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Cyclic Peptides as Non-carboxyl-terminal Ligands of Syntrophin PDZ Domains*

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Syntrophins, a family of intracellular peripheral membrane proteins of the dystrophin-associated protein complex (DAPC), each contain a single PDZ domain. Syntrophin PDZ domains bind C-terminal peptide sequences with the consensus R/K-E-S/T-*X*-V-COOH, an interaction that mediates association of skeletal muscle sodium channels with the DAPC. Here, we have isolated cyclic peptide ligands for syntrophin PDZ domains from a library of combinatorial peptides displayed at the N terminus of protein III of bacteriophage M13. Affinity selection from a library of X_{10}C peptides yielded ligands with the consensus *X*-(R/K)-E-T-C-L/M-A-G-*X*-Ψ-C, where Ψ represents any hydrophobic amino acid. These peptides contain residues (underlined) similar to the C-terminal consensus sequence for binding to syntrophin PDZ domains and bind to the same site on syntrophin PDZ domains as C-terminal peptides, but do not bind to other closely related PDZ domains. PDZ binding is dependent on the formation of an intramolecular disulfide bond in the peptides, since treatment with dithiothreitol, or substitution of either of the two cysteines with alanines, eliminated this activity. Furthermore, amino acid replacements revealed that most residues in the phage-selected peptides are required for binding. Our results define a new mode of binding to PDZ domains and suggest that proteins containing similar conformationally constrained sequences may be ligands for PDZ domains.

PDZ[1] domains are 80–90-amino acid modules present in numerous eukaryotic proteins. They were first described as a series of three internal, repeated elements within the post-synaptic density (PSD)-95 protein (1). In fact, the name PDZ is derived from three proteins first recognized to contain repeats of this domain: PSD-95; the *Drosophila* discs-large tumor suppressor protein, Dlg; and the mammalian tight-junction protein zona occludens-1, ZO-1 (2–5). PDZ domains have since been identified in a large number of multifunctional proteins, many of which are associated with specialized regions of cell to cell contact such as tight junctions, septate junctions, and synaptic junctions (6). The PDZ domain may be an evolutionarily old domain, as it has been detected in mammalian, nematode, yeast, plant, and bacterial genomic sequences by computer analysis (7).

PDZ domains mediate protein-protein interactions by at least two distinct mechanisms. Certain PDZ domains bind directly to specific recognition sequences at the C terminus of transmembrane proteins. For example, the second PDZ domain of PSD-95 interacts with an S/T-*X*-V-COOH motif in N-methyl-D-aspartate receptor 2B subunits (8, 9) and in Shaker-type potassium channels (10). PDZ domains can also form heterotypic dimers with other PDZ domains. For instance, the N-terminal region of nNOS, which itself contains a PDZ domain, binds directly to PDZ domains in both PSD-95 and α_1-syntrophin, a component of the dystrophin-associated protein complex (DAPC) (11). A third possible mode of interaction, in which the consensus binding sequence is located internally, is suggested by the observation that the PDZ domain in the *Drosophila* InaD photoreceptor protein interacts with a S/T-*X*-V sequence near, but not at, the C terminus of the transient receptor potential Ca^{+2} channel (12). More recently, the PDZ domain in the actinin-associated LIM protein has been shown to bind to unidentified sequences within the internal spectrin-like repeats of α-actinin (13). Thus, PDZ domains may take part in several diverse types of interactions.

The structures of several PDZ domains, alone and complexed with peptides, have been deduced (14–16). The PDZ domain has an overall structure very much like the phosphotyrosine binding domain, even though they are unrelated in function (17, 18). Essentially, it is a globular domain formed by six β strands (designated βA–βF) and two α helices (designated αA and αB) arranged into an up-and-down β-barrel (14, 15). Analysis of the crystal structure of the third PDZ domain of Dlg revealed a groove on the surface that ends in a conserved hydrophobic pocket created by the βB strand, the αB helix, and a loop connecting the βA and βB strands (15). The crystal structure of a S/T-*X*-V-COOH peptide ligand complexed with the third PDZ domain of PSD-95 revealed that the peptide binds within this groove and that the terminal carboxylate group of the peptide inserts into the hydrophobic pocket (14). The specificity of binding is determined by the interaction of

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[1] The abbreviations used are: PDZ, PSD-95/Dlg/ZO-1; PSD-95, postsynaptic density protein of 95 kDa; Dlg, *Drosophila* discs-large tumor suppressor protein; ZO-1, zona occludens-1; DTT, dithiothreitol; ELISA, enzyme linked immunoabsorbent assay; GST glutathione *S*-transferase; NR2B, *N*-methyl-D-aspartate receptor 2B subunit; nNOS, neuronal nitric oxide synthase; SH3, Src homology 3; NaCh, sodium channel; AP, alkaline phosphatase; DAPC, dystrophin-associated protein complex.

certain residues in the PDZ domain with side chains of the last three or four residues of the C-terminal peptide.

The specificities of several different PDZ domains have been defined using combinatorial peptide libraries (16, 19, 20). The peptide ligand preferences of one family of nine PDZ domains was shown to be *X*-S/T-*X*-V/I-COOH, where *X* was either any, or a preferred amino acid, depending on the PDZ domain (19). Recently, the PDZ domain of α_1-syntrophin was shown to be selective for peptides with a similar motif, R/K/Q-E-S/T-*X*-V-COOH (16). The C termini of two skeletal muscle sodium channels (NaChs) conform to this consensus and bind directly to the PDZ domains of α_1-, β_1-, and β_2-syntrophins (21). This interaction mediates the association of NaChs with the DAPC and links them to the cortical actin cytoskeleton and the extracellular matrix.

