FEBS Letters 580 (2006) 6948–6954

Binding of Vac14 to neuronal nitric oxide synthase: Characterisation of a new internal PDZ-recognition motif

Jean-François Lemaire, Peter S. McPherson*

Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, 3801 University Street, Montreal, Que., Canada H3A 2B4

Received 11 September 2006; revised 30 October 2006; accepted 24 November 2006

Available online 4 December 2006

Edited by Gianni Cesareni

Abstract PDZ domains mediate protein interactions primarily through either classical recognition of carboxyl-terminal motifs or PDZ/PDZ domain associations. Several studies have also described internal modes of PDZ recognition, most of which depend on β-finger structures. Here, we describe a novel interaction between the PDZ domain of nNOS and Vac14, the activator of the PtdIns(3)*P* 5-kinase PIKfyve. Binding assays using various Vac14 deletion constructs revealed a β-finger independent interaction that is based on a novel internal motif. Mutational analyses reveal essential residues within the motif allowing us to define a new type of PDZ domain interaction.

Keywords: Peptide motif; PDZ; nNOS; Vac14; PIKfyve; 5-HT_{2B} receptor

1. Introduction

PDZ domains are protein interaction modules found in single or multiple copies in a variety of proteins involved in multiprotein signaling complexes. Examples include the postsynaptic density protein PSD-95, the septate junction protein *Drosophila* Discs-large and the epithelial tight junction protein ZO-1, from which the name PDZ originates [1]. Although PDZ/PDZ domain interactions can occur [2,3], the most common mode of PDZ domain recognition is through the last four or five C-terminal residues of interacting partners. Proteins bearing C-terminal peptide motifs generally target specific PDZ domain proteins [4]. In fact, different classes of PDZ domains have been defined based on their preferred C-terminal peptide binding motifs: Class I, -[S/T]-x-Φ*; Class II, -Φ-x-Φ*; Class III, -[D/E/K/R]-x-Φ* and Class IV, -x-Ψ-[D/E]* [1], where * indicates the free carboxyl group at the C-terminus, Φ represents hydrophobic residues, Ψ represents aromatic residues and x is any amino acid.

The neuronal isoform of nitric oxide synthase (nNOS) differs from the two other enzyme paralogs, the endothelial NOS (eNOS) and inducible NOS (iNOS) by an additional 230 residue N-terminal stretch containing a Class III PDZ domain [5]. Given the physiological importance of nNOS in a number of cellular processes including neurotransmitter release, cell survival, muscle contraction, and translocation of the glucose transporter GLUT4, efforts have been made to identify nNOS PDZ domain-binding partners and to define the motifs that mediate the interactions. Peptide library screens have demonstrated a preference of the nNOS PDZ domain for peptides ending with -D-x-V* [6] and more specifically -G-[D/E]-x-V* [4]. Moreover, nNOS uses an internal β-hairpin "finger" structure that mimics a typical C-terminal motif to mediate the interaction of its PDZ domain with the PDZ domains of syntrophin and PSD-95/93 [7–9]. Besides β-fingers, there are only a few examples of internal motifs for PDZ recognition. These include binding of the Dishevelled PDZ domain to internal -K-T-x-x-x-[W/I]- motifs in Frizzled [10] and Idax [11] and the internal sequence -H-R-E-M-A-V- that mediates the Pals1–Par-6 interaction [12].

Vac14 is an evolutionary conserved eukaryotic gene that was originally described in yeast based on its mutation causing vacuole inheritance, acidification and membrane morphology defects [13,14]. Vac14 is involved in the hyperosmotic stress response and controls phosphatidylinositol 3,5-biphosphate (PtdIns(3,5)P_2) synthesis by interacting with and activating the yeast PtdIns3*P* 5-kinase Fab1p, and its mammalian ortholog PIKfyve [14–17]. The phosphoinositide PtdIns(3, 5)P_2 is a non-abundant phospholipid essential for the delivery of cargo into the vacuole/lysosomal compartment via endosomes [18,19]. Intriguingly, we identified Vac14 in a screen for vesicle trafficking proteins [20]. Upon activation with Vac14, PIKfyve but not Fab1p, also produces PtdIns(5)*P*, which has implications in actin remodeling and GLUT4 dynamics [21,22].

Here, we identify nNOS as a Vac14-binding partner and we demonstrate that the nNOS PDZ domain recognizes two peptides motifs in Vac14, one at the C-terminus and a novel motif within the protein chain. The nNOS/Vac14 interaction depends on the internal motif, whereas the C-terminal motif is found to contribute to the interaction but is not sufficient for binding. Through mutational studies, we define critical residues within the internal motif and propose -G-[D/E]-x-Φ-[D/E]- as an internal consensus motif for the nNOS PDZ omain.

