THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 39, Issue of September 24, pp. 27467–27473, 1999

Printed in U.S.A.

PSD-95 Assembles a Ternary Complex with the *N*-Methyl-D-aspartic Acid Receptor and a Bivalent Neuronal NO Synthase PDZ Domain*

(Received for publication, May 5, 1999, and in revised form, June 21, 1999)

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Nitric oxide (NO) biosynthesis in cerebellum is preferentially activated by calcium influx through *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, suggesting that there is a specific link between these receptors and neuronal NO synthase (nNOS). Here, we find that PSD-95 assembles a postsynaptic protein complex containing nNOS and NMDA receptors. Formation of this complex is mediated by the PDZ domains of PSD-95, which bind to the COOH termini of specific NMDA receptor subunits. In contrast, nNOS is recruited to this complex by a novel PDZ-PDZ interaction in which PSD-95 recognizes an internal motif adjacent to the consensus nNOS PDZ domain. This internal motif is a structured "pseudo-peptide" extension of the nNOS PDZ that interacts with the peptide-binding pocket of PSD-95 PDZ2. This asymmetric interaction leaves the peptide-binding pocket of the nNOS PDZ domain available to interact with additional COOH-terminal PDZ ligands. Accordingly, we find that the nNOS PDZ domain can bind PSD-95 PDZ2 and a COOH-terminal peptide simultaneously. This bivalent nature of the nNOS PDZ domain further expands the scope for assembly of protein networks by PDZ domains.

Efficiency and specificity in cellular signaling cascades is often mediated by assembly of multiprotein transduction networks. Signaling by the calcium/calmodulin regulated neuronal nitric oxide synthase (nNOS)[1] in cerebellar neurons is activated by calcium influx through *N*-methyl-D-aspartic acid (NMDA) receptors (1, 2), but nNOS is not efficiently stimulated by activation of non-NMDA receptors that also generate calcium influx (3). Therefore, a mechanism must exist to specifically couple NMDA receptor-mediated calcium influx to nNOS.

In addition to mediating neuronal functions, nNOS is also found in skeletal muscle, where nNOS localizes to the sarcolemma. Membrane association of nNOS in skeletal muscle is mediated by direct interaction with α1-syntrophin, a component of the dystrophin complex. Binding of nNOS to syntrophin occurs through direct interaction of PDZ protein motifs present near the NH_2 termini of both proteins (4). Mice lacking α1-syntrophin lose nNOS protein and enzyme activity from muscle membranes (5). Furthermore, patients with Duchenne muscular dystrophy and *mdx* mice that lack dystrophin evince a selective loss of nNOS from the sarcolemma (6).

In neurons, nNOS is also associated with cell membranes. In a detailed ultrastructural analysis of primate visual cortex, nNOS immunoreactivity in spines was concentrated over thick postsynaptic specializations of plasma membranes, often in association with NMDA receptors (7). This synaptic localization of nNOS in brain may be mediated by association with the postsynaptic density protein, PSD-95. Like α1-syntrophin, PSD-95 binds directly to nNOS through a PDZ-PDZ interaction that involves the second PDZ domain of PSD-95 (4). Moreover, PSD-95 is a member of a larger family of postsynaptic density proteins (PSD-95/SAP-90, PSD-93/chapsyn-110, and SAP-102) that cluster NMDA receptors and may anchor these receptors to the cytoskeleton (4, 8–12).

The mechanism by which PSD-95 interacts with NMDA receptors has been extensively studied. PSD-95 and its relatives contain three NH_2-terminal PDZ motifs (13, 14). Binding studies show that both the first and second PDZ repeats in PSD-95 potently interact with the COOH-terminal tails of specific NMDA receptor subunits and other ion channels that terminate in a consensus Glu-(Ser/Thr)-*X*-(Val/Ile)-COOH (15–18). Crystallographic studies have determined the structural design of this PDZ-peptide interface, which requires: 1) sequence-specific interactions and 2) peptide termination immediately following the valine (19, 20). The fact that PSD-95 PDZ2 can interact with both COOH-terminal peptides and with the nNOS PDZ domain is intriguing. Furthermore, the nNOS PDZ not only binds to certain heterologous PDZ domains, but also to COOH-terminal peptide ligands that terminate in Gly-(Asp/Glu)-*X*-Val-COOH (21, 22).

In this study, we compared the mechanisms of these PDZ-PDZ and PDZ-peptide interactions of nNOS and PSD-95, and pursued the synaptic protein PSD-95 as a candidate scaffold molecule to link nNOS to NMDA receptors at the plasma membrane. We demonstrate that nNOS, PSD-95, and the NMDA receptor subunit 2B (NR2B) coimmunoprecipitate from brain, and that PSD-95 is sufficient to assemble a tight ternary complex with nNOS and an NR2B fusion protein. The mechanism for the assembly of this ternary complex depends on a PDZ-PDZ interaction between PSD-95 and nNOS that is mechanistically distinct from PDZ-peptide interactions in that the tertiary structure of both domains is important. We show that the nNOS PDZ domain contains two non-overlapping binding sites, one that binds other PDZ domains and a second site that binds

* This work was supported by the Muscular Dystrophy Association, the Searle Scholars Program, National Insitutes of Health Grant RO1-NS34822, the National Association for Research on Schizophrenia and Depression, and the Culpeper and Beckman Foundations (to D. S. B.), and grants from the National Institutes of Health and the Howard Hughes Medical Institute (to W. A. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: nNOS, neuronal nitric oxide synthase; NMDA, *N*-methyl-D-aspartic acid; PSD-95, postsynaptic density-95; PDZ, PSD-95, Dlg, ZO-1 homology; SAP-90, synapse-associated protein 90; NR2B, NMDA receptor subunit 2B; INAD, inactivation no afterpotential D; LIM, Lin-11, Isl-1, Mec-3 homolgy; CAPON, carboxyl-terminal PDZ ligand of nNOS; GST, glutathione *S*-transferase; GdnHCl, guanidine hydrochloride.

COOH-terminal peptide ligands. In contrast, the second PDZ domain of PSD-95 uses the same interface to bind either the nNOS PDZ or COOH-terminal peptide partners. Finally, we demonstrate that the two binding interfaces on the nNOS PDZ domain can function simultaneously, a finding that expands the scope for PDZ domains in the assembly of multiprotein networks.