In this report, we utilized phage-displayed combinatorial peptide libraries to define the specificities of the PDZ domains of α_1-, β_1-, and β_2-syntrophin. We have isolated novel peptide ligands for syntrophin PDZ domains which conform to the consensus sequence K/R-E-(S/T)-*X*-(V/L/I/M)-COOH previously determined to bind syntrophin PDZ domains, but lack a C-terminal carboxylate group. Instead, these peptides are able to interact with syntrophin PDZ domains when fused to either the N terminus of a bacteriophage M13 capsid protein or bacterial alkaline phosphatase (AP). Cyclization of these peptides through intramolecular disulfide bond formation was found to be essential for binding to syntrophin PDZ domains since reduction with DTT, or replacement of cysteines with alanine, eliminated this interaction. These cyclic peptide ligands appear to be specific for syntrophin PDZ domains since several other closely related PDZ domains do not bind them. Additional experiments showed that these peptides bind to the same site on syntrophin PDZ domains as C-terminal peptides and suggest that they act as C-terminal peptide mimetics. Our results define a novel mode of binding of peptide ligands to PDZ domains and suggest that proteins containing similar conformationally constrained peptide sequences may be capable of interacting with other PDZ domain-containing proteins.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Syntrophin PDZ Domain-binding Phage—Glutathione *S*-transferase (GST) fusion proteins of the PDZ domain of mouse α_1-, β_1-, and β_2-syntrophins were prepared in the following manner. Oligonucleotides were designed to flank the PDZ domains and the regions were selectively amplified from cDNA clones by polymerase chain reaction (22). The amplified segments were subcloned between the *Bam*HI and *Eco*RI sites of pGEX (Amersham Pharmacia Biotech) and recombinants were verified by DNA sequencing. GST fusion proteins were expressed and purified according to the manufacturer's instructions.

To screen the phage-displayed combinatorial peptide libraries, microtiter plate wells were first coated with 1 μg of a GST fusion protein of the β_1-syntrophin PDZ domain, blocked with SuperBlock® (Pierce), and then 10^{14} recombinant phage particles were added. After incubation for 2 h at room temperature, the wells were washed of excess phage, and the bound phage were recovered by the addition of 50 μl of glycine, pH 2.0. Two libraries were screened in this manner: X_{12} and X_{10}C, where *X* is any amino acid encoded by the degenerate NN(G/T) codons. These peptides were displayed at the N terminus of mature capsid protein III of phage particles (23). After three rounds of affinity selection, isolates were tested for binding to GST-PDZ fusion proteins by ELISA (Amersham Pharmacia Biotech). The DNA sequences of binding phage were determined and the peptide sequences predicted with MacVector® software (IBI-Kodak, New Haven, CT).

The binding properties of isolated phage were determined by ELISA. Microtiter plate wells were coated with equal amounts of various GST fusion proteins, washed, blocked with bovine serum albumin and then incubated with 10^{11} plaque- forming units. The amount of bound phage was quantitated with anti-phage antibodies conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). The ability of the phage to bind to wells was also monitored in the presence of the reducing agent, DTT.

Construction and Testing of AP Fusion Proteins—The insert of the phage isolate F was amplified by the polymerase chain reaction with flanking oligonucleotides, digested with *Sal*I and *Xba*I, and ligated into pMY101 (24). Bacteria, harboring the resulting recombinant plasmid, secreted a chimeric protein with the peptide fused to the N terminus of *Escherichia coli* AP into the culture medium, upon induction with isopropyl-β-D-thiogalactopyranoside. The conditioned medium was then added to microtiter plate wells coated with various proteins, with or without soluble competitor peptides, and the amount of enzyme retained in the wells after 1 h incubation was quantitated with the colorimetric substrate, *p*-nitrophenyl phosphate. Optical absorbance of wells was measured at 405 nm wavelength with a plate reader (Molecular Devices, Sunnyvale, CA).

Alanine Scan of the Syntrophin PDZ Domain Ligand—Each amino acid of one ligand sequence, AKETCLAGYYC, was independently substituted by alanine and fused to the N terminus of AP using standard molecular biological methods. A peptide ligand-AP fusion, in which the two cysteines were substituted with serines, was generated in the same manner. All 12 chimeric proteins were tested for their ability to bind GST fusion proteins of the α1-, β1-, and β2-syntrophin PDZ domains immobilized on microtiter plates. Wells were coated with 0.5 μg of the purified syntrophin PDZ domain fusion proteins or with anti-FLAG M1 antibody (Boehringer Mannheim), and blocked with 1% bovine serum albumin in 0.1 M $NaHCO_3$, pH 8.3, for 1 h at room temperature. Wells were incubated for 2 h at room temperature with bacterial culture media conditioned with each of the peptide ligand-AP fusions (24). After extensive washing with phosphate-buffered saline, pH 7.4, and 0.1% Tween 20, the amount of the AP retained in the wells was quantitated after addition of *p*-nitrophenyl phosphate by measuring optical absorbance at 405-nm wavelength.

Blot Overlay and Affinity Purification Experiments—N-terminal biotinylated peptides corresponding to the phage F insert (AKETCLAGYYCS-COOH), the C terminus of the SkM1 skeletal muscle sodium channel (PGQTVRPGVKESLV-COOH) and the C terminus of Fas (ENSNFRNEIQSLV-COOH) were synthesized. All synthetic peptides contained an N-terminal three amino acid (SGS) linker sequence. The identities of all peptides were determined by mass spectroscopy. Peptides were dissolved either in water, or in Me_2SO, then diluted with water until the organic solvent was less than 1%. The binding of the peptides to various proteins was followed in two ways. Overlay assays with biotinylated peptides (used at a final concentration of 1 μM) were performed as described previously (21). Isolation of syntrophins from skeletal muscle extracts with peptides coupled to streptavidin-linked beads (Sigma) was carried out as described previously (21).

Mass Spectrometry—Measurements of peptide F mass were performed on a Micromass Quattro II triple quadrapole electrospray ionization mass spectrometer. Peptide F was dissolved in neat methanol and infused directly into the electrospray source. The m/z range for data collection was set from 700 to 2000.

