Insights into the C-terminal Peptide Binding Specificity of the PDZ Domain of Neuronal Nitric-oxide Synthase

CHARACTERIZATION OF THE INTERACTION WITH THE TIGHT JUNCTION PROTEIN CLAUDIN-3

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Neuronal nitric-oxide synthase, unlike its endothelial and inducible counterparts, displays a PDZ (PSD-95/Dlg/ZO-1) domain located at its N terminus involved in subcellular targeting. The C termini of various cellular proteins insert within the binding groove of this PDZ domain and determine the subcellular distribution of neuronal NOS (nNOS). The molecular mechanisms underlying these interactions are poorly understood because the PDZ domain of nNOS can apparently exhibit class I, class II, and class III binding specificity. In addition, it has been recently suggested that the PDZ domain of nNOS binds with very low affinity to the C termini of target proteins, and a necessary simultaneous lateral interaction must take place for binding to occur. We describe herein that the PDZ domain of nNOS can behave as a bona fide class III PDZ domain and bind to C-terminal sequences with acidic residues at the P_{-2} position with low micromolar binding constants. Binding to C-terminal sequences with a hydrophobic residue at the P_{-3} position plus an acidic residue at the P_{-3} position (class II) can also occur, although interactions involving residues extending up to the P_{-7} position mediate this type of binding. This promiscuous behavior also extends to its association to class I sequences, which must display a Glu residue at P_{-3} and a Thr residue at P_{-2}. By means of site-directed mutagenesis and NMR spectroscopy, we have been able to identify the residues involved in each specific type of binding and rationalize the mechanisms used to recognize binding partners. Finally, we have analyzed the high affinity association of the PDZ domain of nNOS to claudin-3 and claudin-14, two tight junction tetraspan membrane proteins that are essential components of the paracellular barrier.

Neuronal NOS (nNOS) is expressed constitutively in specific neurons of the brain and in the spinal cord, peripheral nitrergic nerves, epithelial cells of various organs, pancreatic islet cells, and vascular smooth muscle. nNOS differs from the two other mammalian isozymes, endothelial NOS and inducible NOS by an additional ~300-residue N-terminal extension mostly involved in specific subcellular targeting. The N terminus of nNOS contains a PDZ domain (first found in the proteins PSD-95, Dlg, and ZO-1) (2), a β-hairpin module that associates to α1-syntrophin and PSD-95 (3), a DYNLL1 binding site (4), and a stretch known to bind to repeats R16/R17 of dystrophin (5). Brain nNOS is found in particulate and soluble forms in cells, and the differential subcellular localization of nNOS in various tissues may contribute to its diverse functions. Reinforcement of the idea that the subcellular targeting is exquisitely governed by this N-terminal extension came from the observation that an N-terminal deletion mutant of nNOS is an active, mislocalized enzyme (6). In the nervous system, the PDZ domain of PSD-95 is known to couple NMDA receptors to nNOS so that NO release becomes reversibly regulated by Ca^2+/calmodulin binding (1).

PDZ domains are modular protein interaction domains that play a role in protein complex assembly and protein subcellular localization (7–9). These protein modules of ~90 amino acids have remarkable selectivity toward their cellular native targets, which are usually the C termini of proteins. Analysis of the human genome estimates that there are between 270 and 335 non-redundant PDZ domains in more than 150 proteins (10). The structural features of PDZ domains typically consist of six β-strands (β1–β6) and two α-helices (α1 and α2) adopting a β-sandwich that allows them to accommodate the protein C termini that bind as an antiparallel β-strand in a groove between the β2 strand and the α2 helix. The extensive crystallographic and NMR data available today reveal that, at least, the C-terminal 4 residues of PDZ ligands interact directly with the peptide binding groove. In general, PDZ domains show a series of binding pockets involved in the recognition of the P_{-2} and P_{0} ligand residue side chains (where the P_{0} site is defined as the most C-terminal residue of the peptide) as well as the terminal carboxylate ion. C-terminal peptide ligands of class I PDZ domains possess a Ser or Thr residue at P_{-2} position so that the recognition sequence typically falls within a -(S/T)XΦ-COOH motif, whereas class II PDZ domains prefer ligands with a -ΦXΦ-COOH motif in which Φ is a hydrophobic amino acid. However, recent PDZ/ligand interaction studies revealed that residues at the other positions (P_{-1}, P_{-3}, P_{-4}, P_{-5}, and even P_{-7}) of the peptide ligands also contribute to the binding spec-
nNOS PDZ Domain Binding Properties

The binding specificity of the nNOS PDZ domain is poorly understood. Originally, it was considered a class III PDZ domain (7, 13), hence recognizing ligands with the C-terminal sequence -(D/E)XXΦ-COOH. This ascription was obtained when, after screening a random phage peptide library, C-terminal sequences containing the -DXV-COOH motif were retrieved as nNOS tight binders (14). Likewise, a screen with random sequences confronted with the nNOS PDZ domain rendered C-terminal peptides with a -((D/E)XV-COOH) motif as positive interactors (15). In the absence of biochemical information regarding cellular proteins displaying this binding motif, it was suggested that the melatonin receptor -(VDSV-COOH) (16) and the α1c-adrenergic receptor -(GEEV-COOH) (15) were likely candidates to bind to the PDZ domain of nNOS in neurons. Subsequently, more recent results have reported that the C termini of nNOS-interacting DHHC domain-containing protein (NIDD) -(EDIV-COOH), a palmitoyltransferase of the DHHC family (17), C-terminal binding protein-(SDQL-COOH) (18), and cytochrome c oxidase V -(LDKV-COOH) (19) associate to the PDZ domain of nNOS, thus strengthening the conception that the PDZ domain of nNOS displayed a true class III specificity.