EXPERIMENTAL PROCEDURES

Immunoprecipitation—Rat forebrain was homogenized in 20 volumes (w/v) TEE (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA) containing 1 mM phenylmethylsulfonyl fluoride. Sodium deoxycholate was then added to 1% (w/v), and the homogenate was incubated at 4 °C for 30 min. The lysate was cleared by centrifugation at 17,000 × *g* for 15 min, and the supernatant was incubated at 4 °C for 1 h with 6 μg/ml rabbit nNOS antibody (Transduction Labs), rabbit NR2B antibody (Zymed Laboratories Inc.), or a control rabbit antibody to phosphorylated cAMP response element-binding protein (Upstate Biotechnology). Immune complexes were precipitated with 20 μl of protein A-Sepharose (Sigma), washed 3 times with 1 ml of lysis buffer, eluted with SDS and analyzed by Western blotting.

Transfected HEK293 cells were incubated in lysis buffer (TEE with 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) at 4 °C for 30 min. Lysates were cleared by centrifugation at 17,000 × *g* for 15 min and incubated for 1 h at 4 °C with 5 μg of rabbit nNOS antibody (Zymed Laboratories Inc.), rabbit PSD-95 antibody (4), or rabbit GFP antibody (CLONTECH). Immunoprecipitated complexes were washed and detected as above.

Western Blotting Analysis—Protein samples were resolved by SDS-polyacrylamide gel electrophoresis and processed for Western blotting as described (6). The following antisera or antibodies were used: guinea pig PSD-93 antiserum (1:1000 (4)), rabbit PSD-95 antiserum (1:500 (4)), monoclonal NR2B antibody (1:250, Transduction Labs), monoclonal nNOS antibody (1:250, Transduction Labs), monoclonal PSD-95 antibodies MA1–045 and MA1–046 (1:500, Affinity Bioreagents), rabbit α1-syntrophin antiserum (23), monoclonal α-CaM kinase II antibody (1:1000, Zymed Laboratories Inc.), and monoclonal GST antibody B-14 (1:200, Santa Cruz Biotechnology)

Bacterial and Mammalian Expression Vectors—A bacterial expression vector encoding GST-NR2B was generated by cloning the following annealed oligos encoding the last nine amino acids of NR2B into pGEX-4T-1 (Amersham Pharmacia Biotech) digested with *Bam*HI and *Eco*RI: 5′-GATCCAAGCTTTCTAGTATTGAGTCTGATGTCTGAG-3′ and 5′-AATTCTCAGACATCAGACTCAATACTAGAAAGCTTG-3′. GST-nNOS fusion protein constructs were generated by polymerase chain reaction of the appropriate nNOS coding region to incorporate flanking *Bam*HI and *Eco*RI sites followed by ligation into pGEX-4T-1. GST-PSD-95 included amino acids 1–386 of PSD-95 in pGEX-2T (6). Full-length PSD-95 or nNOS 1–130 coding sequences were inserted into the pRSETA bacterial expression vector (Invitrogen) to generate hexahistidine-tagged fusion proteins. Full-length nNOS was expressed in the pCWori bacterial expression vector (24). Mammalian expression vector PSD-95-GW1-CMV was a generous gift from Dr. Morgan Sheng (17) and pRK-NR2B was kindly provided by Dr. Lynn Raymond (25). Full-length nNOS was expressed in the pCIS2 vector (26).

Expression and Purification of Recombinant Proteins—GST fusion proteins were expressed and purified as described (6). Histidine-tagged PSD-95 or nNOS 1–130 were expressed in the bacterial strain BL21-(DE3)pLysS (Novagen), solubilized by sonication in 50 mM sodium phosphate buffer (pH 8.0) containing 6 M GdnHCl, 300 mM NaCl, 10% glycerol and purified by cobalt-iminodiacetic acid Sepharose chromatography (Sigma). Full-length nNOS in pCWori was expressed in BL21(DE)pLysS and purified by 2′,5′-ADP Sepharose chromatography as described (24).

GST Fusion Protein Chromatography—For binding assays using brain extracts, adult rat brain was homogenized in 10 volumes (w/v) of TEE containing 1 mM phenylmethylsulfonyl fluoride and centrifuged at 15,000 × *g* for 15 min. The pellet was solubilized in TEE, 1% Triton X-100, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and cleared by centrifugation at 20,000 × *g* for 20 min. Purified GST fusion proteins (100 μg) bound to glutathione-Sepharose (Amersham Pharmacia Biotech) were incubated in 1 ml of TEE, 0.5% Triton X-100 containing 2 mg of rat brain extract for 1 h at 4 °C. Beads were loaded onto disposable columns, washed with 50 volumes of solubilization buffer, and bound proteins eluted with 100 μl of TEE containing 0.2% SDS. For the peptide competition experiment, 100 μM peptide (KLSSIESDV, VSPDFGDAV or KLSSIEADA; Research Genetics) was added. For binding assays using purified components, 10 μg of each GST fusion protein or control GST was bound to glutathione-Sepharose and incubated with 2 μg of recombinant native nNOS or recombinant His_6-PSD-95 in the absence or presence of 5 or 20 nM His_6-PSD-95 or 35 or 140 nM recombinant nNOS 1–130, respectively.

Cell Culture—HEK293 cells were transfected using the LipofectAMINE Plus method (Life Technologies, Inc.) in 6-well dishes. A total of 2 μg of DNA was used for each well, and co-transfected wells received 1 μg of each expression vector. Cells were harvested 2 days post-transfection.

Circular Dichroism (CD) and Guanidine Hydrochloride Denaturation—CD measurements were taken on an AVIV 62DS spectropolarimeter with protein (cleaved from GST by thrombin) at a concentration of 5 mM in 50 mM sodium phosphate (pH 7.0), 50 mM NaCl, in a 1-cm path length cell. Protein unfolding by guanidine hydrochloride was analyzed by following the decrease in CD ellipticity at 222 nm. For proteins that showed a cooperative unfolding transition, CD ellipticity was fit to a two-state model by nonlinear least-squares analysis using Sigma Plot (Jandel Scientific). The equation used was,

$$q = ((a_N[\mathrm{GdnHCl}] + b_N) + (a_D[\mathrm{GdnHCl}] + b_D)\exp(-\Delta G'/RT))/(1 + \exp(-\Delta G'/RT)) \quad \text{(Eq. 1)}$$

where q is the ellipticity at 222 nm, b_N is the intensity of the native state at 0 M GdnHCl, a_N is the slope of the native baseline, b_D is the (extrapolated) intensity of the denatured state at 0 M GdnHCl, a_D is the slope of the denatured state baseline, and $\Delta G'$ is the free energy change upon unfolding at the given concentration of GdnHCl. $\Delta G'$ is assumed to be a linear function of denaturant concentration, as described by the equation,

$$\Delta G' = \Delta G_{\mathrm{H_2O}} - m[\mathrm{GdnHCl}] \quad \text{(Eq. 2)}$$

where $\Delta G_{H_2}O$ is the free energy of unfolding at 0 M GdnHCl calculated by extrapolation (27).