Surface Plasmon Resonance Measurements—All experiments were performed on a BIAcore 2000 instrument (BIAcore Inc., Piscataway, NJ) at the University of North Carolina Macromolecular Interactions Facility. Biotinylated peptides were immobilized on Neutravidin (Pierce)-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 μl/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 alone was also measured 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). The steady state binding value, R_{eq}, for each PDZ analyte concentration was then plotted as a function of analyte concentration. To derive binding constants, data from these plots were analyzed by the steady state affinity binding model provided in the BIAevaluation software (25–27). The reported values are the average of four separate experiments.

RESULTS

Isolation of Syntrophin PDZ Domain-binding Phage—Three syntrophin isoforms, α_1, β_1, and β_2, are encoded by separate genes but have an identical domain organization (28, 29). Each syntrophin contains a single PDZ domain, which is 78–84%

Name	Peptide Sequence	No. of Isolates
B, C, L	Y R E T C L A G K M C	3
D, E	M R E T C M A G L W C	2
F, I	A K E T C L A G Y Y C	2
Consensus	X R E T C L A G X Ψ C K M	

FIG. 1. **Alignment of amino acid sequences of syntrophin PDZ domain-binding peptides.** Phage were affinity selected from a X_{10}C peptide library (where X is any amino acid encoded by NN(G/T) codons) with a GST fusion protein of the β_1-syntrophin PDZ domain. Three different sequences were present among the seven isolates. The number of times each peptide was represented by a unique phage isolate is indicated at the *right*. The peptides share the motif X-(R/K)-E-T-C-(L/M)-A-G-Ψ-C, where Ψ indicates any hydrophobic amino acid. Invariant residues in the peptide are *shaded*, and the one fixed cysteine residue in the library is *boxed*.

identical in primary structure among the three isoforms (28). To identify peptide ligands for syntrophin PDZ domains, we screened a bacteriophage M13 library displaying combinatorial peptides of the type X_{12} (where X is any amino acid encoded by NN(G/T) codons) with a recombinant GST fusion protein of the β_1-syntrophin PDZ domain. After three rounds of affinity selection, individual phage isolates were tested for binding. No positive isolates were selected using this library. We next screened a library of the type X_{10}C, where every peptide had 10 random residues followed by a fixed cysteine residue. Seven independent phage were isolated from a library of 10^9 different peptides that encoded three different, but related peptide sequences (Fig. 1). The peptides share the consensus sequence X-(R/K)-E-T-C-(L/M)-A-G-X-Ψ-C-COOH, where Ψ represents a hydrophobic residue.

Specificity of Phage Peptide Isolates—Next, we determined the specificity of three phage isolates, each representing a unique peptide sequence, for different PDZ domains. Microtiter wells coated with equivalent amounts of GST fusion proteins of α_1-, β_1-, and β_2-syntrophin PDZ domains, as well as a fusion protein containing the PDZ domain of nNOS (amino acids 1–150), were incubated with equivalent amounts (10^{10} plaque-forming units) of phage particles. The binding of the phage particles was monitored by ELISA. Positive and negative control targets consisted of GST fused to the Src homology 3 (SH3) domain and GST alone, respectively. Phage isolates B, D, and F bound best to the β_2-syntrophin PDZ domain and nearly equivalently to the PDZ domains of α_1- and β_1-syntrophin (Fig. 2*A*). In contrast, the three phage isolates did not bind to either the nNOS PDZ or Src SH3 domains above background (*i.e.* GST alone). Moreover, phage displaying a peptide ligand for the Src SH3 domain bound to the GST-Src SH3 domain but not to any of the PDZ domains tested. Thus, although the peptides were selected with the β_1-syntrophin PDZ domain, they bound better to the β_2-syntrophin PDZ domain than to either α_1 or β_1. These differences in binding may reflect either the different specificities of the PDZ domains or the ability to retain their native conformations when immobilized on plastic.

To demonstrate that the peptide sequences shown in Fig. 1 are necessary and sufficient for binding, one insert sequence (phage F, AKETCLAGYYC) was cloned into the pMY101 vector (24). Bacteria harboring the resulting recombinant produce an enzymatically active *E. coli* AP fusion protein with the peptide at the N terminus and secrete it into the culture supernatant. Equivalent amounts of this fusion protein (designated peptide F-AP) or a Src SH3 ligand-AP fusion protein were incubated in microtiter wells containing a variety of GST fusion proteins. As shown in Fig. 2*B*, peptide F-AP bound best to the PDZ domain of β_2-syntrophin and somewhat less well to the PDZ domains of α_1- and β_1- syntrophin, mirroring the specificity observed in the phage ELISA experiment. Moreover, peptide F-AP did not bind to the PDZ domain of nNOS, the Src SH3 domain, or GST

