The neuronal nitric oxide synthase PDZ motif binds to -G(D,E)XV* carboxyterminal sequences

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Abstract PDZ motifs are small protein–protein interaction modules that are thought to play a role in the clustering of submembranous signalling molecules. The specificity and functional consequences of their associative actions is still largely unknown. Using two-hybrid methodology we here demonstrate that the PDZ motif of neuronal nitric oxide synthase (nNOS) can mediate the binding to several other proteins in brain. Peptide library screening showed that proteins bearing a carboxy-terminal G(D,E)XV* sequence are preferred targets for the nNOS amino-terminal PDZ motif. Potential nNOS targets include a melanoma-associated antigen, cyclophilins and the α1C-adrenergic receptor.

Key words: Adrenergic receptor; Nitric oxide synthase; Protein interaction; PDZ motif; Signal transduction

1. Introduction

Protein–protein interactions are the crucial events in cellular signaling mechanisms and are in many cases mediated via relatively small protein domains. The importance of such defined binding modules came with the recognition of the SH2 and SH3 (src homology) motifs which were later shown to be very specific binding domains for phosphotyrosine-containing peptides and proline-rich stretches [1]. Later, the PH (pleckstrin homology), LIM (Lin-11/lsl-1/Mec-3) and PTB (phosphotyrosine-binding) domain have been added to this list [1–5]. Most recently, the PDZ motif was recognized as a protein-binding module in proteins involved in submembranous signaling processes [6–13]. This 90 amino acid sequence motif was first called GLGF repeat, later renamed to DHR (for Discs-large Homologous Region) and only recently termed PDZ motif (acronym of PSD-95/SAP90, a constituent of synaptic junctions, the *Drosophila* discs-large tumor suppressor gene product DlgA, present in septate junctions, and ZO-1, the major protein component of tight junctions) [14–17]. The motif was suggested to play a role in targeting the protein to the submembranous cytoskeleton or in regulating the intrinsic activity of the enzyme [18]. Currently, more than 50 different proteins are known to contain PDZ motifs [14,19,20]. Most of these proteins are involved in signal transduction events or organization of cytoskeletal structures, all at or close to the plasma membrane.

Other demonstrations of protein–protein interactions that are mediated by PDZ motifs have appeared recently. Several investigations reported on the recognition of a carboxy-terminal T/SXV* motif (where T is threonine, S is serine, X is any amino acid, V is valine, and the asterisk symbolizes the carboxyl end of the protein) by the PDZ domains in multiple members of the PSD-95/SAP90 family of membrane-associated guanylate kinases, MAGUKs [8,9,11]. The T/SXV* motif is found in a diverse series of receptor and channel proteins suggesting that PDZ-containing proteins may be widely involved in membrane specialization by anchoring signaling molecules at specific subcellular sites [15,17]. The in vivo relevance is evident form *InaD*, a PDZ-containing protein that is essential for negative feedback regulation of the light-activated signaling cascade in *Drosophila* photoreceptors by binding the TRP Ca^{2+} channel [6]. Also, the carboxy-terminus of the adenomatous polyposis coli gene product APC was shown to be recognized by the second PDZ domain of the human homolog of the *Drosophila* tumor suppressor *discs-large* [13].

The X-ray crystallographic structure of the third PDZ motifs of hDlg alone and PSD-95 provided the basis for understanding the recognition of the T/SXV* carboxyterminal peptide [21,22]. The peptide binds in a short groove on the surface of the PDZ motif and a prominent cavity, formed by conserved residues in the PDZ motif, stabilizes the carboxylate moiety [21]. In addition, another mode of interaction by PDZ motifs was observed for hDlg PDZ-3; the domain forms a dimer in the crystal albeit that this interaction in solution appeared rather weak [22].

Neuronal nitric oxide synthase (nNOS) differs from the two other NOS family members (endothelial NOS and macrophage NOS) in having an N-terminal segment that harbors a PDZ motif [18] and it has been proposed that this single PDZ motif is responsible for the membrane targeting of nNOS [23]. Indeed it has been demonstrated that its N-terminal PDZ motif determines nNOS association with the sarcolemma in muscle through binding to the dystrophin complex [10]. In brain the submembranous localization of nNOS was independent of the dystrophin complex [10]. To identify the protein-binding sites for the nNOS PDZ motif in brain tissue we used two-hybrid interaction trap methodology [24]. The consensus sequence for carboxy-terminal peptides that are preferentially bound by the nNOS PDZ motif was determined and used to predict potential nNOS-associated proteins in brain.