Yeast Two-hybrid Analysis—Yeast transformations, filter lift assays, and quantitative liquid culture assays were performed as described in the Matchmaker Library protocol (CLONTECH). One β-galactosidase unit = 1000 × OD_{420}/(*t* in min) × *V* in ml × OD_{600}. Yeast strain SFY526 was co-transformed with the appropriate plasmids and co-transformants were selected on plates lacking tryptophan and leucine. nNOS constructs encoding fusion proteins with the Gal4 DNA-binding domain were generated by polymerase chain reaction of the appropriate coding sequence to incorporate *Eco*RI and *Bam*HI flanking sequences (nNOS 1–100; 1–111; 1–130; 1–130 Y77H, D78E; 1–159; 1–159 V93K) and ligation into pGBT9 (CLONTECH). Expression vectors encoding the Gal4 DNA-binding domain fused to a 9-residue peptide ending GESV was generated by cloning the following annealed oligos into pGBT9 digested with *Eco*RI and *Bam*HI: 5′-AATTCTACGCCGGCCAGTGGGGCGAGTCCGTGTAAG-3′ and 5′-GATCCTTACACGGACTCGCCCCACTGGCCGGCGTAG-3′. PSD-95 and PSD-93 constructs encoding fusion proteins with the Gal4 activation domain were generated by polymerase chain reaction of the appropriate coding sequence to incorporate *Eco*RI and *Bam*HI flanking sequences (PSD-93 PDZ2, amino acids 117–371; PSD-93 PDZ2 H258V; PSD-95, amino acids 113–364; PSD-95 L241K) and ligation into pGAD424 (CLONTECH). An expression vector encoding the Gal4 activation domain fused to a 9-residue peptide ending ESDV was generated by cloning the following annealed oligo into pGAD424 digested with *Eco*RI and *Bam*HI: 5′-AATTCAAGCTTTCTAGTATTGAGTCTGATGTCTGAG-3′ and 5′-GATCCTCAGACATCAGACTCAATACTAGAAAGCTTG-3′.

RESULTS

PSD-95 Can Organize a Ternary Complex with nNOS and NR2B—PDZ1 and PDZ2 of PSD-95 can bind to COOH-terminal peptides derived from NR2B, and PDZ2 can bind to the PDZ domain of nNOS, but it is not known whether these interactions can occur simultaneously to form a macromolecular signaling complex. To investigate whether PSD-95 can act as a scaffolding protein to link nNOS and NR2B, we performed immunoprecipitation experiments from brain. Solubilized rat brain extracts were immunoprecipitated with an antibody to nNOS, NR2B, or a control antibody and duplicate immunoblots were analyzed for NR2B, PSD-95, nNOS, or the α subunit of CaM kinase II (CaMKII). Both PSD-95 and NR2B specifically coimmunoprecipitate with nNOS (Fig. 1*A*, *left panel*), and both PSD-95 and nNOS specifically coimmunoprecipitate with NR2B (Fig. 1*A*, *right panel*). However, the α subunit of CaM

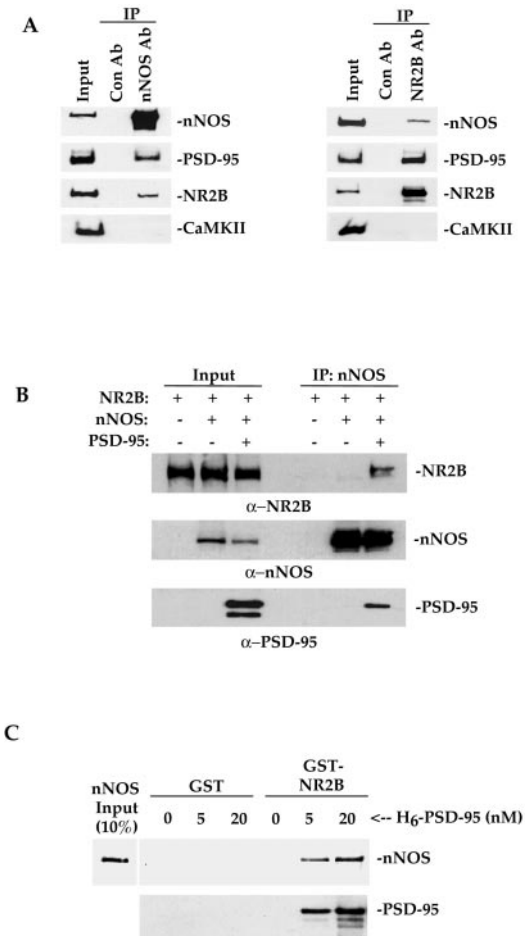

FIG. 1. **PSD-95 links nNOS to an NMDA receptor subunit.** *A*, nNOS, PSD95, and NR2B specifically coimmunoprecipitate from brain. Solubilized rat forebrain homogenates were immunoprecipitated with an antibody to nNOS (*left panel*), NR2B (*right panel*), or a control antibody to phosphorylated cAMP response element-binding protein (*Con Ab*; *both panels*). Identical samples were analyzed by immunoblotting for nNOS, PSD-95, NR2B or the α subunit of Ca^{2+}/calmodulin kinase II (*CaM kinase II*). PSD-95 and NR2B or nNOS co-immunoprecipitate with nNOS or NR2B respectively. *B*, co-immunoprecipitation of NR2B with nNOS is dependent on PSD-95. Lysates of HEK293 cells expressing NR2B alone, NR2B and nNOS, or NR2B, PSD-95 and nNOS were immunoprecipitated with an antibody to nNOS. Identical samples were analyzed by immunoblotting for nNOS, PSD-95, or NR2B. NR2B immunoprecipitates with nNOS only when PSD-95 is coexpressed. *C*, PSD-95 is sufficient to link the COOH terminus of NR2B to nNOS. Recombinant nNOS was incubated alone or with increasing amounts of purified full-length His_6-PSD-95 for 1 h before the addition of purified GST or GST-NR2B bound to glutathione-Sepharose. After extensive washing, bound proteins were eluted with SDS and identical samples were analyzed by immunoblotting with an antibody to nNOS or PSD-95. nNOS does not bind to NR2B directly but associates in a complex when PSD-95 is present.

kinase II, which is also enriched in the postsynaptic density (14), does not coimmunoprecipitate with either nNOS or NR2B.