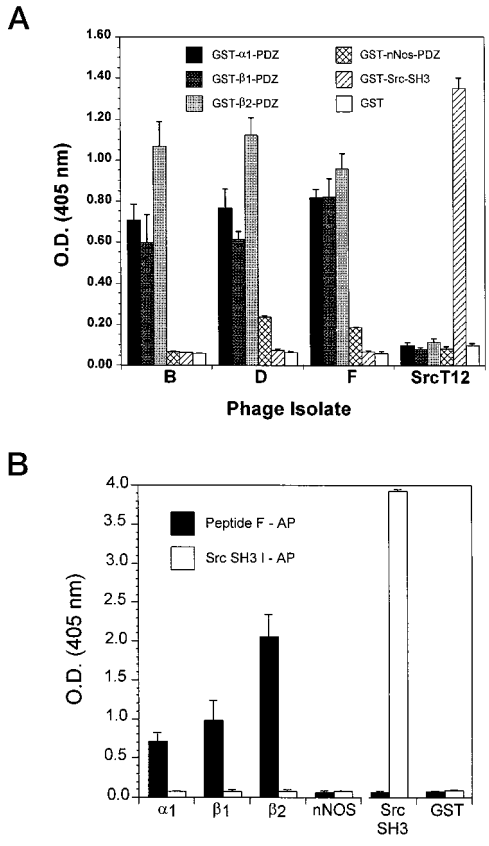

FIG. 2. **Binding specificity of phage isolates.** *A*, phage isolated with the β_1-syntrophin PDZ domain bind to all three syntrophin PDZ domains. Equivalent numbers of phage particles were added to microtiter wells coated with 1 μg each of GST fusion proteins of the PDZ domains of α_1-, β_1-, or β_2-syntrophin (*GST-α_1-PDZ, GST-β_1-PDZ, or GST-β_2-PDZ*), the PDZ domain of nNOS (*GST-nNOS PDZ*), or the SH3 domain of Src (*GST-Src SH3*). The amounts of three β_1-syntrophin PDZ-binding phage (*B, D,* and *F*) and one Src SH3-binding phage (*SrcT12*) bound to these proteins or to GST alone (*GST*) was determined by phage ELISA. *B,* the phage F insert binds to syntrophin PDZ domains when fused to the N terminus of AP. Equivalent amounts of the phage F insert fused to bacterial AP (*Peptide F-AP*) or the Src SH3-ligand fused to AP (*Src SH3 l-AP*) were added to microtiter wells coated with 1 μg of various target GST fusion proteins (noted below the *x axis*). Binding was monitored by optical absorbance at 405 nm after addition of *p*-nitrophenyl phosphate to the wells. For *A* and *B*, values are the average of triplicate determinations from one representative experiment. *Error bars* indicate standard error of the mean.

alone. In contrast, the Src SH3 ligand-AP fusion bound only to the GST-Src SH3 fusion protein. These results demonstrate that the phage F peptide sequence is sufficient for binding to all three syntrophin PDZ domains, even when fused to the N terminus of AP.

Isolated Peptides Require Conformational Constraint for Binding—Even though the location of only one cysteine was specified within the X_{10}C peptides, all three isolates had a second cysteine spaced six residues from the first. To determine whether or not these cysteine residues were disulfide-bridged in the phage-displayed peptides, we tested the effect of the reducing agent DTT on binding. Equivalent numbers of phage particles were incubated in microtiter wells coated with approximately equal amounts of the β_1-syntrophin PDZ domain fusion protein and the extent of binding was determined by phage ELISA. As shown in Fig. 3, binding of three phage isolates (B, E, and F) to the target decreased with increasing DTT concentration. Fifty percent inhibition (IC_{50}) was observed at approximately 2–5 mM DTT. Since PDZ domains lack disulfide bonds, this decreased binding can be attributed to the reduction of disulfide bonds in the displayed peptides by DTT.

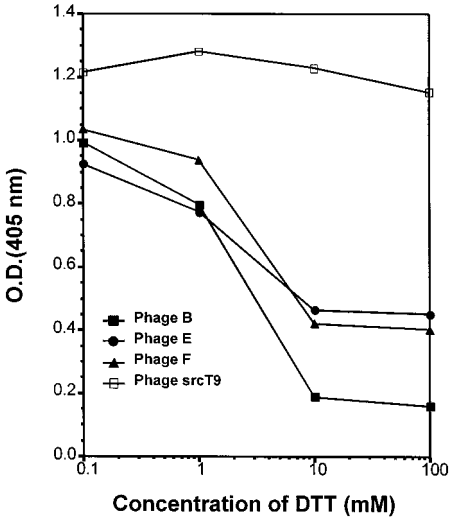

FIG. 3. **Effect of the reducing agent DTT on the binding of phage isolates to the PDZ domain of β_1-syntrophin.** Equivalent amounts (10^{11} plaque-forming units) of phage particles (PDZ binding phage clones B, E, and F and Src SH3 binding clone T9) were added to microtiter wells coated with GST fusion proteins of either the β_1-syntrophin PDZ domain or the Src SH3 domain, in the presence of varying concentrations of DTT. The amount of bound phage particles was monitored by phage ELISA. The optical density (*O.D.*) in single wells was determined at 405 nm. The binding of the PDZ phage, but not the Src SH3 phage (*src T9*), was sensitive to DTT. Note that disulfide-bridged cysteines are not present in either the syntrophin PDZ domain, or the Src SH3 domain and its binding phage.