2. Material and methods

2.1. Antibodies

A polyclonal Vac14 antibody was raised in rabbits against a peptide of human Vac14 ($_{762}$HLEVRHQRSGRGDHLDRRVVL$_{782}$) using a previously described technique [23]. Polyclonal sera against glutathione-*S*-transferase (GST) and NECAP 1 were previously described

*Corresponding author. Fax: +1 514 398 8106.
E-mail address: peter.mcpherson@mcgill.ca (P.S. McPherson).

0014-5793/$32.00
doi:10.1016/j.febslet.2006.11.061

[24,25]. Monoclonal antibodies against synaptophysin and FLAG were from Sigma–Aldrich, nNOS and His6 monoclonal antibodies were from BD Transduction Laboratories and Qiagen, respectively.

2.2. DNA constructs and recombinant proteins

GST-, His- and FLAG-tagged expression constructs were generated by PCR from either Vac14 cDNA (human EST GenBank accession number BM468215) or from human nNOS cDNA (generous gift of Dr. Phil Marsden) using the appropriate primers (Supplementary Table 1) with subsequent cloning into pGEX-4T1 (GE Healthcare), pFO4 (derived from pET15b; kindly provided by Dr. Mirek Cygler) and pCMV-tag2b (Stratagene) vectors, respectively. GST- and His-tagged fusion proteins were expressed in *Escherichia coli* BL-21. N-terminal GST-tagged wild-type and mutated variants of Vac14 $[768–782]_{aa}$ and $[768–777]_{aa}$ and 5-HT_{2B} receptor $[464–479]_{aa}$ were generated by subcloning annealed complementary oligos into pGEX-4T1 (Supplementary Table 1). All constructs were confirmed by sequencing.

2.3. Cell and tissue extracts

HEK 293-T cells expressing FLAG-Vac14 were lysed by sonication in 10 mM HEPES pH 7.4 containing 33 mM NaCl and protease inhibitors (PIs) (0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/mL aprotinin, and 0.5 μg/μL leupeptin). Triton X-100 was added to 1% final concentration and extracts were agitated for 10 min at 4°C, followed by centrifugation at $300\,000 \times g$ for 15 min. The resulting supernatant was analyzed by Western blot. Rat tissues were homogenized in 20 mM Hepes pH 7.4 with PIs and centrifuged at $800 \times g$ for 5 min. Subcellular fractionation of brain extracts was performed as previously described [26]. Equal protein amounts of the cell lysates, tissue extract supernatants and brain subcellular fractions (10, 200 and 100 μg/lane, respectively) were analyzed by SDS–PAGE and Western blot.

2.4. Binding studies

2.4.1. Immunoprecipitation assays. A soluble rat brain extract was prepared in buffer A (10 mM HEPES pH 7.4, 1% Triton X-100, PIs) and pre-cleared by incubation with Sepharose beads as described [27]. The extract (2 mg) was incubated overnight at 4 °C with 2 μl of the preimmune or the Vac14-immune rabbit serum (#3865) precoupled to protein-A Sepharose. Beads were washed three times in buffer A prior to processing by SDS–PAGE and either Coomassie staining or Western blot analysis. Specific bands revealed by Coomassie staining were analyzed by tandem mass spectrometry (MS) as previously described [28].

2.4.2. GST pull-down assays. GST pull-down assays were performed in buffer A, supplemented with 150 mM NaCl, using GST-fusion proteins precoupled to glutathione-Sepharose, and purified His-tagged proteins. For experiments shown in Fig. 3A and C, 1% Triton X-100 was replaced with 1% Tween-20. Protein amounts shown in Fig. 2D were quantified using ImageJ (National Institutes of Health, USA).

2.4.3. Overlay assays. The GST tag from the GST-Vac14 full-length ($[1-782]_{aa}$) fusion protein (GST-Vac14) was cleaved off by thrombin and 3 μg of Vac14 protein was immobilized on nitrocellulose membranes. The membranes were incubated overnight at 4 °C with 10 μg/mL of either eluted GST or GST-nNOS $[1–100]_{aa}$ (GST-nNOS PDZ) in Tris-buffered saline (20 mM Tris, 150 mM NaCl, pH 7.4), 3% bovine serum albumin, 0.1% Tween-20 and 1 mM dithiothreitol (DTT). Bound proteins were revealed by Western blotting using a polyclonal antibody against GST as described [24].