We next transfected heterologous cells to ask whether PSD-95 is sufficient to link nNOS and NR2B. HEK293 cells were transfected with expression constructs encoding either NR2B alone, NR2B and nNOS, or NR2B, nNOS, and PSD-95. Cell lysates were immunoprecipitated with an antibody to nNOS and coimmunoprecipitated proteins were analyzed by Western analysis (Fig. 1*B*). NR2B does not directly associate with nNOS, but does coimmunoprecipitate when PSD-95 is also expressed.

To determine whether the PSD-95 protein alone is sufficient to mediate complex formation, we expressed and purified the three recombinant proteins from *Escherichia coli.* The final nine amino acids of NR2B were expressed as a GST fusion protein, full-length PSD-95 was expressed as a histidine-tagged fusion protein, and nNOS was expressed as the native protein. nNOS binds to PDZ2 of PSD-95 whereas the NR2B tail sequence can bind to both PDZ1 and PDZ2. Therefore a ternary complex could form in which PDZ1 of PSD-95 binds NR2B and PDZ2 binds nNOS. To prevent both PDZ1 and PDZ2 from being bound by the NR2B fusion protein *in vitro*, we first incubated nNOS with PSD-95 for 1 h before adding GST or GST-NR2B bound to glutathione-Sepharose beads. As expected, purified nNOS alone does not bind to the tail of NR2B (Fig. 1*C*). However, when PSD-95 is present in the binding reaction in nanomolar concentrations, the three proteins form a tight ternary complex.

Identification of the Requirements for PDZ-PDZ Binding—To mediate a ternary complex with NR2B and nNOS, the PDZ domains of PSD-95 must participate in both COOH-terminal peptide interactions and PDZ-PDZ interactions. Whereas PDZ-peptide binding is well characterized, the mechanism for PDZ-PDZ associations is less clear. To help define the molecular correlate for PDZ-PDZ binding, we first asked whether a linear epitope in one of the PDZ partners might mimic a COOH-terminal peptide ligand. If this were the case, the ternary structure of such a "ligand PDZ" domain would presumably not be crucial for this interaction. We therefore sought a mutation that disrupts PDZ domain tertiary structure. A previous study showed that a Met to Lys mutation in one of the PDZ domains of the *Drosophila* inactivation no afterpotential D (INAD) protein disrupts binding of INAD to the COOH-terminal tail of the transient receptor potential calcium channel (28). The corresponding amino acid in the crystal structure of the third PDZ domain of PSD-95 (Ile-388) occurs within a hydrophobic core (19), indicating that insertion of a charged residue could generally affect domain structure and function. We made the corresponding mutation in the PDZ domain of nNOS (V93K) and in PDZ2 of PSD-95 (L241K). When this mutation is introduced into the nNOS PDZ domain, it completely disrupts the tertiary structure as determined by CD spectroscopy. The mutant nNOS protein shows a significantly reduced CD ellipticity at 222 nm, corresponding to a loss of structure (Fig. 2*A*). Additionally, no cooperative unfolding transition is observed with chemical denaturation, indicating that the mutant protein is largely unfolded (Fig. 2*B*). We analyzed the effect of this mutation on PDZ binding in the yeast two-hybrid assay, and found that when this point mutation is introduced into the PDZ domain of nNOS, it completely eliminates binding to a PDZ partner, PDZ2 of PSD-95 (Fig. 2*C*). In addition, introducing this mutation into PDZ2 of PSD-95 also blocks the PDZ-PDZ interaction with nNOS, suggesting that the tertiary structure of both partners is critical for recognition.

We next defined the minimal region of the nNOS PDZ necessary to bind to partner PDZ domains. We constructed a set of 8 fusion proteins containing fragments of the NH_2 terminus of nNOS and evaluated the binding of each of these constructs to PSD-95, PSD-93, and α1-syntrophin proteins from rat brain extracts. We find that a fusion protein encompassing the canonical PDZ domain of nNOS (amino acids 1–99) is not competent for binding. Instead, binding requires a core region of nNOS amino acids 16–130 that contains additional residues COOH-terminal to the consensus PDZ domain (Fig. 3). Deletion of 17 amino acids from the NH_2-terminal side or 14 amino acids from the COOH-terminal side of this core completely eliminates binding. As a control we confirmed that a fusion protein containing the final 9 residues of NR2B binds to both PSD-95 and PSD-93 but not to α1-syntrophin. The *bottom panel* in Fig. 3 verifies that equal amounts of GST-nNOS fusion proteins were present in the binding assays.