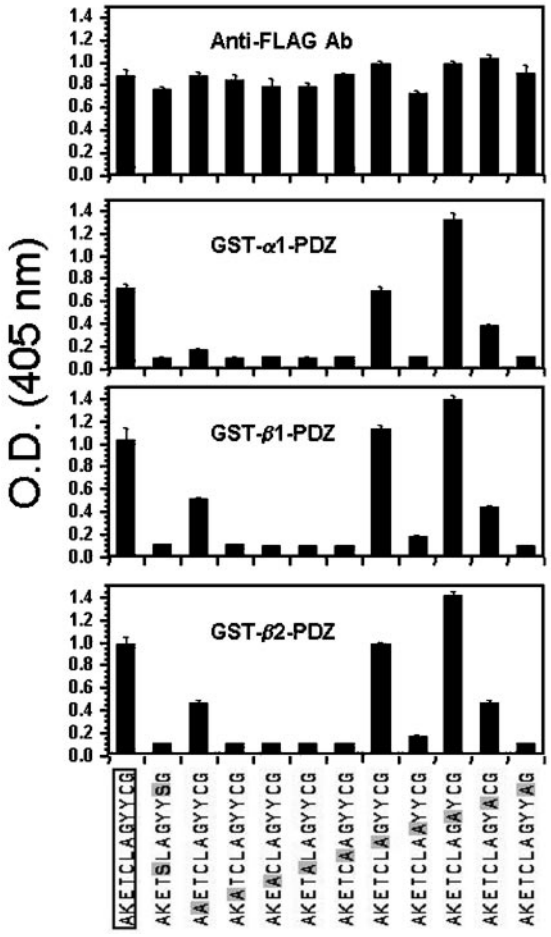

FIG. 4. **Alanine scan of the phage F peptide sequence.** Equivalent amounts (0.5 μg/well) of the α_1-, β_1-, and β_2-syntrophin PDZ domain fusion proteins or anti-FLAG M1 antibody were immobilized in microtiter plate wells and incubated with culture media conditioned by the indicated variants of the phage F peptide sequence (A-K-E-T-C-L-A-G-Y-Y-C) fused to a secreted bacterial AP. All the peptide-AP fusions carried the FLAG epitope at their N termini. Interaction between the FLAG epitope and anti-FLAG M1 antibodies was used to assure exposure of the wells to equivalent amounts of AP fusion protein. The amount of the AP enzyme activity retained in the wells was determined spectrophotometrically by optical absorbance at 405 nm for triplicate samples. *Error bars* indicate standard error of the mean. The wild type peptide F sequence is *boxed*. Alanine and serine substitutions in the peptide ligand sequence are *shaded*.

In contrast, the binding of a Src SH3 domain-binding phage (30) to the Src SH3 domain was unaffected over the same range of DTT concentrations. In this case neither the SH3 domain nor the peptide ligand have cysteines. The binding of the peptide F-AP fusion protein to syntrophin PDZ domains was also DTT sensitive (not shown) suggesting that it forms disulfide bonds as well. These data suggest that conformational constraint imparted by the disulfide bond in these peptides is required for binding to syntrophin PDZ domains.

To test this idea directly, we substituted both cysteines in the peptide F sequence with serines. This simultaneous replacement completely abolished binding of the peptide F-AP fusion to all three syntrophin PDZ domains (Fig. 4), confirming the importance of the structural constraint of the peptide for binding. To evaluate the contribution of each residue in the peptide to the binding of syntrophin PDZ domains, we produced peptides with alanine substitutions at each position. Consistent with the results of the serine replacement, alanine-substitution of either cysteine resulted in a complete loss of binding (Fig. 4). Furthermore, substitution of most residues in the peptide greatly diminished binding to all three PDZ domains, whereas alanine-substitution of the lysine at position 2 (numbering from the N terminus) and the tyrosine at position 10 resulted in only a partial loss of binding. Interestingly, alanine-substitution of the tyrosine at position 9 improved the apparent binding to all three syntrophin PDZ domains. This residue was the least conserved among the three initial peptide ligands for the β_1- syntrophin PDZ domain, suggesting that there is potential to improve the binding efficiency of this ligand further by varying the identity of the residue at this position.

Isolated Cyclic Peptides are Structural Mimics of C-terminal Peptides with an S/T-X-V Motif—We recently reported that peptides corresponding to the C terminus of two skeletal muscle sodium channels (designated SkM1 and SkM2) with the motif R/K-E-S/T-*X*-V-COOH, bound strongly to syntrophin PDZ domains (21). Schultz *et al.* (16) obtained a similar consensus (R/K/Q-E-S/T-*X*-V-COOH) for α_1-syntrophin PDZ domain ligands using combinatorial peptide libraries. Together, our results define the ligand preferences of syntrophin PDZ domains. Close inspection of the consensus sequence in Fig. 1 (*X*-R/K-E-T-C-L/M-A-G-*X*-Ψ-C) revealed a remarkable likeness (residues underlined) to the C-terminal consensus for binding to syntrophin PDZ domains. To test the hypothesis that the phage-displayed peptides isolated here bind syntrophin PDZ domains at the same site as C-terminal peptides, peptide F-AP and Src SH3 ligand-AP fusion proteins were added to microtiter wells coated with the β_1-syntrophin PDZ domain fusion protein in the presence of increasing concentrations of soluble C-terminal peptides. Binding of peptide F-AP to the β_1- syntrophin PDZ domain was blocked by the SkM1 peptide (K-E-S-L-V-COOH) but not by a peptide corresponding to the C terminus of the Fas antigen (I-Q-S-L-V-COOH), a ligand for the PDZ domain of the Fas-associated protein tyrosine phosphatase PTPL1/Fas-associated protein-1 (31). The IC_{50} value of the SkM1 peptide was ~30 μM. Neither of the two peptides blocked, at any concentration tested, the binding of the Src SH3 ligand-AP to the Src SH3 domain fusion protein.

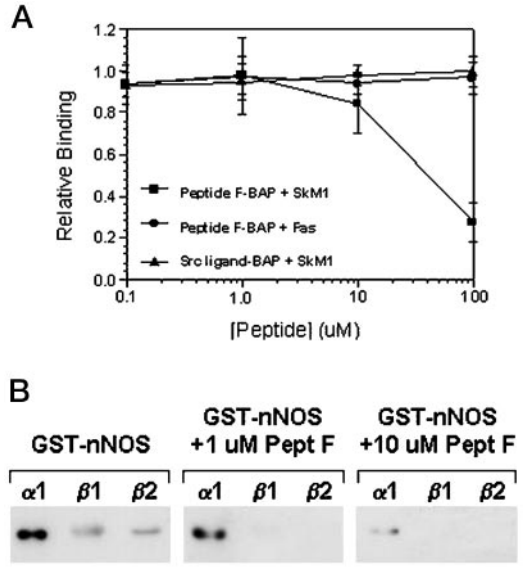

FIG. 5. **Peptide F competes with C-terminal peptides and nNOS for binding to syntrophin PDZ domains.** *A*, competition of peptide F-AP binding to syntrophin PDZ domains by the C terminus of the SkM1 NaCh. Equivalent amounts of the peptide F-AP or Src SH3 ligand-AP fusion proteins were added to microtiter plate wells coated with 1 μg each of GST fusion proteins of the β_1-syntrophin PDZ domain or the Src SH3 domain. Wells were incubated with soluble peptides corresponding to the C-terminal 13 amino acids of the Fas antigen or the SkM1 NaCh. The amount of bound AP fusion proteins was determined colorimetrically by optical absorbance at 405 nm for triplicate samples. *Error bars* indicate standard error of the mean. Values were normalized to the binding levels measured in the absence of added peptide (not shown). *B,* competition of nNOS binding to syntrophin PDZ domains by peptide F. Purified syntrophin PDZ domain fusion proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Identical blots were incubated with a GST-nNOS (1–150) fusion protein either alone (*left panel*) or in the presence of 1 μM (*middle panel*) or 10 μM (*right panel*) peptide F. Bound nNOS was detected with a monclonal antibody to nNOS followed by an horseradish peroxidase-conjugated goat anti-mouse secondary antibody and chemiluminescence.