2. Materials and methods

2.1. Interaction-trap assay

Plasmid DNAs and the yeast strain used for the interaction-trap assay were provided by Drs. Finley, Brent and colleagues (Massachusetts General Hospital, Boston, MA) and used as described [24]. The bait vector, which contains sequences encoding the protein of interest fused to the DNA-binding domain, prey vector, containing inserts

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Abbreviations: MAGUK, membrane-associated guanylate kinase; nNOS, neuronal nitric oxide synthase; PDZ-PSD-95/Discs-large/ZO-1

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Table 1
Interactors of nNOS

Clone	Accession number
= Human cDNA clone 147773 5′ (127)	R 81609
~ Mouse cDNA clone 605767 5′ (321)	AA 154912
= Human thyroid receptor interactor mRNA TRIP13 (375)	L 40384
= Human thyroid receptor interactor mRNA TRIP13 (157)	L 40384
= Human cDNA clone 45462 5′ (477)	H 10486
= Human cDNA clone 530635 5′ (91)	AA 071112
= Human cDNA clone 143050 5′ (161)	R 71284

=, Identical; ~, 90% homologous. Between brackets the position of the starting nucleotide of the obtained cDNA in the database sequence entry is given.

encoding protein fused to the transcription activation domain, and lacZ reporter plasmid were introduced into yeast strain EGY48 by transformation. Interaction was tested on minimal agar plates lacking leucine, tryptophan, uracil and histidine, and containing 2% galactose, 1% raffinose and 80 μg/ml X-gal [24].

2.2. Generation of nNOS subclones

The full-length rat nNOS cDNA, cloned in plasmid pBluescript KS, was a gift of Dr. Snyder (Baltimore, MD). The insert of subclone nNOS(aa 1–111) was generated by the polymerase chain reaction (PCR) using oligonucleotide primers nos1 (5′-TTGAATTCTGAAG-GACACAGATACCAT-3′) and nos2 (5′-TAAGATCTGAAGGTG-GTCTCCAGATG-3′). The amplified product was blunt-ended with Klenow (large fragment) DNA polymerase and subcloned in pEG202 vector. Subclone nNOS(aa 1–195) is the product of the amplification with primers nos1 and nos3 (5′-TTTCTCGAGCATCATGTTCCCC-GATGT-3′). After digestion with *Eco*RI, this fragment was blunt-ended with Klenow, digested with *Xho*I and also subcloned in pEG202 vector. The fragment nNOS(aa 99–418) that is missing the PDZ motif was excised from pBlue-nNOS using *Apa*I and *Bam*HI restriction enzymes, blunt-ended and subcloned in pEG202.

2.3. Generation of PSD-95/SAP90, Fas and α1C-adrenergic receptor subclones

Total RNA was isolated using the LiCl/urea method [25]. SAP90 cDNA fragment was generated by reverse transcriptase (RT)-PCR as described [26]. Specific oligonucleotide primers SAP90-1 (5′-CCA-GATCTGCAGTGGAGGCCC-3′) and SAP90-2 (5′-AGGCTCGAG-GTCCTTGGCCAC-3′) were deduced from the published sequence [27]. The amplification product was digested with *Bgl*II and *Xho*I and subcloned in plasmid pJGBr2 (a modified prey vector) and a pEG202-derived bait vector.

Prey plasmid pJG-hFas was constructed by subcloning a blunt-ended *Bgl*II–*Apa*I fragment derived from pCMV-hFas (kindly provided by Dr. P. Anderson, Boston, MA) into the pJGBr2 interaction trap prey vector.

A cDNA fragment encoding the last 114 amino acid residues of the rat α1C-adrenergic receptor [28] was generated by reverse transcriptase (RT)-PCR [26] using oligonucleotide primers α1CAR-1 (5′-GGAATTCAAGCATGCCCTGGG-3′) and α1CAR-2 (5′-ATACTC-GAGCTAGACTTCCTCCCCGTT-3′). The PCR product was digested with *Eco*RI and *Xho*I and subcloned in the prey vector pJG4-5.