We also evaluated binding of these nNOS fragments to fu-

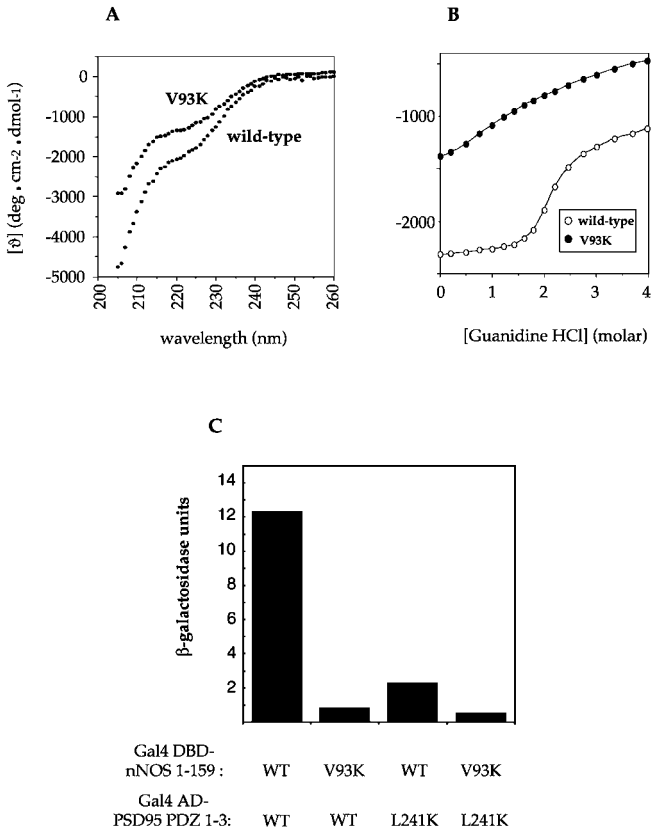

FIG. 2. **PDZ-PDZ interactions require the tertiary structures of both PDZ domains.** *A,* CD spectra of wild-type nNOS PDZ domain (amino acids 1–159) and a point mutant (V93K). The mutant protein shows dramatically reduced ellipticity at 222 nm, indicative of a loss of helical structure. *B*, GdnHCl denaturation of wild-type and V93K nNOS, amino acids 1–159. The mutant protein does not show a cooperative unfolding transition, indicating that it is already largely unfolded at 0 M GdnHCl. *C,* point mutations in either PSD-95 or nNOS that disrupt PDZ domain structure block PDZ/PDZ binding. Yeast strain SFY526 was co-transformed with the indicated constructs. β-Galactosidase expression was assayed by quantitative liquid culture assay and values shown are averages of duplicates that varied by <10%. Assays were repeated with similar results.

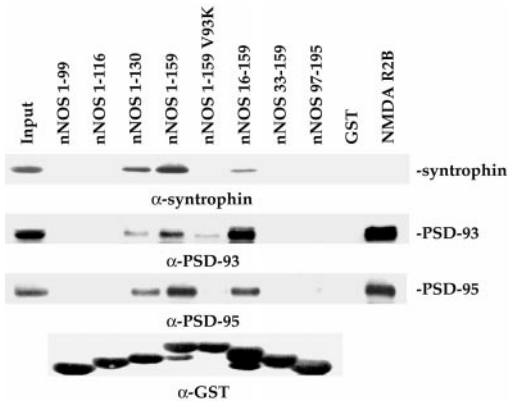

FIG. 3. **PDZ/PDZ binding requires additional residues adjacent to the nNOS PDZ consensus motif.** Fifty micrograms each of GST-nNOS or GST-NR2B fusion protein bound to glutathione-Sepharose was incubated with solubilized rat brain extract. After extensive washing, bound proteins were eluted with SDS and analyzed by Western blotting with an antibody to α-syntrophin, PSD-93, PSD-95, or GST. *Input* = 10% of protein present in each binding reaction.

sion proteins containing PSD-93, amino acids 117–371 (PDZ2), or PSD-95, amino acids 20–364 (PDZ1–3) by the yeast two-hybrid system. The yeast results were consistent with the fusion protein binding experiments (data not shown). Again,

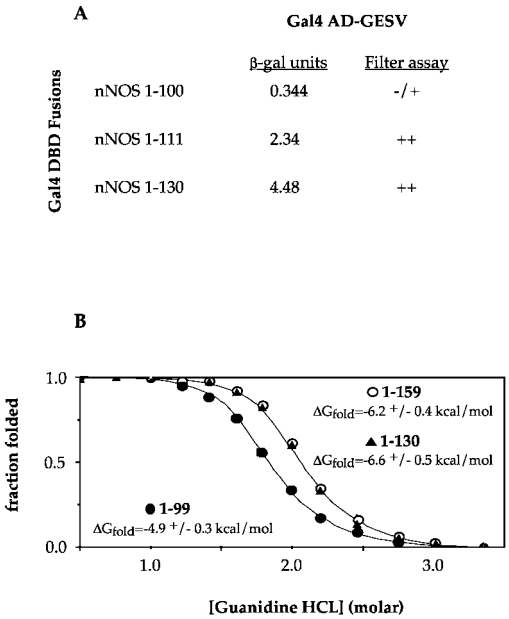

FIG. 4. **The canonical nNOS PDZ domain is sufficient to interact with COOH-terminal peptides.** *A*, yeast strain SFY526 was co-transformed with a construct encoding the GAL4 DNA-binding domain fused to domains of nNOS and a construct encoding the Gal4 activation domain fused to a 9-residue peptide terminating GESV. β-Galactosidase expression was assayed by both filter lift assay (++, 90–180 min; +, 180–360 min; −/+, >360 min) and quantitative liquid culture assay (values shown are averages of duplicates that varied by <10%). Assays were repeated with similar results. *B*, GdnHCl denaturation of functionally active nNOS domains (1–130 and 1–159) shows that both constructs have a stability of ~6.5 kcal/mol. An inactive construct (1–99) is destabilized by ~2 kcal/mol.

amino acids 16–130 were implicated as the minimal PDZ interaction domain of nNOS. Therefore, the interactions between the nNOS PDZ and other PDZ domains require: 1) intact tertiary structures in both partners, and 2) an additional 16–30 residues following the canonical nNOS PDZ.

Distinct nNOS Domains for PDZ-PDZ versus PDZ-Peptide Binding—In addition to binding PDZ2 of PSD-95/93 and the PDZ domain of α-syntrophin, the nNOS PDZ also binds short linear peptides terminating with the consensus Gly-(Asp/Glu)-*X*-Val-COOH (21, 22). We previously reported that, similar to the above nNOS PDZ-PDZ interactions, the extended nNOS PDZ domain (nNOS 1–130) is also required to bind COOH-terminal peptides *in vitro* (22). However, using the yeast two-hybrid assay others have reported that an nNOS construct containing only the consensus PDZ domain (amino acids 1–111) is sufficient for peptide binding (21). We wondered whether this discrepancy could be explained by the different methods used (*i.e. in vitro* binding *versus* yeast two hybrid). Indeed, using the yeast two-hybrid system we find that nNOS 1–111 does bind to COOH-terminal peptides, in contrast to our *in vitro* results (Fig. 4*A*). However, a smaller construct that also contains the consensus PDZ domain (nNOS 1–100) binds more weakly than nNOS 1–111, whereas a larger construct (nNOS 1–130) binds more strongly than nNOS 1–111.

