Binding to β 2-PDZ is shown for comparison.

domains is incompatible with nNOS binding. Interestingly, γ 1-PDZ does not bind to peptide ligands that interact strongly with α 1-, β 1-, and β 2-syntrophin PDZ domains, but is able to bind to C-terminal peptide ligands with related sequences.³

To determine if Arg-62 is a critical determinant for the lack of nNOS binding of γ 1-PDZ, we analyzed the effect of replacing it with a Lys (R62K). In overlay assays, we found that this substitution partially “restored” nNOS binding, although the binding appeared to be weaker than for other syntrophin isoforms (Figure 4B). The fact that this amino acid change only partially restored nNOS binding suggests that the carboxylate-binding loop Lys or Arg residue is a critical, but not the only, determinant of nNOS binding. Other specificity-determining residues must also contribute to the absence of nNOS binding of this syntrophin isoform (see below).

Tertiary Interactions in PDZ–PDZ Heterodimerization. Membrane association of nNOS in skeletal muscle is mediated by the heterodimerization of the nNOS PDZ domain with the PDZ domain of α 1-syntrophin, an interaction that requires at least 30 additional amino acids downstream of the canonical nNOS PDZ domain (amino acids 1–99) (11, 16). The three-dimensional structure of the complex has revealed that the downstream residues form a stabilized β -hairpin finger that docks in the syntrophin PDZ peptide binding groove, mimicking a peptide ligand (12). Other residues in the syntrophin PDZ domain (Ser-95, Asn-102, Ser-109, His-142, and Asp-143) participate in tertiary interactions with the canonical nNOS PDZ domain to stabilize the β -finger interaction. To directly determine the contribution of one of these residues to the binding of the extended nNOS PDZ domain, we assayed the effect of mutating Asp-143 to glycine (D143G) in α 1-PDZ. As shown in Figure 5A, the level of nNOS binding to D143G was decreased by about 30% in comparison with that of wild-type α 1-PDZ. These data demonstrate that tertiary interactions contribute significantly to the binding of nNOS to α 1-PDZ and may explain the relatively minor effect of the Lys to Arg mutation on nNOS binding to α 1-PDZ.

To determine if the Lys to Arg mutation would have a more severe effect in the absence of this tertiary interaction, we analyzed the effect of mutating both Lys-86 and Asp-143 in α 1-PDZ. The double mutant (K86R/D143G) was expressed at levels equivalent to the wild-type level (Figure 5B, protein). In overlay assays, the level of binding of nNOS to K86R/D143G was decreased by more than 70% compared

³ S. Quenneville, A. Hogan, and S. Gee, unpublished observations.

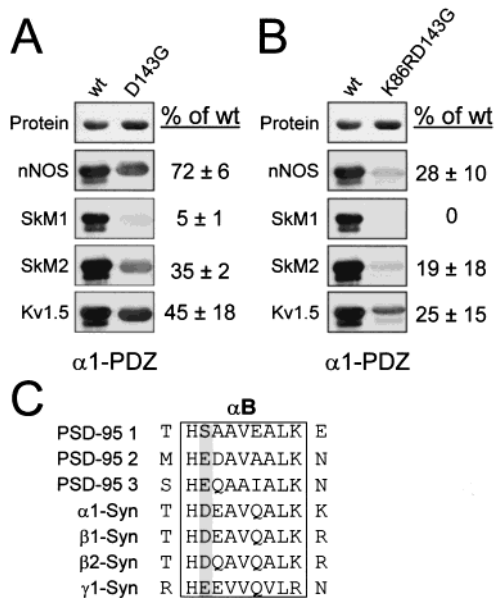


FIGURE 5: Mutation of Asp-143 in the α 1-syntrophin PDZ domain affects binding to both nNOS and peptide ligands. (A) The binding of nNOS and peptide ligands to the wild-type α 1-PDZ domain (α 1-PDZ) and the Asp-143 mutant (D143G) was compared by blot overlay analysis. Purified recombinant PDZ domains were overlaid with an nNOS fusion protein (amino acids 1–150) or with C-terminal peptides of SkM1, SkM2, and Kv1.5. (B) Binding of nNOS and peptide ligands to the wild type and K86R/D143G double mutant. The combination of both mutations dramatically decreases the level of binding to all the ligands that were tested. For both panels A and B, the amount of each ligand bound to the mutant is indicated as a percentage of that bound to the wild-type domain and is shown at the right of each representative blot. Results are the average of three separate experiments \pm the standard deviation. (C) Comparison of α B helix sequences from PDZ domains of PSD-95 and syntrophins. The amino acids corresponding to Asp-143 of α 1-syntrophin are shaded.

to that of wild-type α 1-PDZ (Figure 5B), comparable to the change in nNOS binding to PSD-95 PDZ2 induced by the Lys to Arg substitution. These results indicate that Asp-143 can largely compensate for the Lys to Arg substitution in α 1-PDZ. In the absence of this interaction, the level of nNOS binding is dramatically reduced. Taken together, our results suggest that tertiary interactions contribute more to the binding of nNOS to α 1-PDZ than to PSD-95 PDZ2.