2.4. Construction of peptide library

The sequences of the oligonucleotides used in the generation of the peptide library are 5′-CCGGATCC(NNB)$_{20}$CGAATTCC-3′ (where N is a mixture of A, G, C and T and B a mixture of G, T and C) and 5′-GGAATTCG-3′ (for antisense priming). The primers were annealed at room temperature and filled in using Klenow (large fragment) DNA polymerase in the presence of 0.1 mM of each dNTP. After removal of unincorporated nucleotides and primers, the amplified product was digested with *Bam*HI and *Eco*RI. After size-separation on 4% NuSieve GTG agarose (FMC) the product was recovered from the agarose using Gelase (Biozym) and then precipitated. Subsequently, the fragments were ligated in *Bgl*II–*Eco*RI-digested pJGBr2 prey vector, transferred to TG-1 cells by electroporation and plated on TYE/glucose plates. The resulting library contained 8×10^8 independent transformants of which 95% contained the peptide-encoding insert. Colonies on the plates were collected by scraping and plasmid DNA was isolated using the Qiagen plasmid Mega Kit (Westburg).

2.5. DNA sequence analysis

Nucleotide sequences were determined by the double-stranded DNA dideoxy sequencing method using radioactive nucleotides [29] or by cycle sequencing employing fluorochrome-tagged primers according to the manufacturer's instructions (Amersham, UK). DNA sequence gel readings were analyzed using the GCG package [30] provided by the Dutch CAOS/CAMM Center. The OWL database (a nonredundant sum of Swiss-Prot, PIR, GenBank and NRL-3D databases), release 29.1 (January 1997), was searched for -G(D,E,N)XV stop-containing proteins using FINDPATTERNS [30].

3. Results

In muscle tissue nNOS can be targeted to the dystrophin glycoprotein complex on the sarcolemma by virtue of its PDZ motif [10]. In brain, a dystrophin glycoprotein complex is absent but still nNOS is localized submembranously [10] suggestive of other nNOS targets in brain. Reasoning that the nNOS PDZ motif was responsible for the subcellular distribution [23] we set out to clone the nNOS PDZ motif targets in brain using the two-hybrid interaction trap methodology [24]. To this end, the rat nNOS PDZ motif (amino acids 1–111) as fused to the LexA DNA-binding domain and used as a bait to screen a human fetal brain cDNA library. Several interacting clones were obtained from 10^6 transfected yeast colonies and for most cDNAs the interaction could be confirmed after re-introduction of the involved plasmids in yeast. Subsequently, end-in sequencing of the isolated interacting cDNAs was performed to enable screening of the nucleotide sequence databases for possible homologies to known genes. The results are summarized in Table 1. Five clones appeared identical or homologous to expressed sequence tags (EST), database entries for which no functional data at the protein level are available. Also, two independent but overlapping cDNAs were isolated that encode a thyroid receptor interacting protein called TRIP13 [31]. This suggests an nNOS PDZ-mediated submembranous docking of TRIP13 and, consequently, of the thyroid receptor that is awaiting its ligand. After steroid binding the receptor is released from TRIP13 and could

Table 2
Neuronal NOS PDZ motif specificity deduced through two-hybrid screening of a random peptide library

Peptide	Sequence	Interaction
1	GAGERGDSV*	++
2	YAGQWGESV*	++++
3	GDAV*	++
4	RRWGDPV*	++
5	R. PAGNPV*	++
6	RGRDQRDDAV*	+

Interaction results are reflected using plus and minus signs: ++++, very strong binding; ++, strong binding; +, weak but significant interaction.

Table 3
PDZ motif specificity for distinct carboxyl-terminal peptide sequences

Preys	Baits		
	NOS PDZ	SAP90 PDZ2	PTP-BL PDZ2
Peptide 2 (-GSEV*)	++++	−	−
hFAS (-QSLV*)	−	++	+
MAGE-11 (-GEGV*)	+/−	−	−
α1C-AR (-GEEV*)	++++	−	−
Empty pJG vector	−	−	−

Interaction results are reflected using plus and minus signs: ++++, very strong interaction; ++, strong interaction; +, weak but significant interaction; +/−, very weak interaction; −, no interaction detectable.

move to the nucleus to perform its role as a transcription factor. Such a model does not unravel, however, the mechanism by which nNOS itself is targeted to the submembranous space in brain cells. Perhaps PDZ motifs can mediate multiple interactions simultaneously, which would imply independent binding interfaces on this small protein domain.