The progressive attenuation in binding observed with nNOS 1–111 and nNOS 1–100, together with our inability to detect binding with purified nNOS 1–111 *in vitro*, suggested that the PDZ domain may become less structurally stable as the COOH-terminal extension is truncated. To test this, we evaluated the thermodynamic stability of bacterially expressed nNOS PDZ domain proteins using a guanidine hydrochloride denaturation assay. Protein unfolding by guanidine hydrochloride was analyzed by following the decrease in ellipticity at 222 nm (29),

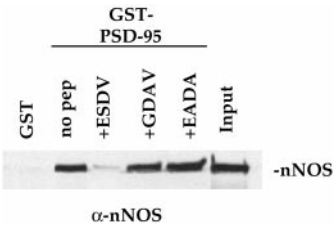

FIG. 5. **Peptides terminating in ESDV but not GDAV block nNOS/PSD-95 binding.** Glutathione-Sepharose-bound GST-PSD-95 was incubated with rat brain extract in the presence or absence of the indicated 9-residue peptides (100 μM). Peptide ESDV is derived from the COOH terminus of NR2B and binds PDZ1 and 2 of PSD-95/93, GDAV is an nNOS PDZ-binding peptide, and EADA is a control peptide. After extensive washing, bound proteins were eluted with SDS and analyzed by Western blotting with an antibody to nNOS. *Input* = 10% of protein present in each binding reaction.

which presumably corresponds to the loss of the two α-helices observed in known PDZ domain structures. Using this assay we find that the canonical nNOS PDZ domain (amino acids 1–99) is significantly less stable than larger nNOS constructs which contain the entire extended domain (amino acids 1–130 and 1–159; Fig. 4*B*). Furthermore, nNOS 1–116 shows an unfolding transition that is intermediate between nNOS 1–99 and 1–130 (data not shown), indicating that residues adjacent to the canonical nNOS PDZ (1–99) increase stability. Therefore, the canonical nNOS PDZ is sufficient to bind COOH-terminal ligands, although the instability of this minimal construct makes it more difficult to assay.

The above results demonstrate that residues 100–130 of nNOS are necessary for PDZ-PDZ interactions but not for PDZ-peptide interactions. This difference suggests that either: 1) the increased stability of the larger nNOS constructs is required for PDZ domain but not peptide binding, or 2) there are two distinct binding interfaces on nNOS, and only the PDZ-PDZ interface incorporates residues 100–130. To evaluate this latter possibility, we tested whether a COOH-terminal nNOS PDZ-binding peptide, Gly-Asp-Ala-Val-COOH, competes with PSD-95 for binding to nNOS. We find that this Gly-Asp-Ala-Val-COOH peptide does not disrupt the nNOS/PSD-95 interaction (GDAV; Fig. 5), providing evidence that the PDZ-PDZ and PDZ-peptide binding interfaces of nNOS are distinct. By contrast, a COOH-terminal PSD-95-binding peptide that terminates Glu-Ser-Asp-Val-COOH (ESDV; Fig. 5) does disrupt the nNOS/PSD-95 interaction.

Taken together, these data suggest that the nNOS PDZ domain has distinct binding sites for COOH-terminal peptides and for other PDZ domains, whereas these interfaces are overlapping or identical in PDZ2 from PSD-95. To confirm these results, we sought a point mutation in the nNOS PDZ that would differentiate between the two modes of binding. In the co-crystal structure of PSD-95 PDZ3 bound to a COOH-terminal peptide, the critical threonine at the −2 position of the peptide makes a hydrogen bond with His-372 (19). The equivalent residue in the nNOS PDZ is Tyr-77, and we have previously shown that changing this residue to His converts the nNOS peptide-binding specificity from Asp-*X*-Val-COOH to Ser-*X*-Val-COOH (22). Whereas this mutation in nNOS alters PDZ-peptide binding, it does not disrupt PDZ-PDZ associations (nNOS 1–130 Y77H, D78E; Fig. 6*A*). In contrast, mutating the analogous residue in PSD-93 PDZ2 disrupts interactions with both COOH-terminal peptides and with the nNOS PDZ (H258V; Fig. 6*B*), consistent with the idea that PDZ2 of both PSD-93 and PSD-95 use overlapping or identical binding interfaces to contact the nNOS PDZ and COOH-terminal peptides.

Very recently, we determined the crystal structure of nNOS (amino acids 1–130) complexed with the PDZ domain of α1-syntrophin (30). This structure demonstrates that the 30 additional amino acids following the PDZ domain of nNOS that are specifically required for PDZ-PDZ binding form a β-hairpin "finger." This finger contains the tetrapeptide Glu-Thr-Thr-Phe (amino acids 108–111), which inserts into the peptide binding groove of the syntrophin PDZ domain, mimicking the typical COOH-terminal peptide ligand Glu-(Thr/Ser)-*X*-Val-COOH.

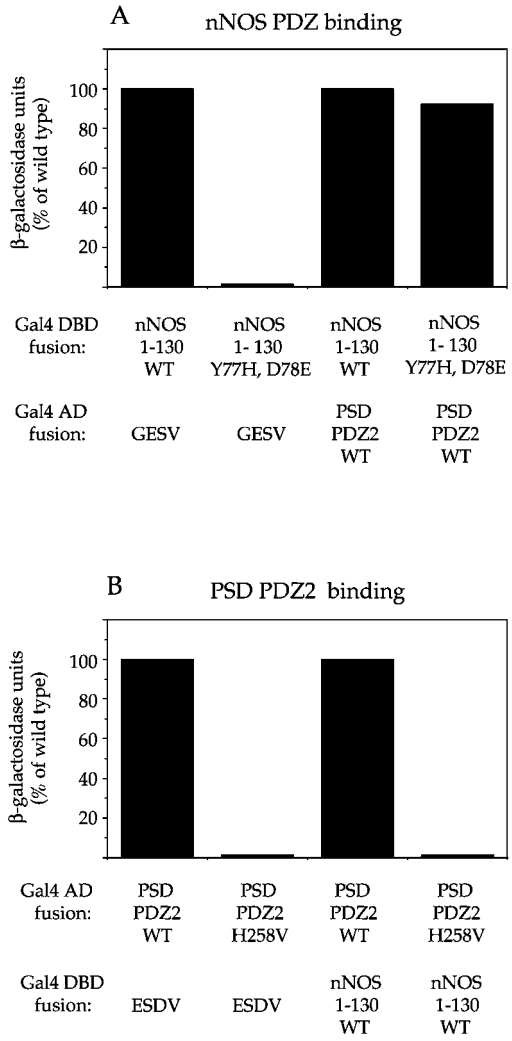

FIG. 6. **The nNOS PDZ domain contains two separate interfaces for binding COOH-terminal peptides and other PDZ domains.** Yeast strain SFY526 was co-transformed with a plasmid encoding nNOS 1–130, nNOS 1–130 Y77H, D78E, or a 9-residue peptide terminating ESDV as a fusion protein with the GAL4 DNA-binding domain and a plasmid encoding PSD-93 PDZ2, PSD-93 PDZ2 H258V, or a 9-residue peptide terminating GESV as a fusion protein with the Gal4 activation domain. β-Galactosidase expression was quantitated by liquid culture assay and values shown are averages of duplicates that varied by <10%. Assays were repeated with similar results. *A*, a mutation in the peptide-binding pocket of nNOS does not affect association with other PDZ domains. *B*, a mutation in the peptide-binding pocket of PDZ2 of PSD-93 abolishes interaction with the nNOS PDZ.