Effect of D143G Mutation on C-Terminal Peptide Binding. We also analyzed the effect of the D143G mutation on C-terminal peptide binding. The binding of SkM1 to D143G was almost completely abolished, and the level of binding of SkM2 and Kv1.5 to D143G was reduced by \sim 65 and \sim 55%, respectively, compared to that of wild-type α 1-PDZ (Figure 5A). These results are in agreement with the solution structure of α 1-PDZ in complex with the SkM1 C-terminal peptide (KESLV), which predicts a salt bridge between the Lys at position -4 of the peptide and Asp-143 (9). The finding that SkM2 (RESIV) and Kv1.5 (RETDL) were less affected by the D143G mutation suggests that an Arg residue at position -4 contributes to a stronger interaction with the syntrophin PDZ domain and is consistent with their higher affinity for wild-type α 1-PDZ (Table 1).

The level of binding of C-terminal peptide ligands to the double mutant was even more dramatically reduced; the binding of SkM1 was not detectable, and the level of binding of SkM2 and Kv1.5 was reduced by \sim 70–80% (Figure 5B).

This is consistent with the more than 10-fold reduction in the affinity of SkM2 for K86R/D143G compared to wild-type α 1-PDZ as determined by SPR (Table 1). The binding of Kv1.5 and SkM1 peptides to the double mutant was too weak to determine affinity constants using SPR. However, at a concentration of 2.5 μ M, the binding of Kv1.5 was reduced to virtually zero (Figure 3B, K86R/D143G). Thus, together, these two substitutions greatly reduce the level of binding to all syntrophin ligands.

One possible explanation for the reduced level of binding of the mutants is that their tertiary structure is partially disrupted. To confirm that the observed reduction in the level of binding was not due to a nonspecific structural alteration in the protein caused by the introduced mutations, we compared the wild-type and mutant domains by circular dichroism (CD) spectroscopy. As shown in Figure 6A, under experimental conditions similar to those used for the SPR measurements, the CD spectra of wild-type α 1-PDZ, K86R, and K86R/D143G were virtually identical, excluding the possibility of gross structural alterations in the mutants. The K116R mutant also appeared to be folded correctly (Figure 6B).

Specificity of Syntrophin PDZ–Peptide Ligand Interactions. Since syntrophin PDZ domains share certain specificity-determining residues with PSD-95 (7), we determined whether PSD-95 PDZ domains could interact with identified C-terminal peptide ligands for syntrophins, like, for example, Kv1.5 and SkM2. When tested in overlay assays, the SkM2 and Kv1.5 peptides bound exclusively to syntrophin PDZ domains and not to PDZ domains of PSD-95 (PDZ1–3), Chapsyn-110 (PDZ3), or nNOS (Figure 7). The absence of binding of these syntrophin ligands to these other PDZ domains further demonstrates the specificity of these interactions.

We next determined whether C-terminal peptide ligands for PSD-95 would interact with syntrophins. Inward rectifying K⁺ channels (Kir) of the 2.x subfamily (Kir2.1 and 2.3) have been reported to bind to PDZ2 of PSD-95 (24). This interaction, which is inhibited by phosphorylation of the Ser residue at position -2 of the peptide, mediates the association of PSD-95 with Kir channels in the brain. Kir2.x channels have C-terminal sequences that are similar to SkM2 and Kv1.5 channels and conform to the consensus sequence previously identified for strong binding to syntrophin PDZ domains (9, 14). In overlay assays, we found that a biotinylated peptide corresponding to the C terminus of Kir2.1 (R-E-S-E-I) bound strongly to α 1- and β 2-PDZ and weakly to β 1-PDZ (Figure 7). Unexpectedly, no binding to PSD-95 PDZ2 was observed, even at longer exposure times. There was, however, weak binding to PDZ1 and -3 and to PDZ3 of Chapsyn-110. To rule out the possibility that PDZ2 was improperly folded, we overlaid a parallel blot with Kv1.4 peptide. Kv1.4 bound strongly to PDZ2 and PDZ1 of PSD-95 as expected. Thus, the inability of PDZ2 to bind Kir2.1 is not due to improper folding of the recombinant protein. From these data, we conclude that PDZ2 does not interact directly with the C terminus of Kir2.1. As an additional test of specificity, we examined whether the C terminus of the Kv2.1 channel (R-D-Q-S-I) could bind to syntrophin PDZ domains. As shown in Figure 7, Kv2.1 did not bind to any of the PDZ domains that were tested.