Nevertheless, the best characterized neuronal protein known to associate to the PDZ domain of nNOS and to mediate N-methyl-D-aspartate receptor-driven actions is NOS1AP/(NOS1AP/carboxy-terminal PDZ ligand of nNOS(CAPON)) (20, 21), which ends in -EIAV-COOH, a consensus class II motif -(ΦΧΦ-COOH). Likewise, Vac14 -(RVVL-COOH) (22) and phosphofructokinase-M -(EAAV-COOH) (23), two proposed nNOS binding partners, display a class II PDZ domain binding motif at their C termini as well. To make things even more complicated, other proposed binding partners of the PDZ domain of nNOS seem to possess class I -(X(S/T)ΧΦ-COOH) PDZ domain binding motifs. That would be the case for the C terminus of the plasma membrane calcium/calmodulin-dependent calcium ATPase (PMCA-4b) -(ETSV-COOH) (24).

Because some of the proposed interacting partners of the nNOS PDZ domain have been identified in pulldown assays, the question remains whether a direct interaction is indeed taking place or if a bridging protein is responsible for the reported association. It is interesting, in this context, that several proteins described to associate to the nNOS PDZ domain such as C-terminal binding protein and phosphofructokinase-M failed to do so in a large screening of binding partners for PDZ domains present in the mouse proteome (9). Furthermore, it has been recently proposed that binding of the PDZ domain of nNOS to the C termini of protein targets does not proceed as a canonical peptide/PDZ domain interaction because the low affinity of this association might not be physiologically relevant (25). Thus, a heterodivalent interaction between NOS1AP/CAPON and the PDZ domain of nNOS has been put forward in which the insertion of the C terminus within the PDZ binding groove becomes accompanied by a secondary, lateral interaction that increases the overall binding affinity.

We have therefore investigated the binding properties of the PDZ domain of nNOS toward multiple C-terminal sequences. Our data specifically suggest that binding in the low micromolar range can occur with peptides falling within class I, class III, and class II, although for the latter interactions mediated by residues up to position P -7 must be present. Finally, we have focused on the yet uncharacterized association of the PDZ domain of nNOS with claudin-3, a tight junction protein, and described a binding mechanism regulated by tyrosine phosphorylation.

Experimental Procedures

Reagents—15N-labeled (NH4)Cl was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Buffers, chemicals, oligonucleotides, and common laboratory reagents were obtained from Sigma-Aldrich if not otherwise indicated. Pfu polymerase, T4 DNA ligase, restriction endonucleases, and molecular mass markers were obtained from Fermentas. Nickel-nitrilotriacetic acid resin was from Qiagen, and Sepharose 4B from GE Healthcare. Synthetic peptides (described in detail in supplemental Fig. S4) were purchased from Thermo Scientific (Waltham, MA) and were at least 90% pure. d (+)-Lactose monohydrate was from Scharlau (Barcelona, Spain). Cell culture medium (Dulbecco’s modified Eagle’s medium), antibiotics, glutamine, and X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranose; B4252) were purchased from Sigma-Aldrich. Trypsin-EDTA and fetal bovine serum were from BioWhittaker Europe (Veviers, Belgium). HEK-293 cells (human embryonic kidney) were from the ATCC.

Constructs—The cDNA corresponding to residues 14–131 of rat nNOS N terminus was amplified and cloned into the yeast two-hybrid vectors pGAD and pGBT9 as well as in the recombinant expression vector pKLSt (26). This same construct was used to create the Y77A and the S44A mutants. The cDNA of sequences corresponding to C-terminal sequences of proteins suspected to bind to the nNOS PDZ domain were created using long annealing oligonucleotides and ligated with protruding overhangs into pGBT9 or pGAD vectors. YFP-tagged claudin-3 was a kind gift from Dorothea Günzel. mCherry-claudin-3 was obtained by inserting the claudin-3 cDNA between BsrGI and BamHI restriction sites. The PDZ domain of α1-syntrophin (residues 80–180) was amplified and cloned into the recombinant expression vector pKLSt between EcoRI and BamHI restriction sites. GFP-tagged nNOS clones were obtained after amplification of the desired fragment and its insertion between the EcoRI and Sall sites of the mammalian expression vector pEGFP-C2.

Protein Expression and Purification—Both the rat nNOS (27) and the α1-syntrophin (28) cDNAs were cloned into the recombinant expression vector pKLSt (26, 29). Briefly, the pKLSt-nNOS(14–131) or the pKLSt-α1-syntrophin(80–180) plasmid was used to transform BL21 DE3 (Escherichia coli). 2 liters of bacterial culture in 2× yeast extract Tryptone (YT) medium were routinely used for recombinant expression. When the protein was used for NMR experiments, bacteria were grown in M9 minimal medium supplemented with 15N-labeled (NH4)Cl plus glucose as the carbon source. Protein expression was induced by addition of 0.5 mM isopropyl 1-thio-
β-D-galactopyranoside and incubation overnight at 20 °C with 150 rpm aeration rate. Bacterial cells were pelleted and frozen at −20 °C until used for protein purification. Briefly, the bacterial cell lysis was performed on ice in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, pH 7.5) with continuous stirring in the presence of protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, and 200 μM PMSF), 200 mg/ml lysozyme, and 5 mM β-mercaptoethanol followed by four cycles of sonication on ice. The cell lysate was clarified by centrifugation at 10,000 × g and filtered through porous paper. The recombinant protein was purified using a Sepharose 4B column. Recombinant full-length nNOS tagged with a His6 sequence was expressed and purified as described previously (4).

Yeast Two-hybrid Assays—Essentially, we followed our own published protocol (30, 31). We used plasmids containing GAL4 activation domain that were confronted with plasmids containing the GAL4 binding domain as described previously (29). Double transformants were plated in Leu−/Trp−/His− synthetic defined medium plates in the presence of 12 mM 3-amino triazole (triple dropout plates) as well as in Leu−/Trp−/His+. Interacting proteins expressed within the same yeast resulted in colonies that could rescue growth in the absence of His. These colonies were subsequently screened in the X-Gal assay. Blue colonies corresponded to a positive interaction, whereas white colonies corresponded to absence of interaction. The various constructs are described in detail in supplemental Fig. S4.