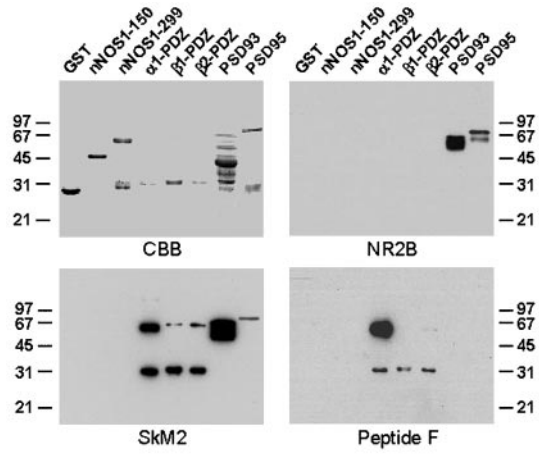

FIG. 6. **Specificity of peptide F.** Approximately 5 μg each of the indicated PDZ domain fusion proteins were resolved by SDS-polyacrylamide gel electrophoresis and were either stained with Coomassie Brilliant Blue (*CBB*), to determine the position and relative amount of each fusion protein, or transferred to nitrocellulose membranes. Identical blots were overlaid with biotinylated peptides corresponding to the C terminus of the NR2B, the SkM2 skeletal muscle NaCh (*SkM2*), or peptide F that had been preconjugated to streptavidin-horseradish peroxidase. Bound peptide-streptavidin-horseradish peroxidase complexes were detected by chemiluminescence. Peptide F binds to both the monomer and dimer forms of syntrophin PDZ domains, but not to the PDZ domains of nNOS, PSD-93, or PSD-95. Molecular mass markers (kilodaltons) are indicated at the *left* of each blot.

The PDZ domain of α_1-syntrophin binds directly to nNOS, presumably by forming PDZ:PDZ heterodimers (11). As previously reported, the SkM2 NaCh C-terminal peptide inhibited the binding of nNOS to syntrophin PDZ domains (21). We tested whether the phage F peptide sequence could similarly block nNOS binding. For these studies we chemically synthesized a peptide corresponding to the phage F insert (designated peptide F). To confirm that the two cysteine residues in the synthetic peptide were disulfide-bonded, the peptide was analyzed by mass spectrometry. The presence of a peak in the mass spectral analysis, corresponding in mass to the peptide lacking two H atoms, confirmed that the peptide was cyclized (data not shown). As shown in Fig. 5*B,* addition of 1 μM peptide F substantially reduced the interaction between nNOS and all three syntrophin PDZ domains, with 10 μM peptide producing near complete inhibition. Since peptide F does not bind to the nNOS PDZ domain (Fig. 2, *A* and *B*), antagonism of the nNOS-syntrophin PDZ interaction presumably occurs via binding of the peptide to the same site of the syntrophin PDZ domain as the nNOS PDZ domain. These data add further support to the conclusion that peptide F binds to the same site as C-terminal syntrophin PDZ ligands.

Peptide F Binds Specifically and with High Affinity to Syntrophin PDZ Domains—We further defined the specificity of the peptide F in overlay experiments using several different PDZ domains, α_1-, β_1-, and β_2-syntrophin PDZ domain fusion proteins, two overlapping nNOS fusion proteins containing the PDZ domain, and fusion proteins of PSD-93 and PSD-95 containing two and three PDZ domains, respectively. When fusion proteins, that had been resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, were incubated with a peptide corresponding to the C terminus of the *N*-methyl-D-aspartate receptor 2B subunit (NR2B), a strong signal corresponding to the position of the intact PSD-93 and PSD-95 fusion proteins was observed, as expected (8, 9, 32) (Fig. 6, *panels CBB* and *NR2B*). In comparison, binding of the NR2B peptide to syntrophin PDZ domains was observed only with longer exposures (not shown), consistent with previous data showing that NR2B peptide binds weakly to the PDZ domain of syntrophins. As shown in Fig. 6 (*panels SkM2* and *Peptide F*), a significant fraction of all three syntrophin PDZ domain fusion proteins consistently migrated in SDS-polyacrylamide gels as ~60-kDa bands, twice the size expected. The basis for this apparent dimerization is not known. As reported previously, a peptide corresponding to the SkM2 NaCh C terminus bound to both the monomeric and dimeric forms of all three syntrophin PDZ domain fusion proteins (Fig. 6, *panel SkM2*) (21). SkM2 also bound to fusion proteins of both PSD-93 and PSD-95. In contrast, peptide F bound to all three syntrophin PDZ domains, but not to the PDZ domains of nNOS, PSD-93, or PSD-95 (Fig. 6, *panel Peptide F*). None of the peptides tested bound to either of the nNOS PDZ domain fusion proteins or to GST alone. These results further demonstrate the specificity of peptide F for syntrophin PDZ domains and that the peptide F sequence can bind syntrophin PDZ domains when removed from the context of a fusion protein.