Guided by this structure, we made alanine mutations of specific residues in the nNOS finger. Mutations of either Glu-108 (E108A) or Thr-109 (T109A) disrupt PDZ-PDZ binding but do not affect PDZ-peptide binding (Table I). In contrast, mutation of Thr-110 (T110A), which is not predicted to be essential for PDZ binding, does not affect either interaction. Mutations of the critical hydrophobic residue, Phe-111 (F111A and F111S), disrupt both PDZ-PDZ and PDZ-peptide binding, suggesting that mutations of this residue may perturb the structure of the nNOS PDZ domain.

These data clearly demonstrate that the nNOS PDZ domain

TABLE I
Mutational analysis of the "PDZ ligand" domain of nNOS

Yeast strain SFY526 was co-transformed with pGBT9 and pGAD424 (Clontech) constructs expressing the appropriate Gal4 fusion proteins. The Gal4 DNA-binding domain was fused to nNOS amino acids 1–130 wild-type or 1–130 containing one of the above mutations. The Gal4 activation domain was fused to PSD-93 (amino acids 117–371) or to the nine residue peptide LSSYYGESV*. Transformants were selected on plates lacking tryptophan and leucine, and assayed for β-galactosidase expression by both filter lift assay (++++, <30 min; ++, 90–180 min; +, 180–360 min) and quantitative liquid culture assay (β-galactosidase units = $1000 \times OD_{420}/(t \text{ in min} \times V \text{ in ml} \times OD_{600})$ as described in the Matchmaker Libary protocol (CLONTECH). Liquid assay numbers are averages of duplicates that varied by <10%.

	PSD PDZ2		GESV	
	β-Gal units	Filter assay	β-Gal units	Filter assay
nNOS WT	44.9	++++	5.12	++
E108A	1.41	+	6.00	++
T109A	0.940	+	4.12	++
T110A	44.8	++++	4.61	++
F111A	0.481	+/−	1.41	+
F111S	0.610	+/−	1.45	+

contains distinct binding interfaces for PDZ and COOH-terminal peptide binding partners. We therefore asked whether both binding sites can be simultaneously occupied. For this experiment, we used purified protein components and tested whether the nNOS PDZ can form a ternary complex with both PSD-95 and a COOH-terminal peptide ligand. We first verified that PSD-95 does not directly interact with a GST fusion protein terminating Gly-Asp-Ala-Val-COOH. However, when we titrate in the extended nNOS PDZ domain (amino acids 1–130) at 35 to 140 nM, we readily detect formation of a ternary complex (Fig. 7*A*).

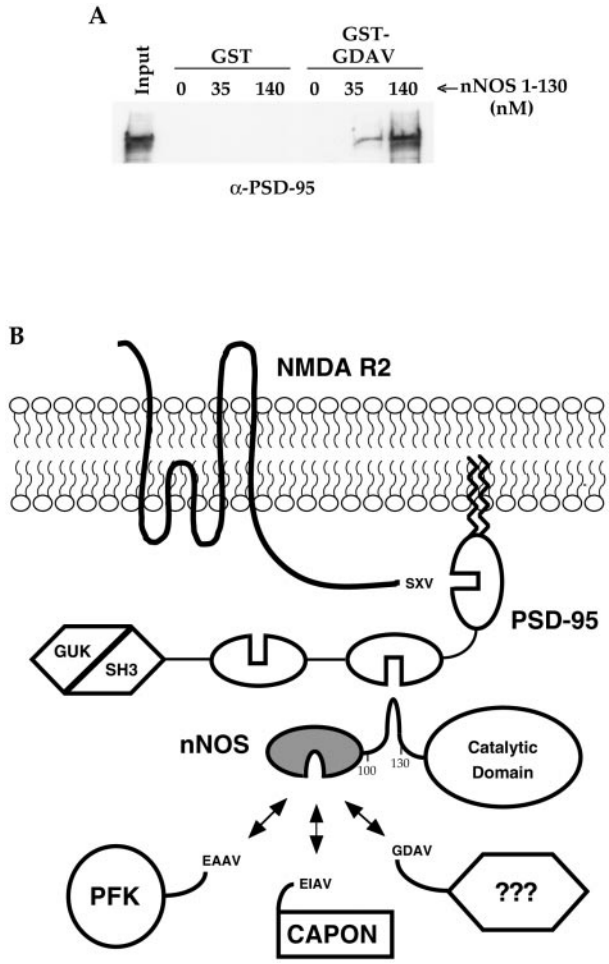

FIG. 7. **The two binding domains on nNOS can function simultaneously.** *A*, the extended nNOS PDZ domain can simultaneously interact with a heterologous PDZ protein and a COOH-terminal peptide ligand. A GST fusion protein containing a 9 residue nNOS PDZ-binding peptide (*GST-GDAV*) or GST control was bound to glutathione-Sepharose and incubated with full-length purified His_6-PSD-95 in the absence or presence of increasing amounts of purified nNOS 1–130. After extensive washing, bound proteins were eluted with SDS and analyzed by immunoblotting. *Input* = 10% of His_6-PSD-95 present in each binding reaction. *B*, a schematic model for protein complex assembly at the synaptic membrane. PSD-95 functions to bring nNOS to the NMDA receptor, allowing specific activation of nNOS in response to glutamate-induced calcium influx. Binding of nNOS to PSD-95 leaves the nNOS PDZ domain peptide-binding pocket free to interact with other proteins such as CAPON and phosphofructokinase (*PFK*). nNOS exists as a stable dimer (not pictured) which further increases the complexity of molecules that can be brought into this complex through the nNOS PDZ domain.