Isothermal Titration Calorimetry—The interaction between the PDZ domain of nNOS and C-terminal peptides was measured using a VP-ITC microcalorimeter (MicroCal, Northampton, MA) in 20 mM Hepes buffer, pH 7.0, containing 0.15 M NaCl at 25 °C. Commercial peptides were dissolved in water at a 2 mM concentration and subsequently diluted to 0.5–1 mM in the aforementioned buffer when put in the syringe. The protein solutions were introduced into the sample cells, and the peptides were in the syringe. All samples were degassed for at least 5 min in a ThermoVac (MicroCal). Control experiments were performed whereby peptides were titrated into buffer and buffer–titrated into nNOS PDZ domain. As a rule, each experiment consisted of an initial injection of 2.5 μl followed by 30 7.5–μl injections. The heat released in each injection was calculated from the raw data by integration of the peaks after subtraction of the baseline. All data were analyzed using the Origin®7 software program.

Fluorescence Polarization Assays—Fluorescence polarization (FP) was performed in a PerkinElmer Life Sciences MPF 44-E spectrophuorometer. Saturation binding experiments were performed for measuring binding affinity (Kd) between FITC-labeled peptides and the PDZ domain of nNOS by applying an increasing amount of recombinant protein (typically 0–150 μM) to a fixed and low concentration of probe (5–100 nM). Incubation time was 10–15 min (room temperature), and the assay was performed in 20 mM Hepes, 150 mM NaCl, pH 7.0, in a final volume of 0.5 ml. Polarization of the FITC-labeled peptides was measured at excitation/emission values of 488/530 nm (bandwidth, 10 nm). The fluorescence anisotropy (r) values were obtained using the fluorescence polarization (P) values with the equation r = 2P/(3 − P). The initial anisotropy (r0) in the absence of added protein was measured. The FP values were fitted to the equation (FP − FP0) = (FPmax − FP0)[PDZ domain]/(Kd + [PDZ domain]) where FP is the measured fluorescence polarization, FPmax is the maximal fluorescence polarization value, FP0 is the fluorescence polarization in the absence of added PDZ domain, and Kd is the dissociation constant (32). As long as the concentration of labeled peptide is well below the true Kd during the assay, the Kd can be directly derived from this saturation curve as described previously (32–34).

NMR Spectroscopy—Recombinant protein was expressed in bacteria and labeled with 15NH4Cl as reported previously (35, 36). Solutions of the PDZ domain with final concentrations in the range of 100–200 μM were prepared in water with 10% D2O in buffer 100 mM KH2PO4, pH 6.5. Concentrated solutions of the peptides were prepared in the same buffer. Titration experiments were performed by recording 15N HSQC spectra of the 15N-labeled PDZ domain sample in the absence and presence of different unlabeled peptides. All experiments were acquired at 25 °C in a Bruker AVIII 700-MHz NMR spectrometer equipped with a z-gradient cryoprobe. The protein/peptide ratio was ~1:10. Amide proton resonances of free PDZ domain were assigned from published data recorded in similar conditions, and most of the signal changes (16, 37) could be followed during the titration. Chemical shift perturbation analysis was performed with 15N and 1H weighted average chemical shift values.

Determination of Protein Concentration—Aliquots of each purified recombinant protein were dried and subsequently hydrolyzed at 110 °C in 5.9 N HCl, 0.1% phenol for 24 h in vacuum-sealed tubes. Then samples were dried, an internal control of norleucine was added, and the samples were processed in an automatic Biochrom 30 amino acid analyzer. In addition, when needed, the concentration of every preparation of recombinant protein in solution was obtained using the individual F0.1% (280 nm, 1 cm) coefficient.

Circular Dichroism—Far-UV circular dichroism (CD) spectra were obtained on a Jasco 715 spectropolarimeter at 20 nm/min scanning speed. Proteins were dissolved in 15 mM MOPS buffer, pH 7.0, containing 100 mM NaCl (0.2 mg/ml protein concentration). At least four spectra were averaged to obtain the final spectrum. CD measurements were also used to study the thermal stability of the mutants. Tm values correspond to the temperature at the midpoint of the monophasic thermal denaturation transition.

Cell Transfection, Immunofluorescence, and Pulldown Assays—We followed the procedures described previously by our group (38, 39). The pulldown assays of transfected nNOS fused to the LSLt lectin were performed using Sepharose 4B in the absence of added antibodies (29). The beads were subsequently extensively washed to avoid nonspecific interactions and processed as in a conventional immunoprecipitation.

Results

Upstream Residues Mediate the Interaction between the nNOS PDZ Domain and Class II Ligands—The list of nNOS PDZ interaction partners is based on observations from yeast two-hybrid screens, GST pulldown assays, and co-immunoprecipitation studies. Consequently, some interactions might be direct and others indirect, that is mediated by bridging pro-
nNOS PDZ Domain Binding Properties

**FIGURE 1. Binding of the PDZ domain of nNOS to the C terminus of NOS1AP/CAPON.** A, C-terminal sequences of the three proteins reported to be nNOS PDZ class II ligands. B, representative melting curve of the PDZ domain of nNOS in the absence (trace 1) or presence (trace 2) of 50 μM NOS1AP peptide (ELGDSLDDEIAV*) in the 20–80 °C range recording the circular dichroism signal at 222 nm. Independent melting experiments of any condition differed less than ±0.3 °C between one another. C, yeast two-hybrid assay using the nNOS PDZ domain in the bait plasmid and various NOS1AP constructs in the prey plasmid. Both the wild-type and the single mutants were confronted with nNOS(1–131). The columns indicate the yeast growth in the absence of the amino acid His (in the presence of 3-aminotriazole) and X-Gal activity (blue denotes a positive interaction). D, representative binding curve for the FITC-labeled NOS1AP peptide (f-ELGDSLDDEIAV*) to the nNOS PDZ domain measured by fluorescence polarization. A calculated $K_d$ of 117.4 ± 2.2 μM could be obtained. E, isothermal titration calorimetric analysis of the binding of three peptides of different length corresponding to the C terminus of NOS1AP to the PDZ domain of nNOS. The thermogram is shown in the **upper panels**, and the binding isotherms are shown in the **bottom panels**. The thermodynamic parameters for the dodecapeptide/nNOS PDZ domain interaction are also shown to the right. Data are representative of four independent titrations. mP, millipolarization units; mdeg, millidegrees; PFK-M, phosphofructokinase-M.
nNOS PDZ domain, although upstream binding motifs are necessary for this interaction to occur. This is in agreement with the binding data reported for Vac14, a protein involved in hyperosmotic stress response, in which an internal binding motif up to residue Asp + 8 was reported (22) and emphasizes the observation that, at least in the case of class II peptides, interactions outside the boundaries of the canonical binding groove of the nNOS PDZ domain influence binding. Using the aforementioned techniques, we could also observe a strong interaction of long Vac14 peptides with the nNOS PDZ domain (data not shown). Conversely, we and others (9) failed to obtain any significant binding of the C terminus of phosphofructokinase, a class II sequence lacking an internal binding motif (Fig. 1A), to the nNOS PDZ domain, a fact that might indicate that the reported association of these two proteins in pulldown assays may be indirect.