The affinity of syntrophin PDZ domains for peptide F was measured by surface plasmon resonance. In these experiments, increasing concentrations (31 nM to 4 μM) of syntrophin PDZ domain fusion proteins were allowed to bind to peptide F (Fig. 7*A*) and the SkM2 peptide (Fig. 7*B*) immobilized on a sensor chip. Analysis of the steady-state binding yielded overall K_d values of ~300–800 and ~100–300 nM, respectively. These results are summarized in Table I. In both cases, the β_2-PDZ fusion protein had a somewhat lower affinity than either the α_1-PDZ or β_1-PDZ fusion proteins, even though the β_2-PDZ

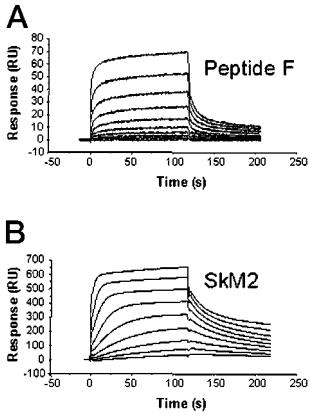

FIG. 7. **Surface plasmon resonance analysis of the peptide F-syntrophin PDZ domain interaction.** The binding of recombinant syntrophin PDZ domains to biotinylated peptides immobilized on the surface of a sensor chip was detected by changes in resonance units (*RU*) over time. *A,* superimposed sensorgrams of the binding of the α_1-syntrophin PDZ domain fusion protein to peptide F at different concentrations (31 nM to 4 μM). *B,* binding of the α_1-syntrophin PDZ fusion protein to the SkM2 NaCh C-terminal peptide over the same range of concentrations. Binding constants derived from these sensorgrams are summarized in Table I.

TABLE I
Dissociation constants for syntrophin PDZ-binding peptides

The binding of various concentrations of the indicated syntrophin PDZ domain fusion proteins to the SkM2 NaCh C-terminal peptide and to peptide F was measured by surface plasmon resonance. The steady-state binding values for each PDZ analyte concentration were plotted as a function of analyte concentration and binding constants were derived using a steady state affinity binding model.

Peptide	Syntrophin PDZ domain fusion protein		
	α_1-PDZ	β_1-PDZ	β_2-PDZ
		nM	
F	359 ± 40[a]	382 ± 88	786 ± 100
SkM2	112 ± 10	217 ± 23	321 ± 18

[a] Values are shown ± S.D. and are the average of four separate determinations from two different experiments. Each determination comprises 10 different fusion protein concentrations.

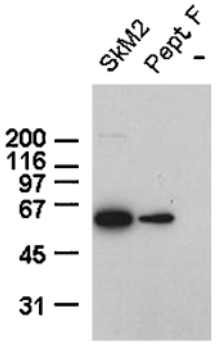

FIG. 8. **Affinity isolation of syntrophins from muscle extracts with peptide F.** Mouse skeletal muscle extracts were incubated with approximately equal amounts of the SkM2 NaCh C-terminal peptide (*SkM2*) or peptide F (*Pept. F*) bound to streptavidin-agarose beads, or with beads alone (−). Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted for syntrophins with a pan-specific monoclonal antibody.

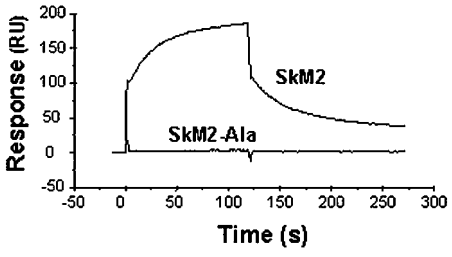

FIG. 9. **The addition of an alanine to the C terminus of the SkM2 NaCh peptide abolishes binding to the α_1-syntrophin PDZ domain.** The binding of purified α_1-syntrophin PDZ domain fusion protein to the SkM2 NaCh C-terminal peptide and to the same sequence with a C-terminal alanine extension (*SkM2-Ala*) was measured by surface plasmon resonance. Robust binding of the α_1-PDZ domain to the SkM2 peptide (*upper trace*) was observed. In contrast, there was no detectable binding of the α_1-PDZ domain to SkM2-Ala (*lower trace*).

domain bound more phage than did α_1 or β_1 in ELISA assays (Fig. 2, *A* and *B*). It is possible that these results reflect differences in the ability of the fusion proteins to retain their native conformation when adsorbed to plastic as compared with that in solution.

We next asked if the affinity of peptide F for syntrophin PDZ domains was sufficient to bind native syntrophins from tissue extracts. Approximately equal amounts of the SkM2 peptide or peptide F were coupled to streptavidin-agarose and used to isolate syntrophins from Triton X-100 extracts of mouse skeletal muscle membranes. Fig. 8 shows that peptide F isolated approximately half the amount of syntrophin than did the SkM2 peptide. Although not strictly quantitative, these results are consistent with the approximately 2-fold higher affinity of SkM2 than peptide F for recombinant syntrophin PDZ domains (Table I). Streptavidin-agarose alone did not bind syntrophins detectably.

Syntrophin PDZ Domains Cannot Accommodate Additional Residues following the Hydrophobic Residue in Linear Peptides—Collectively, our results suggest that peptide F binds to the same site on syntrophin PDZ domains as C-terminal peptide ligands. However, previous studies have shown that PDZ domains cannot accommodate additional residues following the C-terminal hydrophobic residue. For example, the addition of an alanine to the C terminus of Fas abolished binding to the PDZ domains of the protein-tyrosine phosphatase PTPL1/Fas-associated protein-1 (33). One possibility is that the peptide binding groove in syntrophin PDZ domains is long or deep enough to contain additional residues. To test this possibility, we synthesized a peptide corresponding to the C terminus of SkM2 with an additional alanine residue at the C terminus. As shown in Fig. 9, this peptide did not bind to the PDZ domain of α_1-syntrophin. Thus, syntrophin PDZ domains, like other PDZ domains, cannot accommodate additional residues following the hydrophobic amino acid at the C terminus.