DISCUSSION

This study identifies a ternary complex containing nNOS, PSD-95, and the NMDA receptor. nNOS is incorporated into the PSD-95·NMDA receptor complex through a PDZ-PDZ interaction with PDZ2 of PSD-95. This interaction requires the intact tertiary structure of both domains and a 30-amino acid extension beyond the canonical nNOS PDZ domain. Several other examples of PDZ-PDZ interactions have been identified. The neuronal proteins ABP (AMPA receptor-binding protein) and the related glutamate receptor-interacting protein contain seven PDZ domains and can form homomultimers and heteromultimers with each other through a subset of these repeats (31, 32). In *Drosophila*, the multi-PDZ proteins INAD and Discs Lost also form homomultimers (33, 34). Interestingly, homomultimerization of PDZ3 of INAD requires additional amino acids COOH-terminal to the consensus PDZ domain (33). Therefore, the nNOS/PSD-95 interaction defined here may be a general mechanism for PDZ/PDZ binding.

Because nNOS is efficiently stimulated by calcium influx through the NMDA receptor but not by other calcium influx pathways (1–3), the ternary complex formed between nNOS, PSD-95, and NR2B has important implications for the biology of nNOS. Ultrastructural studies have previously shown that NMDA receptor subunits and a subpopulation of nNOS are co-localized at the postsynaptic density of synaptic spines (7, 35–37). Taken together, these studies suggest that PSD-95 may function to link plasma membrane receptors to second messenger pathways, in addition to the proposed role for PSD-95 in ion channel clustering. The abnormalities in synaptic plasticity and learning detected in PSD-95 mutant mice (38) are consistent with a role for PSD-95 in assembly of functional synaptic transduction cascades. Another PDZ-containing protein that has been shown to assemble signaling components is *Drosophila* INAD, which contains five PDZ domains that interact with multiple components of the visual signal transduction machinery to assemble a functional transduction unit (28, 39, 40).

In terms of the molecular mechanism for the nNOS/PSD-95 interaction, nNOS appears to play the role of a ligand PDZ and contains an extended region that interacts with the peptide-binding pocket of the PSD-95 "receptor PDZ." In support of this model, mutation of the peptide-binding pocket of PSD-95 blocks PDZ-PDZ association, whereas the interaction is not sensitive to mutation of the nNOS peptide-binding site. Furthermore, COOH-terminal peptides that bind PSD-95 block the PDZ-PDZ interaction, but nNOS-binding peptides do not.

The extended "ligand" region of the nNOS PDZ contains a "pseudo-peptide" sequence Glu-Thr-Thr-Phe that closely resembles the consensus for peptides binding to PSD-95 Glu-(Thr/Ser)-*X*-(Val/Ile)-COOH. A major difference is that the previously characterized peptide ligands for PSD-95 require chain termination following the hydrophobic residue (Val). Our recent determination of the crystal structure of the nNOS PDZ

demonstrates that this ligand domain is a structurally constrained β-hairpin (30). Unlike typical COOH-terminal PDZ ligands, precise orientation of this nNOS pseudo-peptide appears to be critical, as the complete structured PDZ domain of nNOS is necessary. Interaction of PSD-95 with the nNOS pseudo-peptide is reminiscent of the binding of the α1-syntrophin PDZ domain to engineered cyclic peptides containing an internal recognition sequence, Glu-Thr-Thr-(Leu/Met) (41). As we find for the nNOS pseudo-peptide, conformational restriction, in this case peptide cyclization, is necessary to present the critical contact residues in the proper structural context.

In addition to making interactions with the peptide-binding groove of α1-syntrophin, the nNOS PDZ domain forms additional "tertiary interactions," hence this is truly a PDZ-PDZ association. Most of the tertiary contacts come from the most NH_2-terminal strand of the nNOS PDZ domain (strand A), which in the complex packs against the syntrophin αβ-helix (30). Residues that participate in this ensemble of primary and tertiary interactions (Ser-95, Asn-102, Ser-109, His-142, and Asp-143) are uniquely conserved in α1-syntrophin, PSD-95 (PDZ2), and PSD-93 (PDZ2), perhaps explaining why only these three PDZ domains heterodimerize with nNOS.

The mechanism for PDZ-PDZ binding may to be a prototype for a broader class of interactions with PDZ domains, recognition of internal sequences. Examples of other PDZ proteins that participate in PDZ-internal peptide interactions have been identified. The third PDZ domain of INAD binds to transient receptor potential at an internal Ser-Thr-Val motif (28), and PDZ5 of INAD binds an internal motif in phospholipase C-β (42). In addition, the second PDZ domain of protein tyrosine phosphatase-BAS-like and the PDZ domain of reversion-induced LIM gene both bind to internal sequences in the LIM domain of reversion-induced LIM gene (43). It will be important to determine whether these interactions are also mediated by insertion of a constrained pseudo-peptide ligand into the PDZ domain peptide-binding pocket.

Whereas nNOS and α1-syntrophin contain only a single PDZ domain, tandem PDZ domains such as the triplet in PSD-95 are also common. This concatamerization of PDZ domains is likely to facilitate the assembly of multiprotein complexes. Our demonstration that the single nNOS PDZ domain can bind both a COOH-terminal peptide and a heterologous PDZ domain simultaneously identifies an important additional mode for protein scaffolding by PDZ domains. Identified ligands of the peptide-binding site for nNOS *in vivo* include CAPON (44) and the glycolytic enzyme phosphofructokinase (45). The bivalent nature of the nNOS PDZ domain implies that it may recruit these proteins or other appropriate COOH-terminal ligands to the PSD-95 complex. Furthermore, the fact that nNOS is a stable dimer in solution (46–48) enhances the possibilities for protein assembly.

Our finding that two binding sites exist within the nNOS PDZ may explain the mislocalization of nNOS in Becker's muscular dystrophy (14). In human patients and mouse models of Becker's dystrophy, which results from deletions of the central rod-like domain of the dystrophin protein, nNOS is absent from the sarcolemma even though α-syntrophin is properly localized (49). Our data suggest that proper localization of nNOS in skeletal muscle may also require interaction with another component of the dystrophin complex through the peptide-binding interface of the nNOS PDZ domain.

Acknowledgment—We thank Dr. Stanley Froehner for the α1-syntrophin antiserum.

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