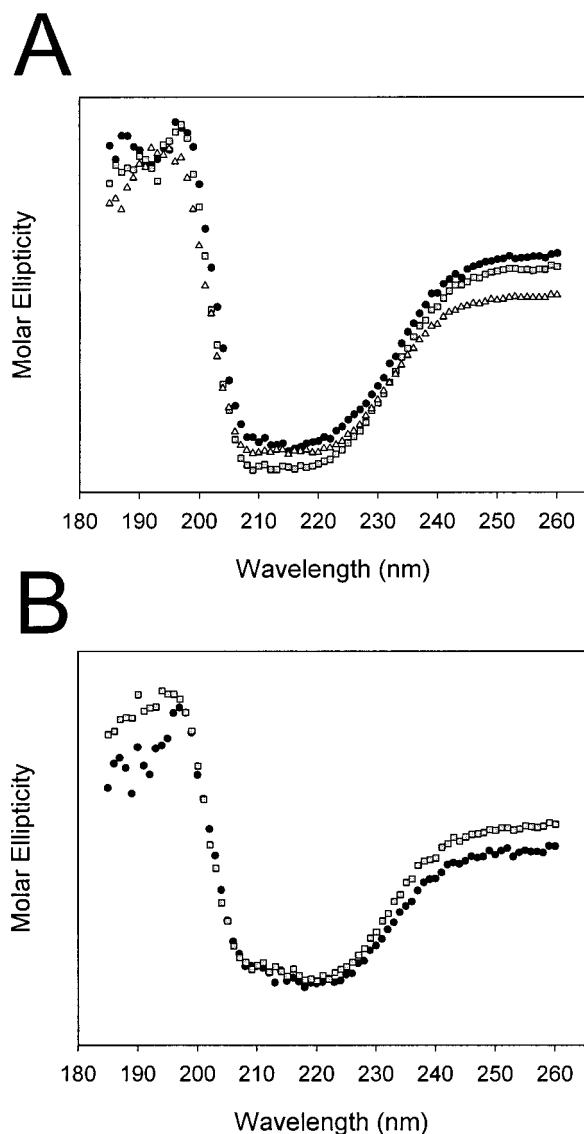


FIGURE 6: CD spectra of wild-type and mutant syntrophin PDZ domains. (A) Spectra of wild-type α 1-PDZ (●), K86R (□), and K86R/D143G (△) are essentially identical. (B) Spectra of β 1-PDZ (●) and K116R (■) are also identical. CD spectra were collected from 190 to 260 nm at 1 nm intervals using an averaging time of 5 s. Spectra were recorded at 20 °C in 10 mM potassium phosphate buffer (pH 8.0). Three individual measurements were averaged and expressed as molar mean residue ellipticity values. The buffer was subtracted from the protein scans. Individual curves were superimposed on one another; therefore, no units are shown.

DISCUSSION

In this report, we have used *in vitro* mutagenesis to examine the importance of a conserved Lys or Arg residue in the carboxylate binding loop of PDZ domains. By analyzing the binding properties of mutant PDZ domains, we found that the identity of this residue is a critical determinant of ligand binding specificity, in particular for heterodimerization with the extended PDZ domain of nNOS. We also examined the importance of Asp-143 in the α B helix of the α 1-syntrophin PDZ domain. This residue is important for tertiary interactions with the PDZ domain of nNOS and also for the binding of C-terminal peptide ligands. Below we discuss our results on the basis of the known structures of PDZ domains in complex with their cognate ligands.