DISCUSSION

We have used an N-terminal phage-displayed peptide library to identify cyclic peptides with the motif *X*-R/K-E-T-C-L/M-A-G-X-W/Y-C that bind specifically to syntrophin PDZ domains. These conformationally constrained peptide ligands are similar to the carboxyl-terminal sequence R/K/Q-E-S/T-*X*-V-COOH previously demonstrated to bind syntrophin PDZ domains (16, 21), except that, in these peptides, this sequence lacks a free carboxylate group which has been shown to contribute significantly to the peptide-PDZ domain interaction (14). The binding of the phage-displayed peptides is dependent on cyclization of this sequence by a pair of disulfide-bridged cysteines. Our data suggest that these cyclic peptides mimic C-terminal peptides and may be useful as specific inhibitors of syntrophin PDZ domain interactions.

Phage-displayed libraries have been used to isolate high affinity cyclic ligands for other proteins including integrins (34), the erythropoietin receptor (35), the Grb2 SH2 domain (36), and calmodulin (37). In most cases these peptide ligands do not resemble the primary sequence of the natural interact-

ing proteins. Instead, they act as structural mimics. The intrachain disulfide bonds of these peptides constrain them into a conformation that is favorable for strong binding. Accordingly, we found that substitution of both cysteines with serines, or of either cysteine with alanine, completely eliminated the syntrophin PDZ-binding activity of one cyclic peptide. Consistent with the idea that the cyclic conformation of these peptides is important, we also found that syntrophin PDZ domains did not bind any peptides from a library (X_{12}) encoding linear peptides. This negative result is in agreement with Songyang *et al.* (19), who found that none of the nine PDZ domains they investigated retained specific peptides from a library encoding non-C-terminal linear peptide sequences.

Although the affinity of peptide F for syntrophin PDZ domains was 2–3-fold lower than the SkM2 peptide, the interaction was of sufficient strength to isolate native syntrophins from detergent extracts (Fig. 8). Alanine-substitution of the tyrosine at position 9 of the peptide produced a peptide that bound stronger to all three recombinant syntrophin PDZ domains than the parental sequence. These data suggest that it may be possible to isolate higher affinity peptide ligands for syntrophin PDZ domains by varying the identity of the amino acid at this position. Moreover, these peptides should serve as an excellent starting point for the design of compounds which mimic C-terminal ligands of syntrophin PDZ domains.

Our results are entirely consistent with the idea that the cyclic peptides isolated here bind to syntrophin PDZ domains in the same manner as homologous C-terminal peptides, even though they lack a free carboxylate group. This binding is contingent upon a constrained conformation provided by an intramolecular disulfide bond which may allow residues following the hydrophobic residue (L/M; which is normally at the C terminus) to bend away from the peptide binding groove. This is likely to be an essential feature of these cyclic peptides since additional residues following the hydrophobic residue in linear peptides cannot be accommodated (Fig. 9). Syntrophin PDZ domains also bind to regions of nNOS that contain a PDZ domain (21). Since the PDZ domain of nNOS is not at the C terminus, this interaction cannot occur via a C-terminal peptide sequence. One possibility is that a constrained sequence in nNOS mediates the binding of the nNOS PDZ domain to syntrophin PDZ domains. However, nNOS does not appear to contain any obvious R/K-E-S/T-*X*-L/M/V-related sequence within its PDZ domain, or in sequences upstream of the PDZ domain, which are necessary for strong binding to syntrophin PDZ domains (38),[2] nor does nNOS contain any cysteines in this region which could potentially form intramolecular disulfide bonds. Nevertheless, it should be kept in mind that syntrophin PDZ domains may bind to sequences where flanking amino acids other than cysteines are capable of inducing a secondary structure conducive to binding.

Collectively, our results suggest that the peptide F sequence is specific for syntrophin PDZ domains. Rational explanations for this specificity can be deduced by comparing the solution structure of the α_1-syntrophin PDZ domain complexed with a C-terminal peptide (G-V-K-E-S-L-V-COOH) (16) to the crystal structure of the third PDZ domain of PSD-95 complexed with its peptide ligand (14). Consistent with the idea that syntrophin PDZ domains bind C-terminal peptide ligands in a manner similar to PSD-95, amino acids whose side chains are known to be involved in binding to the general S/T-*X*-V motif in PSD-95 are well conserved in syntrophin PDZ domains (14). Indeed, Schultz *et al.* (16) have shown that the syntrophin PDZ domain binding site is structurally conserved. In contrast, residues that are proposed to determine ligand specificity are strongly conserved in syntrophin PDZ domains, but vary considerably among PDZ domains from different families (16). These findings account for the identical ligand preferences of the three syntrophin PDZ domains (21). Moreover, small differences in these key residues are likely to explain the unique ability of syntrophin PDZ domains to bind peptide F.

An unexpected finding of this study was that the SkM2 NaCh C-terminal peptide bound to the PSD-93 and PSD-95 fusion proteins (Fig. 6, *SkM2*). Since these fusion proteins contain two and three PDZ domains, respectively, we cannot conclude at this time which PDZ domain binds the SkM2 peptide. Interestingly though, of the three PDZ domains in PSD-93 and PSD-95, the second PDZ domain in each most closely resembles syntrophin PDZ domains at the residues described by Schultz *et al.* (16). These similarities may explain why syntrophin PDZ domains bind to some extent to many of the same C-terminal peptide sequences as the second PDZ domain of PSD-93 and PSD-95, including NR2B, Kv1.4, and SkM2 (21)[2] and to the nNOS PDZ domain (11). This observation raises the possibility that the PDZ domains of syntrophins, PSD-93, and PSD-95 compete for similar ligands.

In summary, our results suggest that syntrophin PDZ domains can potentially interact with target proteins via three different modes of binding. (i) Syntrophins bind skeletal muscle NaChs and target them to the DAPC through the interaction of the PDZ domain with C-terminal S/T-*X*-V-COOH sequences (21). (ii) Syntrophins also mediate the membrane localization of nNOS in skeletal muscle by forming presumptive heterodimers with a N-terminal PDZ domain in nNOS (11). (iii) Syntrophin PDZ domains may also be capable of interacting with internal, conformationally constrained, sequences in other proteins. Such proteins may play a role in modulating the localization or function of nNOS and NaChs in skeletal muscle or brain.

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