As an alternative strategy to identify target sites for nNOS we constructed a library that directed the synthesis in yeast of constrained peptides of random sequences fused to the protein moiety encoded by the interaction trap prey vector and screened it using the nNOS PDZ motif as a bait. Oligonucleotides were used to produce DNA fragments harboring 20 random codons of the type NNB (N is any nucleotide; B is G, C, or T) flanked by *Bam*HI and *Eco*RI sites. The codon bias was chosen in such a way that two-thirds of the encoding oligonucleotides would lack internal stop codons. The resulting library contained 8×10^8 individual recombinants. From 0.25×10^6 yeast transformants, we isolated six plasmids that encoded peptides interacting with the nNOS PDZ motif (Table 2) but not with control proteins. From these peptide sequences a C-terminal consensus target site, -G(D,E)XV*, emerged.

We next investigated the sequence specificity in the nNOS PDZ-mediated interaction in a two-hybrid assay using different baits (Table 3). The nNOS PDZ motif, which has a strong affinity for the peptide GESV*, does not interact with the (T,S)XV* MAGUK target sequence as present in human Fas. The second PDZ motif of the MAGUK protein PSD-95 does bind to this hFas C-terminal peptide (Table 3). The same holds true for the PTP-BL PDZ motif that was reported to bind to human Fas [12]. Both PDZ motifs, however, failed to interact with the nNOS PDZ-specific peptide GESV* (Table 3).

To look for proteins that do contain a G(D,E,N)XV* carboxyterminus and thus are prime candidates for nNOS-associated proteins, we performed a database search and identified four different types of mammalian proteins. The liver-specific nuclear protein acylneuraminate cytidylyl transferase (acc. no. P29188; carboxy-terminal sequence -GNGV*) from rat was considered not relevant on the basis of its restricted localization. The other mammalian proteins were the human melanoma-associated antigen MAGE-11 (acc. no. P43364; sequence -GEGV*), the α1-adrenergic receptor (AR) subtype C (acc. no. P43140; sequence -GEEV*), and peptidylprolyl isomerases or cyclophilins (acc. no. S61070 and S66681; sequence -GEYV*). To test these predicted interactions a MAGE-11-encoding prey clone (available through an unrelated two-hybrid screen; W.H.A. van den Maagdenberg, B. Pepers; unpublished data) and the carboxyterminal tail of the rat α1C-AR were used in the interaction trap assay. Indeed we could observe a weak interaction for MAGE-11 and a strong one for α1C-AR with the nNOS PDZ motif (Table 3).

Table 4
Binding of nNOS to the SAP90 PDZ2 motif requires sequences C-terminal of the nNOS PDZ motif

Bait	Prey: SAP90-PDZ 2	Prey: hFAS
nNOS (amino acid 1–111)	−	−
nNOS (1–195)	++	−
nNOS (99–418)	−	−
SAP90-PDZ 2	ND	+

Interaction results are reflected using plus and minus signs: ++, strong binding; +, weak but significant interaction; −, no interaction detectable. ND, not determined.

In our search for submembranous nNOS-associating proteins in brain we did not obtain MAGUK-encoding cDNA clones as reported by Bredt and coworkers [7]. They described the binding of the second PDZ motif of PSD-95/SAP90 and of the related protein PSD-93 to amino acids 1–195 of nNOS, suggestive of PDZ-PDZ homotypic interactions. The nNOS fragment that was used in their experiment spans not only the PDZ motif (as in our study; residues 1–111) but contains an additional 87 residues C-terminally. Thus, the observed interactions of nNOS with PSD-95/SAP90 and PSD-93 could be mediated through binding of stretches just C-terminal of the nNOS PDZ motif, spanning residues 112–195. To investigate this possibility we generated a cDNA fragment encoding the second PDZ motif of PSD-95 by RT-PCR on brain mRNA and inserted the fragment into the interaction trap prey vector. Subsequently, various nNOS N-terminal fragments were produced as baits for two-hybrid analysis (Table 4). The experiment showed that no interaction with PSD-95 PDZ-2 is observed for the nNOS PDZ motif alone (aa 1–111). Also, no binding with PSD-95 was found if only the additional 84 residues just C-terminal of the PDZ domain were present. By contrast, employing the entire stretch of 195 N-terminal residues of nNOS (including the PDZ motif) in the two-hybrid assay resulted in an interaction with the second PSD-95 PDZ motif (Table 4).