The nNOS PDZ Domain Binds Class III Ligands upon Recognition of the 3 C-Terminal Residues—Next, we proceeded to analyze the binding to class III sequences (-D/E)Xφ-COOH. Previous published data have shown that screening of an unbiased library of 13 billion C-terminal peptides resulted in the selective binding of the nNOS PDZ domain to C-terminal peptides displaying a -DXX-COOH motif where X was predominantly Ala, Ile, Leu, Tyr, Trp, or Pro (14). With that in mind, we validated the binding of the PDZ domain of nNOS to C-terminal sequences terminating in class III sequences (Fig. 2). We chose three sequences from those originally reported as tight binders, NBP-44, NBP-123, and NBP-161 (14) (Fig. 2A), and analyzed their interaction with the nNOS PDZ domain in thermal denaturation experiments using CD (Fig. 2B). Interestingly, a melting curve of the nNOS PDZ domain upon monitoring the circular dichroism signal at 222 nm revealed that binding to peptide NBP-44 and to a short peptide with the sequence GGGDAV-COOH displaced the T_m by 2.7 and 2.5 °C, respectively (Fig. 2B). This result indicates that class III peptides are bona fide nNOS PDZ domain binders and that the final 3 residues establish most of the binding interactions. Likewise, a yeast two-hybrid experiment (Fig. 2C) revealed that NBP-44, NBP-123, and NBP-161 sequences bound to the PDZ domain of nNOS because all three grew in the absence of His and metabolized X-Gal in less than 10 min, hence indicating a strong interaction. To use a more quantitative methodology, we analyzed the fluorescence polarization of NBP-123 peptide labeled with FITC at its C terminus (both NBP-44 and NBP-161 labeled with FITC were less soluble). Saturation curves were then created by applying increasing concentrations of the nNOS PDZ domain and fixed concentration of the probe, and the K_d value between the probe and PDZ domain was determined to be 5.7 ± 1.3 μM (Fig. 2D). Next, we obtained thermodynamic parameters of the interaction of the nNOS PDZ domain with class III peptides using isothermal titration calorimetry. When the nNOS PDZ domain preparation was titrated with the NBP-44 C-terminal 12-amino acid peptide, the resulting thermodynamic parameters were comparable with those of the GGGDAV peptide with both in the low micromolar range (K_d = 2.2 ± 0.5 μM, ΔH = −5.53 kcal/mol, and −TD S = 2.19 kcal/mol for the ARLNLSYYYGDAV peptide versus K_d = 15.4 μM, ΔH = −2.46 kcal/mol, and −TD S = 4.12 kcal/mol for the GGGDAV peptide). In summary, class III peptides bind effectively to the nNOS PDZ domain, but unlike class II peptides the binding interactions are mostly mediated through residues located at positions P_−2, P_−1, and P_0.

No cellular protein known to associate to the PDZ domain of nNOS displays exactly a GDAV motif at its C terminus. However, several nNOS binding partners do have class III binding motifs at their C termini. Specifically, we analyzed the binding of C-terminal peptides corresponding to α-adrenergic receptor, melatonin receptor, C-terminal binding protein, NIDD, and cytochrome c oxidase (Fig. 2A). For these five sequences, we detected a very weak association using yeast two-hybrid analysis and very modest changes in the T_m of the denaturation curve followed by the ellipticity values at 222 nm. Essentially, no displacement in the T_m of the denaturation of the nNOS PDZ domain could be observed at 50 μM peptide, and only at high concentrations (500 μM peptide) could displacements of 1.4–2.8 °C be attained (supplemental Fig. S2). In addition, we could not detect a measurable heat signal in ITC experiments using any of these five peptides, probably due to the fact that their K_d values toward the nNOS PDZ domain are significantly greater than 100 μM. Typically, very few interactions are that weak because ~90% of PDZ domain/peptide interactions have a K_d <50 μM, and ~60% have a K_d <20 μM (41). It must also be mentioned that some of these sequences have also failed to bind to the nNOS PDZ domain in a systematic screen of the mouse PDZ domain interactome (9). Thus, some of the reported interactions of these proteins with the nNOS PDZ domain might be indirect, or alternatively, besides the insertion of a C-terminal stretch within the PDZ moiety, a secondary interaction surface might be promoting their association in a cellular context.