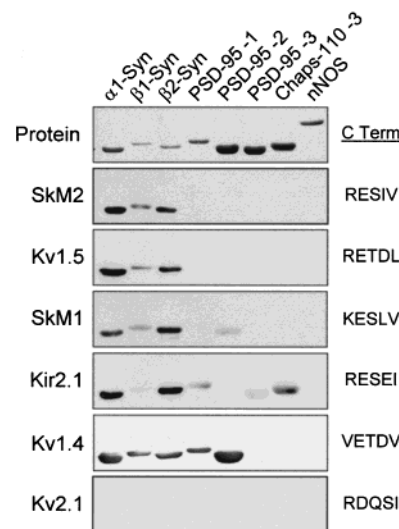


FIGURE 7: Specificity of C-terminal peptides for syntrophin PDZ domains. PDZ domain fusion proteins of syntrophins, PSD-95 (PDZ-1–3), Chapsyn-110 (PDZ3), and nNOS were overlaid with the indicated C-terminal peptides. The amount of each protein that was loaded was determined by Ponceau S staining (Protein). Of the peptides that were tested, only SkM2 and Kv1.5 bound uniquely to syntrophin PDZ domains. The five C-terminal amino acids of each peptide are shown at the right.

Role of the Carboxylate Binding Loop Lys or Arg Residue in PDZ Heterodimerization. A major finding of this study is that a Lys residue in the carboxylate-binding loop of PDZ domains is permissive for heterodimerization with the nNOS PDZ domain, whereas a corresponding Arg residue is restrictive. This conclusion is based on the three following lines of evidence. The first is the observation that PDZ domains with a Lys residue (PSD-95 PDZ2 and α - and β -syntrophins) bind to nNOS, whereas those with an Arg residue (PSD-95 PDZ1, PDZ3, and γ 1-syntrophin) do not. Second, substitution of an Arg for Lys reduced or completely eliminated binding of PDZ domains to nNOS. Third, substitution of a Lys residue for Arg-62 in the PDZ domain of γ 1-syntrophin was able to partially “restore” nNOS binding. Collectively, our results indicate that the Lys or Arg residue in the carboxylate-binding loop plays a dominant role in the selection of ligands, but they also underscore the idea that additional contact sites within the PDZ domain contribute to ligand specificity (8, 9, 12, 25).

Rational explanations for these results can be obtained by analyzing the structure of the extended nNOS PDZ domain in complex with the α 1-syntrophin PDZ domain. The 30 amino acids downstream of the canonical nNOS PDZ domain form a stabilized β -finger that inserts into the syntrophin PDZ domain peptide binding pocket and mimics a C-terminal peptide ligand (12, 25). The structure also reveals that Lys-86 in the carboxylate-binding loop of α 1-syntrophin forms hydrogen bonds with Thr-152 and Gly-153, residues in the opposing part of the loop. These interactions may help to stabilize the loop structure. The substitution of the Lys with an Arg residue, which is longer by one methyl group, may destabilize the conformation of the carboxylate-binding loop and consequently lower the affinity for nNOS.

In the case of PSD-95, a Lys to Arg substitution in PDZ2 completely abolished its ability to heterodimerize with nNOS, without significantly affecting its ability to interact with the C terminus of Kv1.4. The same Lys to Arg substitution had

a much less dramatic effect on the binding of syntrophin PDZ domains to nNOS. This difference may be due to the compensatory effect of tertiary interactions with the canonical nNOS PDZ domain (discussed below). The absence of strong tertiary interactions with the canonical nNOS PDZ domain may explain the more pronounced effect of the Lys to Arg substitution on nNOS binding to PSD-95 PDZ2. Additionally, differences in the carboxylate-binding loop residues may also play a role. Unexpectedly, we found that substitution of an Ala for Arg-70 in PSD-95 PDZ1 bestowed upon the mutant domain the ability to bind nNOS, even though the wild-type PDZ1 domain does not. From these results, we surmise that the smaller Ala residue does not alter the structure of the carboxylate-binding loop, nor does it hinder the binding of the nNOS β -finger.

Role of the Lys or Arg Residue in PDZ–Peptide Interactions. The identity of the carboxylate-binding loop Lys or Arg residue is also important for PDZ–peptide interactions (8, 9). This was clearly demonstrated by the substitution of Ala for Arg-70 in PSD-95 PDZ1 (R70A), the effect of which was complete abolishment of Kv1.4 binding. This is consistent with the critical role of this residue in the coordination of a water molecule that directly interacts with the carboxylate group of the peptide (8). However, whether this residue is an Arg or Lys appears to be less critical for peptide ligand binding. For instance, there was no observable effect of the Lys to Arg substitution on Kv1.4 binding to PSD-95 PDZ2. Moreover, there was only a small difference in the binding of Kv1.5 and SkM2 peptides to the α 1-syntrophin PDZ domain variant (K86R). However, the effect was more pronounced for SkM1, reflecting the lower affinity of this ligand for wild-type α 1-PDZ.