4. Discussion

It is becoming increasingly clear that PDZ motifs are well-tailored to bind to the carboxy-termini of proteins [15,17,20,32,33]. Use of the oriented peptide library technique has resulted in a catalogue of unique optimal sequences for multiple individual PDZ motifs, defined primarily by the carboxyl terminal three to seven residues of the target peptide [34]. Using two-hybrid methodology we have identified the carboxyterminal consensus sequence that is recognized specifically by the neuronal NOS PDZ motif (Table 2). This target site differs from the ones determined for mouse discs-large, PTP-BAS, p55, LIN-2, Tiam-1, and AF-6 [34] and the (T,S)XV* peptide recognized by MAGUK family member PDZ motifs [15,17]. Carboxyterminal sequence recognition,

however, cannot explain the observed interaction between nNOS and PSD-95/SAP90 [7] and also a pure PDZ-PDZ homotypic interaction can be excluded (Table 4). As a matter of fact, using a variety of PDZ motifs in the interaction trap assay, including those of PSD-95, nNOS and PTP-BL [19], we have never observed any significant PDZ-PDZ association (data not shown). Nevertheless, the affinity of PSD-95 PDZ motif 2 for an nNOS internal protein segment convincingly demonstrates that PDZ-mediated interactions are not limited to C-terminal peptide recognition only.

We re-examined the clones isolated from the brain cDNA library using the nNOS PDZ motif (Table 1) for the carboxyterminal sequence they encoded but this was hampered by the limited sequence data that are available for ESTs. No 3′ sequence entries have been deposited in the database for the EST cDNA clones 605767 and 143050. For EST clones 147773, 45462 and 530635 sequences have been determined at the 3′ end (acc. nrs. R81352, H09728 and AA071113) but, provided that coding sequences are at all present in these 3′ sequence segments, no G(D,E,N)XV*-like carboxyterminus was found. Also the TRIP13 carboxyterminus (-LSLA*) lacks homology to the peptide target binding site preferred by the nNOS PDZ motif.

A database search with the preferred nNOS PDZ target site revealed three interesting candidate partner proteins: the melanoma-associated antigen 11, cyclophilins and the α1C-adrenergic receptor (Table 3). The human melanoma-associated antigen MAGE-11 is a member of a large family of tumor rejection antigens that are normally only expressed in testis and placenta. The cellular function of this protein class is completely obscure and, in combination with the highly restricted expression profile, precludes a further discussion. The prototype members of the *cis–trans* peptidyl-prolyl isomerases, cyclophilin A and FKBP12, were discovered on the basis of their ability to bind and mediate the immunosuppressive effects of the drugs cyclosporin, FK506 and rapamycin. Cyclophylin is localized submembranously and in brain cyclosporin blocks the IL-2-induced release of CRH (corticotropin-releasing hormone) by preventing the dephosphorylation and thus activation of nNOS [35]. This would, however, not require a direct interaction between the cyclophylin carboxy-terminal tail and nNOS, and can be explained by the cyclosporin–cyclophylin complex binding to calcineurin, the calcium-dependent protein phosphatase that should dephosphorylate nNOS [36]. Finally, the α1-adrenergic receptor (AR) subtype C is expressed in multiple tissues including brain and its carboxyterminal tail of the rat α1C-AR strongly and specifically interacted with the nNOS PDZ motif (Table 3). Interestingly, NOS is heavily involved in the regulation of hormone and neuropeptide release in different areas of the brain and is acting downstream of the α1-AR in certain signaling pathways [37,38]. A physical link between the α1-AR and NOS would be an ideal setting for such a signal cascade and, moreover, might explain why inhibition of NOS does not affect α2-AR signaling [39]. The interaction of the nNOS PDZ motif and the α1C-AR reported here now enables to test the occurrence in vivo and, of course, to determine its functional implications.

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