Analysis of the Structural Determinants of Site P_−2 and P_−3 Specificity—Next, we wondered how the nNOS PDZ domain might be able to bind alternatively to C-terminal sequences displaying an acidic residue at the P_−2 position (such as GDAV) but also to sequences with a hydrophobic residue at the P_−2 position together with an acidic residue at the P_−3 position (such as EIAV). To understand the molecular details underlying these interactions, we used NMR spectroscopy to determine the solution structure of the nNOS PDZ domain in solution and in complex with an NOS1AP peptide, ELGDSLDEIAV*, or an NBP-44 peptide, RLNLSSYYGDGA*. A canonical PDZ/ligand interaction is defined as one in which the ligand main chain adopts a β-strand conformation by interacting in an antiparallel fashion with strand β2. This conformation places the P_−2 side chain proximal to the side chains at positions α2-1 and α2-5, which serve to determine the ligand residue types that are favored at this position. In addition, the residue at position β2-2 also contributes to site P_−2 specificity by helping to orient the incoming peptide in the binding groove. In contrast, in most of PDZ domains studied to date, the predominant specificity at site P_−3 is for hydrophobic residues; albeit a few prefer Asp/Glu, and some prefer Thr/Ser (11, 42). Within canonical PDZ domains, site −3 is always occupied by the P_−3 ligand residue, and in most cases peptides interacting with the Asp_−3 or Glu_−3 residue use a similar mechanism for ligand recognition (42). In general, positions β2-2, β3-4, and β3-5 are occupied by Lys,
Arg, or Ser, all of which are capable of forming salt bridges or hydrogen bonds with the carboxylate group of the acidic residue at P$_{-2}$. Sequence comparison of the nNOS PDZ domain with those of the PDZ domains of ZO-1–3 and MUPP1–10, two proteins known to bind to peptides with acidic residues at P$_{-2}$ reveals that, instead of a polar residue, a Leu residue occupies the nNOS P$_{-2}$ position. A Tyr residue occupies position P$_{-1}$ in nNOS instead of Arg or His such as in ZO-1–3 and MUPP1–10 (supplemental Fig. S3). Hence, the nNOS domain only partially fulfills the sequence requirements necessary for acidic P$_{-3}$ recognition. Likewise, sequence comparison of nNOS PDZ domain with those of ERBB2IP1, Dlg1-2, Dlg4-3, and SNT1A, four proteins known to recognize peptides with acidic residues occupying P$_{-3}$ position, reveals that nNOS lacks a Ser or Asn residue in P$_{-2}$ position capable of hydrogen bonding to the incoming peptide. In addition, only nNOS Ser-44 at
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β3-4 could be involved in the recognition of peptides with a Glu at P$_{-3}$ such as in that present in the NOS1AP peptide (supplemental Fig. S3).

To understand the binding modes in atomic detail, we analyzed by NMR spectroscopy the free nNOS PDZ domain in solution and in complex with peptides corresponding to the 12 C-terminal residues of the ligands NBP-44 and NOS1AP. Homogeneous $^{15}$N-labeled samples were prepared, and $^{1H-15}$N HSQC spectra of the three samples were recorded (Fig. 3A). In all cases, the measurements resulted in well-resolved $^{1H-15}$N HSQC NMR spectra characteristic of folded proteins. Because both peptides occupy the P$_0$ site with a Val residue, we identified differences in backbone NH chemical shifts between the free nNOS PDZ domain and when bound to the NBP-44 and NOS1AP peptides. All 3 residues, Leu-84, Ile-87, and Val-94, present in the hydrophobic binding pocket display changes in the backbone NH chemical shifts, hence indicating that the canonical binding groove is occupied in both cases with Val$_0$ inserted in the expected position (Fig. 3A). In contrast, Tyr-77, a residue predicted to hydrogen bond to Asp$_{-2}$, does not in fact display changes in the backbone NH chemical shifts more pronounced in the nNOS PDZ-NBP-44 complex than in the nNOS PDZ-NOS1AP complex (Fig. 3B) in agreement with the former ending in GDAV-COOH and the latter ending in EIAV-COOH. Regarding nNOS Ser-44, a residue expected to show changes in the backbone NH chemical shifts upon binding to peptides with an acidic at the P$_{-3}$ position, no signal could be observed with either of the two peptides due to exchange broadening. Nevertheless, the preceding residues Ile-42 and Ile-43 did show more significant changes in the backbone NH chemical shifts upon binding to the NOS1AP peptide than when binding to the NBP-44 peptide (Fig. 3C). Finally, binding of the NOS1AP peptide to the nNOS PDZ domain also resulted in a significant backbone NH chemical shift of Ala-51 (Fig. 3D), a residue lying outside the canonical binding groove. This observation might be indicative of a forward displacement of this peptide upon binding (see below). Hence, NMR spectroscopy can be a useful tool to discriminate between different binding modes.

**PDZ Domain Tyr-77 Is Involved in the Recognition of Class III Sequences**—To determine the importance of Tyr-77 and Ser-44 in class III peptide binding, we prepared site-directed mutants and determined their binding to NBP-44 peptide labeled with FITC. The calculated $K_d$ values for the Y77A and S44A mutants were 35.56 ± 3.42 and 8.36 ± 2.03 μM, respectively, whereas the value for the wild-type PDZ domain was $K_d = 5.7 ± 1.3 \mu M$ (Fig. 4A). This result underlines the importance of the hydrogen bond between the hydroxyl group of Tyr-77 and the peptide Asp positioned at P$_{-2}$. Taken together, our results indicate that the nNOS PDZ domain does not entirely fall into the category of proteins that can recognize acidic residues at P$_{-2}$ or at P$_{-3}$ but instead has evolved, presenting certain amino acids that allow for both of these dissimilar classes of C-terminal peptides to bind.

The β-Hairpin and the Hydrophobic Groove of the PDZ Domain Are Independent Binding Entities—The nNOS PDZ domain is the only reported example of a PDZ domain with an appended β-hairpin motif capable of binding to other PDZ domains such as PSD-95 or α-syntrophin (3, 43, 44). Next, we wondered whether binding of a PDZ domain to the β-hairpin of nNOS could somehow alter the binding of C-terminal peptides to the canonical binding groove of the PDZ domain itself. We expressed and purified the PDZ domain of α-syntrophin and preformed a complex with nNOS(14–131) before testing its association to a class III C-terminal peptide. The possibility of the formation of a tripartite complex between the nNOS PDZ domain saturated with a C-terminal peptide and another PDZ domain attached to its β-hairpin has remained controversial. Whereas one report suggested that ligand binding releases