Tertiary Interactions in PDZ Domain Heterodimerization. The structure of the nNOS–syntrophin heterodimer reveals the importance of tertiary interactions in PDZ recognition (12). We have examined the role of Asp-143 in the α B helix of the α 1-syntrophin PDZ domain, a residue that forms a tertiary contact with Arg-19 in strand A of the nNOS PDZ domain. Substitution of Asp-143 with Gly substantially reduced the level of binding to nNOS, confirming the critical role of tertiary interactions in the PDZ heterodimer complex. Moreover, Asp-143 interaction with Arg-19 was able to compensate for the Lys-86 to Arg substitution in α 1-PDZ (K86R), effectively reducing the effect of this mutation. The equivalent interaction was not able to compensate for the Lys to Arg mutation in PSD-95 PDZ2 (K165R), which completely abolished binding to nNOS, perhaps because the corresponding residue (Glu-226) does not bind as strongly to Arg-19.

Role of Asp-143 in Peptide Ligand Recognition. We have shown that Asp-143 in the α B helix of α 1-syntrophin is also a critical determinant of C-terminal peptide recognition. Mutation of this residue virtually abolished binding to SkM1 and dramatically reduced the level of binding to both SkM2 and Kv1.5. Rational explanations for these effects can be deduced by analyzing the solution structure of the α 1-syntrophin PDZ domain in complex with the peptide G-V-K-E-S-L-V (SkM1) (9). The structure reveals that Asp-143 contacts the Lys residue at position -4 of the peptide, explaining the dependence and the preference of syntrophin PDZ domains for peptides with a positively charged residue at position -4 (9, 14). Consistent with these previous

findings, we now demonstrate that α - and β -syntrophin PDZ domains bind with the highest affinity to peptides with an Arg at position -4 such as SkM2 (R-E-S-I-V) and Kv1.5 (R-E-T-D-L). Peptides with a Lys at position -4 such as SkM1 (K-E-S-L-V) bind less strongly, and those with other residues such as Kv1.4 (V-E-T-D-V) bind less strongly still. Thus, an Arg residue at position -4 of the peptide appears to be optimal for strong interaction with Asp-143.

The differences in the affinity of these ligands for syntrophin PDZ domains may reflect the distinct functional roles of the interactions. For example, it has been shown that mutating the C-terminal Val of Kv1.4 disrupts clustering induced by PSD-95, but does not affect immobilization of Kv1.4 channels (26). Thus, one possibility is that syntrophins “cluster” strongly interacting ligands and only “immobilize” more weakly interacting ligands. The consequences of binding may depend additionally on protein interactions mediated by other syntrophin domains.

Consistent with a preference of syntrophin PDZ domains for peptides with an Arg at position -4 , we found that a peptide corresponding to the 10 C-terminal amino acids of Kir2.1 (R-E-S-E-I) bound strongly to syntrophin PDZ domains. This finding raises the possibility that these channels are binding partners for syntrophins in the brain or elsewhere. The lack of binding of the Kir2.1 C terminus to PSD-95 PDZ2 in overlay assays is surprising given previous results showing association of a fusion protein containing the Kir2.1 C terminus with PSD-95 PDZ2 (24). However, there is at least one report which suggests that additional internal sequences in Kir2.1 mediate the interaction with PDZ2 and, therefore, that the C-terminal T/S-X-V motif is not the sole determinant of its association with PDZ domains (27).

The finding that peptides with an Arg at position -4 bind preferentially to PDZ domains of α - and β -syntrophins and not to other closely related PDZ domains, including those of PSD-95 and γ 1-syntrophin (data not shown), is consistent with Asp-143 being conserved in α - and β -syntrophins; Asp-143 is replaced with a Glu in PSD-95 PDZ2 and γ 1-syntrophin (Figure 5C). Thus, our data support the structure prediction that Asp-143 is a key determinant of peptide binding and demonstrate that interactions with the residue at position -4 of the peptide affect binding affinity and specificity.

In summary, we have shown that single-amino acid substitutions can profoundly affect the binding properties of PDZ domains. Perhaps more importantly, we have identified specific mutations that alter one mode of PDZ recognition without affecting another. Schneider et al. (28) used random mutagenesis and selection procedures to produce PDZ domains with novel recognition properties and showed that these “artificial” PDZ domains could be used to target ligands to different subcellular compartments. Combining these approaches makes it possible to specifically alter the localization or function of one PDZ ligand without affecting another interacting with the same domain.

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