![FIGURE 4. Binding of NBP-123 peptide to WT nNOS PDZ domain, Y77A, S44A, and complex nNOS PDZ domain-α-syntrophin. FITC-labeled NBP-123 peptide (f-DRLRNRVHDGAV*) (50 nM) was titrated with increasing concentrations of wild-type nNOS PDZ domain (solid line), its Y77A mutant (dotted line), or its S44A mutant (dashed line). This FITC-labeled NBP-123 peptide was also titrated with wild-type nNOS PDZ domain preincubated with 250 μM purified α-syntrophin, which is known to bind to the β-hairpin (dashed-dotted line). A Coomassie Brilliant Blue-stained polyacrylamide-SDS gel with the purified recombinant PDZ domain of α1-syntrophin is shown in the inset. Data are representative of four independent measurements with variability below 1 millilipopartition unit (mp) in all cases in individual experiments of identical conditions.](https://jcb.asm.org/content/early/2016/05/27/jcb.201512015/Fig4.large.jpg)
PSD-95 from nNOS (21), other reports have suggested that nNOS is able to assemble a ternary complex (40, 45). Binding of the NBP-123 class III peptide to an nNOS PDZ domain-syntrophin complex showed a $K_d$ of $11.5 \pm 0.8 \mu M$, which is very similar to the $K_d$ value of $5.7 \pm 1.3 \mu M$ obtained for the PDZ domain by itself (Fig. 4B). The appearance of an increased maximal fluorescence polarization value is also consistent with the formation of a ternary complex and clearly indicates that both interaction regions work independently. Finally, we wondered whether the presence of 0.5 mM Ca$^{2+}$ or 0.5 mM calcium chelator EDTA might result in changes in the peptide-nNOS PDZ domain association. In neither of these conditions could we observe significant variations in the $K_d$ value of $5.7 \pm 1.3 \mu M$, indicating that, very likely, the intracellular calcium concentrations do not regulate the association of the nNOS PDZ domain with target peptides.

**Binding of the nNOS PDZ Domain to Class I Sequences**—

Next, we analyzed whether the nNOS PDZ domain could bind to class I C-terminal sequences. Both the C terminus of PMCA-4b, a plasma membrane calcium/calmodulin-dependent calcium ATPase (24), and that of the receptor tyrosine phosphatase-like protein ICA512 (46) have been reported to associate to the nNOS PDZ domain. Interestingly, both proteins display a C-terminal -ETXV-COOH motif (Fig. 5A). We analyzed in detail the interaction with PMCA-4b. Our yeast two-hybrid assay (Fig. 5B) showed a modest binding, whereas the melting profile of the nNOS PDZ associated to a PMCA-4b C-terminal peptide indicated a displacement of +2 °C (Fig. 5C). The exploration of this interaction by ITC rendered a $K_d$ of $59.9 \pm 2.5 \mu M$, whereas the resulting thermodynamic parameters were $\Delta H = -1.15$ kcal/mol and $\Delta S = -4.47$ kcal/mol (Fig. 5D). These data indicate that the binding partners of the nNOS PDZ domain also include class I C-terminal sequences. In addition, the value obtained for $K_d$ albeit modest, is also consistent with a physiological binding. It is possible, nevertheless, that this interaction in a cellular environment might be assisted by other types of interactions. Finally, the presence of an acidic residue in the P$_3$ position is reminiscent of the class II peptides aforementioned and might very likely contribute to the binding.

**Characterization of Novel nNOS PDZ-interacting Proteins**—

All the interactions with C-terminal peptides that we have characterized so far have been described in reports that analyzed the association to the nNOS PDZ domain using a great variety of techniques. For instance, in some reports, mass spectrometry techniques were used to identify cellular proteins that co-elute with the nNOS PDZ domain when recombinantly expressed and immobilized in a resin (18, 23). Alternatively, nNOS-associated proteins have been identified in immunoprecipitated samples using specific antibodies (24). Neither of these two approaches can rule out indirect associations. In contrast, a high scale analysis that identified C-terminal binding peptides interacting with 157 distinct PDZ domains suggested

![FIGURE5. Binding of the PDZ domain of nNOS to the C terminus of PMCA-4b, a class I peptide. A, C-terminal sequences of two proteins reported to be nNOS PDZ class I ligands. B, yeast two-hybrid assay using the nNOS PDZ domain (residues 1–131) in the bait plasmid and the C terminus of PMCA-4b in the prey plasmid. The columns indicate the yeast growth in the absence of the amino acid His and X-Gal activity (blue denotes a positive interaction). C, representative melting curve of the PDZ domain of nNOS PDZ domain in the absence (trace 1) or presence (trace 2) of 50 $\mu M$ PMCA-4b peptide (SSLQSLETSV*) in the 20–80 °C range recording the circular dichroism signal at 222 nm. Independent melting experiments of any condition differed less than ±0.3 °C between one another. D, isothermal titration calorimetric analysis of the binding of the PMCA-4b peptide (SSLQSLETSV*) to the PDZ domain of nNOS. The thermogram is shown in the upper panels, and the binding isotherms are shown in the bottom panels. The thermodynamics parameters for the peptide/nNOS PDZ domain interaction are also shown to the right. Data are representative of four independent titrations. mdeg, millidegrees.](https://doi.org/10.1074/jbc.M115.679684)

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three novel interacting proteins that could associate to the nNOS PDZ domain that are uncharacterized so far: the tight junction proteins claudin-3 and claudin-14 and the voltage-dependent sodium channel Nav1.4 (9). Both claudin-3 and claudin-14 would fall within the class III ligand (both ending in -DYV-), whereas Nav1.4 would fall within the class I ligands (ending in ESLV-COOH). To address whether these three proteins could indeed bind to the PDZ domain of nNOS, we used the biophysical techniques described above. All three bound to the nNOS PDZ domain in yeast two-hybrid assays (see below) and could displace the $T_m$ of the nNOS PDZ domain by $\sim 1.3$ °C in all cases (Fig. 6A). Our ITC analysis also showed a strong binding (Fig. 6B). Calculated $K_d$ values were $25 \pm 1.5 \mu M$ for claudin-3, $40 \pm 3.3 \mu M$ for claudin-14, and $10 \pm 0.9 \mu M$ for Nav1.4 C-terminal peptides. Hence, these three proteins expand the list of known ligands for the nNOS PDZ domain.

**Binding of the PDZ Domain of nNOS to Claudin-3**—Given these findings, we performed a detailed analysis of the interaction of the C terminus of claudin-3 with the PDZ domain of nNOS. Claudins are tight junction proteins that have four transmembrane domains, and their extracellular loops connecting the transmembrane domains form the paracellular barrier (47). Lung epithelial cells interconnected by tight junctions provide a barrier to the free diffusion of solutes into airspaces, and claudin-3 expression is known to regulate alveolar epithelial barrier function (48, 49). Conversely, nNOS is also enriched in the lung epithelium (50, 51). Furthermore, regulation of trafficking of Claudins has been attributed to interactions in their C-terminal intracellular tails (52). To address whether nNOS and claudin-3 colocalized in mammalian cells, we transfected mCherry-tagged claudin-3 and GFP nNOS (residues 1–771). In the absence of claudin-3 coexpression, nNOS(1–771) displayed a cytoplasmic distribution when transfected in HEK cells but became translocated to the plasma membrane tight junctions when associated to claudin-3 (Fig. 7A). Neither GFP by itself nor an nNOS construct that lacked the PDZ domain were able to become translocated to tight junctions where we could observe the claudin-3 localization. Although a claudin-3 C-terminal peptide bound tightly to the nNOS PDZ domain (Fig. 6), we wondered whether this interaction also occurred with full-length claudin-3. YFP-tagged Claudin-3 transfected in HEK cells was allowed to interact with full-length nNOS in a pulldown assay. Whereas YFP could not associate to nNOS, YFP-claudin-3 bound to the nNOS-saturated Sepharose beads (Fig. 7B). Hence, full-length nNOS and full-length claudin-3 can indeed form a complex.

The intriguing possibility that claudin-3 binding to the nNOS PDZ domain might be regulated through Tyr phosphorylation was also explored. The C termini of various claudin isoforms are known to display one or two phosphorylatable Tyr residues (Fig. 7C), and the activity of cellular tyrosine kinases is known to regulate the association of claudins and interacting PDZ domains (53, 54). The tyrosine residue at the $-1$ position is conserved among all claudin family members commonly expressed in epithelial cells except claudin-11 and claudin-12, whereas the $-6$ tyrosine is present only in certain members. In the case of claudin-3, it has been described that phosphorylation of both Tyr$_{-1}$ and Tyr$_{-6}$ might regulate its association with yet unrecognized PDZ domains (55). Moreover, in the tail of claudin-3, these 2 tyrosine residues were found to be phosphorylated in proteomics analysis of non-small cell lung cancer cells (56), and their phosphorylation was reduced upon treatment with the c-MET inhibitor gefitinib (57). To further dissect whether phosphorylation of claudin-3 C-terminal Tyr residues could affect its association to the nNOS PDZ domain, we created the phosphomimetic mutations Tyr$_{-1} \rightarrow$ Asp and Tyr$_{-6} \rightarrow$ Asp and analyzed the binding of these claudin-3 mutant sequences in a yeast two-hybrid assay. Whereas the Tyr$_{-1} \rightarrow$ Asp mutation completely abrogated binding, the binding of the Tyr$_{-6} \rightarrow$ Asp mutant was only marginally affected. In summary, claudin-3 behaves as a novel ligand for the nNOS PDZ domain, and this interaction might be regulated by the phosphorylation state of the 2 Tyr residues positioned at the claudin-3 C terminus.

**nNOS PDZ Domain Binding Properties**

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FIGURE 6. Binding of the PDZ domain of nNOS to the C terminus of three novel protein targets, claudin-3, claudin-14, and Nav1.4. **A**, representative melting curve of the PDZ domain of nNOS in the absence (trace 1) or presence of 50 $\mu M$ claudin-3 peptide (GTAYDRKDVY*) (trace 2), claudin-14 peptide (HSGYRLNDYV*) (trace 3), and the sodium channel Nav1.4 peptide (RPGVKESLV*) (trace 4) in the 20–80 °C range recording the circular dichroism signal at 222 nm. Independent melting experiments of any condition differed less that $\pm 0.3$ °C between one another. **B**, isothermal titration calorimetric analysis of the binding of the C-terminal peptides of claudin-3 (left panel), claudin-14 (middle panel), and the sodium channel Nav1.4 (right panel) to the PDZ domain of nNOS. The thermogram is shown in the upper panels, and the binding isotherms are shown in the bottom panels. Data are representative of four independent titrations. mdeg, milidegrees.
Protein/protein interactions mediated by PDZ domains are vital for numerous cellular functions and represent an attractive target for putative pharmacological intervention. The molecular details that govern specificity of nNOS PDZ domain interactions are not fully understood. We describe herein the systematic analysis using yeast two-hybrid assays of over 20 C-terminal sequences of proteins reported to bind to the PDZ domain of nNOS. The binding of synthetic peptides corresponding to selected sequences were also analyzed using ITC.

**Discussion**

Protein/protein interactions mediated by PDZ domains are vital for numerous cellular functions and represent an attractive target for putative pharmacological intervention. The molecular details that govern specificity of nNOS PDZ domain interactions are not fully understood. We describe herein the systematic analysis using yeast two-hybrid assays of over 20 C-terminal sequences of proteins reported to bind to the PDZ domain of nNOS. The binding of synthetic peptides corresponding to selected sequences were also analyzed using ITC.
FP, and CD. Our data indicate that the nNOS PDZ domain promiscuously binds to class I, class II, and class III C-terminal sequences. In addition, every single interacting sequence must possess an acidic residue. This applies to the internal binding motif found in class II sequences (-GDXΦΦD-) and to the C-terminal motifs found in both class III (-(D/E)XΦ-Φ-COOH) and class I (-(E/S/T)XΦ-COOH) sequences. Our data clearly show that synthetic peptides corresponding to all three classes can bind with $K_d$ values in the low micromolar range. NMR titration also indicates that Ser-44 and Tyr-77 differentially form hydrogen bonds with the side chain of the acidic residues of the interacting peptide depending on whether it is positioned in the $P_{-2}$ or $P_{-3}$ binding pocket (Fig. 8A). Class III peptides would bind in a canonical way with the hydrophobic Val$_6$ occupying Site S$_3$.

**FIGURE 8.** Proposed docking modes of C-terminal peptides when binding to the nNOS PDZ domain. A, based on the chemical shift changes, the NOS1AP peptide ELGDSLDEIAV* could bind within the hydrophobic groove through a canonical class II binding mode in which Val$_6$ would occupy site S$_3$ and Glu$_8$ would occupy site S$_4$, forming a hydrogen bond with Ser-44 (left panel). Alternatively, the upstream binding mode would proceed with Val$_6$ occupying site S$_1$ (outside the canonical groove and in the proximity of Ala-51), Leu$_6$ positioned inside the hydrophobic site S$_0$, and Asp$_8$ forming a hydrogen bond with Tyr-77 (right panel). B, class III sequences fall typically within the distribution -DXXV-COOH, whereas class II sequences (strictly -DXXD-COOH) usually also have an internal -GDLD motif. Finally, class I sequences have an -E(S/T)XXV-COOH motif. Proposed models depicting the various binding modes and the nNOS residues that stabilize each class are also shown. CtBP, C-terminal binding protein.
the S₀ pocket and the peptide Asp₋₋₋₋ hydrogen-bonding PDZ 
Tyr-77. Additionally, we have shown that proteins with C termi-
mini displaying class II motifs (such as Vac14 or NOS1AP) 
extend the interactions up to residues at position –₈ using an 
amino acid distribution that is almost identical in both cases 
(Fig. 8B). For this to occur, we propose that the backbone chem-
ical shift analysis supports a second discriminating binding 
mode. Our data suggest that the NOS1AP peptide, in addition 
to a class II-like insertion mode (Fig. 8A, left panel), is likely to 
move forward and have a different noncanonical, internal 
insertion mode, which partially relies on interactions outside 
the defined PDZ binding groove. It is then possible that the Leu 
residue at P₋₋₋₋ is packed into the hydrophobic pocket S₀ and the 
Val residue at P₋₋₋₋ fills an additional hydrophobic pocket, very 
likely affecting the side chain of Ala-51 (Fig. 8A, right panel). 
The presence of upstream binding motifs in C-terminal 
sequences that occupy the canonical binding groove of PDZ 
domains with the final residues being displaced forward is not 
without precedent (12). Our results also support the previous 
observation that an internal sequence of Vac14 might insert in 
the canonical binding groove of the nNOS PDZ domain (22). 
Furthermore, non C-terminal internal interactions have been 
observed in several other cases, including the PDZ domain of 
PICK1 when binding to the C terminus of AS161a (12), the cell 
polarity protein Par-6 that binds an internal sequence of Pals1 
(58), the C-terminal extension of neuronal NOS PDZ forming a 
β-hairpin when bound to syntrophin PDZ or PSD-95 (43, 44), 
and Dishevelled, which was suggested to bind an internal 
sequence of Frizzled (59).

Our data also indicate that cellular proteins displaying class 
III sequences never show Kᵥ values as low as those of the 
sequences retrieved in phage display experiments. As previ-
ously observed for numerous PDZ domains, phage display 
selects high affinity peptide ligands through an iterative plan-
ning process; some of these might be physiologically relevant. 
However, the in vivo ligand interactions for any given PDZ 
domain depend on the concentration and context of the protein 
in which it is located, its intrinsic peptide specificity, and the 
range and concentration of accessible ligands. Also, some phys-
iological ligands may interact with suboptimal affinities to regu-
late specific biological processes. Thus, endogenous C termi-
ni closely matching -DXV-COOH are likely to bind the nNOS 
PDZ domain in vivo.

In addition, we have provided a detailed description of the 
binding of the nNOS PDZ domain to three novel targets: clau-
din-3, claudin-14, and the sodium channel Nav1.4. The major-
ity of claudins possess PDZ binding motifs at their C terminus 
with which they are able to bind to various tight junction-asso-
ciated scaffolding/adapter proteins that link them to the cyto-
skeleton (47, 60). We have dissected in detail the molecular 
determinants of the nNOS PDZ domain/claudin-3 interaction, 
two proteins known to be present in the lung epithelium (50, 
51). We have shown that claudin-3 is able to induce the trans-
location of nNOS to the plasma membrane when claudin-3 is 
present. This association was also shown in pulldown assays. 
Furthermore, our results suggest that this association is regu-
lated through the tyrosine phosphorylation of the claudin-3 C 
terminus. Although phosphorylation of C-terminal sequences 
and the concomitant disruption of binding to PDZ domains are 
perhaps better characterized in the case of Ser and Thr residues, 
several precedents exist in which Tyr residues are involved. In 
this regard, Tyr-7 phosphorylation of the ErbB2 peptide EYL-
GLDVPV is known to abrogate binding to the β₂-β₃ loop of 
ErbB PDZ domain, hence significantly diminishing the binding 
affinity (61). Likewise, Tyr₋₋₋₋ phosphorylation in the C termi-

n of Syndecan-1 (sequence TKQEEFYA) prevents its binding 
to the PDZ domain of Syntenin-1 (62). Somehow, expectedly, 
our results indicate that phosphorylation of claudin-3 Tyr₋₋₋₋ might abrogate the binding of the C terminus of claudin-3 to 
the nNOS PDZ domain. The partial inhibition in the binding 
observed in the phosphomimetic Tyr₋₋₋₋ → Asp construct indi-

cates the presence of contacts of claudin-3 with the PDZ 
domain of nNOS that include residues distal from the C termi-

nus. This is reminiscent of the phosphorylation-dependent 
interaction between ZO-1 PDZ1 and claudin-2, which also con-
tains a phosphorylatable Tyr₋₋₋₋ residue (53). Because nitric 
oxide is known to mediate the degradation of claudin-5 (63) and 
to induce claudin-2 nitration in an experimental type 1 diabetic 
nephropathy model (64), it will be important to determine how 
claudin-3 functionality becomes regulated when attached to 
active nNOS.

In summary, our studies, presenting a detailed structural 
analysis of the binding of nNOS to C-terminal sequences, have 
revealed the molecular basis for the promiscuous behavior of 
the PDZ domain of nNOS and have identified novel binding 
partners. Finally, we have described herein the first nNOS PDZ 

binding partner known to interact in a phosphorylation-
regulated process.

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