3. Results

To better characterize mammalian Vac14, we raised an anti-peptide Vac14 polyclonal antibody in rabbits. Reactivity of the antibody against Vac14 was verified using extracts of either

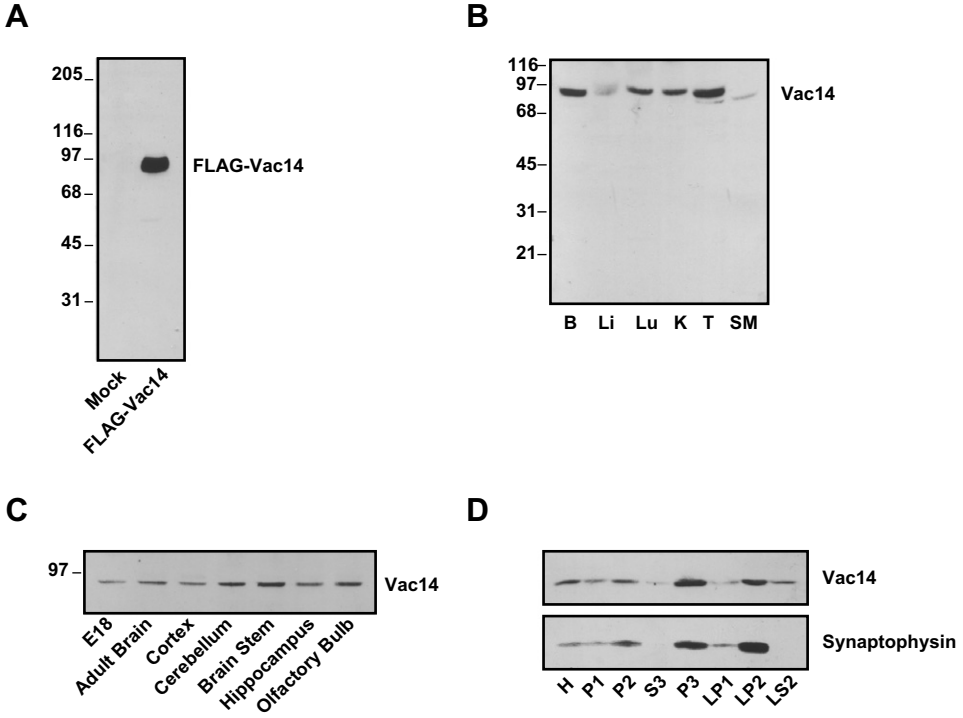

Fig. 1. Expression pattern of Vac14. (A) Lysates (10 μg/lane) from FLAG and FLAG-Vac14 transfected HEK-293 cells were blotted with affinity-purified Vac14 antibody. (B) Equal protein aliquots (200 μg/lane) of rat brain (B), liver (Li), lung (Lu), kidney (K), testis (T) and skeletal muscle (SM) extracts were blotted with affinity-purified Vac14 antibody. (C) Equal protein aliquots (200 μg/lane) of extracts from various adult rat brain regions and embryonic day 18 (E18) whole brain were blotted with affinity-purified Vac14 antibody. (D) Proteins of brain subcellular fractions (100 μg/fraction) were blotted with antibodies against Vac14 and synaptophysin as indicated (homogenate, H; pellet, P; supernatant, S; lysed pellet, LP; lysed supernatant, LS).

mock or FLAG-Vac14-transfected cells (Fig. 1A). The antibody detected a single band in tissue extracts at approximately 85 kDa, the predicted molecular mass of Vac14. Vac14 was found to be expressed in brain, lung, kidney and testis with highest levels in brain and testis (Fig. 1B). For brain, Vac14 is detected in all regions of adult brain tested and is also present in embryonic brain (Fig. 1C). Subcellular fractionation of rat brain shows that Vac14 is present in a microsomal membrane fraction (P3) and also enriches in LP2, a fraction composed of microsomal membranes from synapses, suggesting a neuronal function for Vac14 (Fig. 1D). The synaptic vesicle protein synaptophysin shows the expected fractionation profile (Fig. 1D).

We immunoprecipitated Vac14 from solubilized rat brain extracts to identify protein binding partners. Coomassie staining revealed specifically immunoprecipitated bands at approximately 85 kDa and 160 kDa (data not shown), and tandem MS analysis identified the 85 kDa band as Vac14 (21 peptides) and the 160 kDa band as nNOS (7 peptides). The interaction was confirmed by Western blot of the immunoprecipitates (Fig. 2A). In contrast, the abundant brain protein NECAP 1 [29] does not co-immunoprecipitate with Vac14 (Fig. 2A).

nNOS differs from the other enzyme paralogs, eNOS and iNOS by the presence of an N-terminal PDZ domain. To test if this domain mediates interaction with Vac14, we generated constructs encoding the first 100 residues of nNOS (nNOS PDZ), which includes the complete PDZ domain but does not harbour the β-finger structure just C-terminal of the PDZ domain. The binding of His-Vac14 to GST-nNOS PDZ demonstrates a direct interaction through the nNOS PDZ domain (Fig. 2B), which was confirmed in overlay assays (Fig. 2C). When testing a range of His-Vac14 concentrations for nNOS binding, we found saturation at approximately 20 μg (0.23 μM) (Fig. 2D and E).

The detection of Vac14/nNOS binding by overlay suggests an interaction through a peptide motif, as secondary and tertiary structures in Vac14 are likely denatured during SDS–PAGE. To identify the nNOS binding sequences in Vac14, we tested a series of deletion constructs in pull-down assays for binding to GST-nNOS PDZ (Fig. 3A–C). GST-nNOS

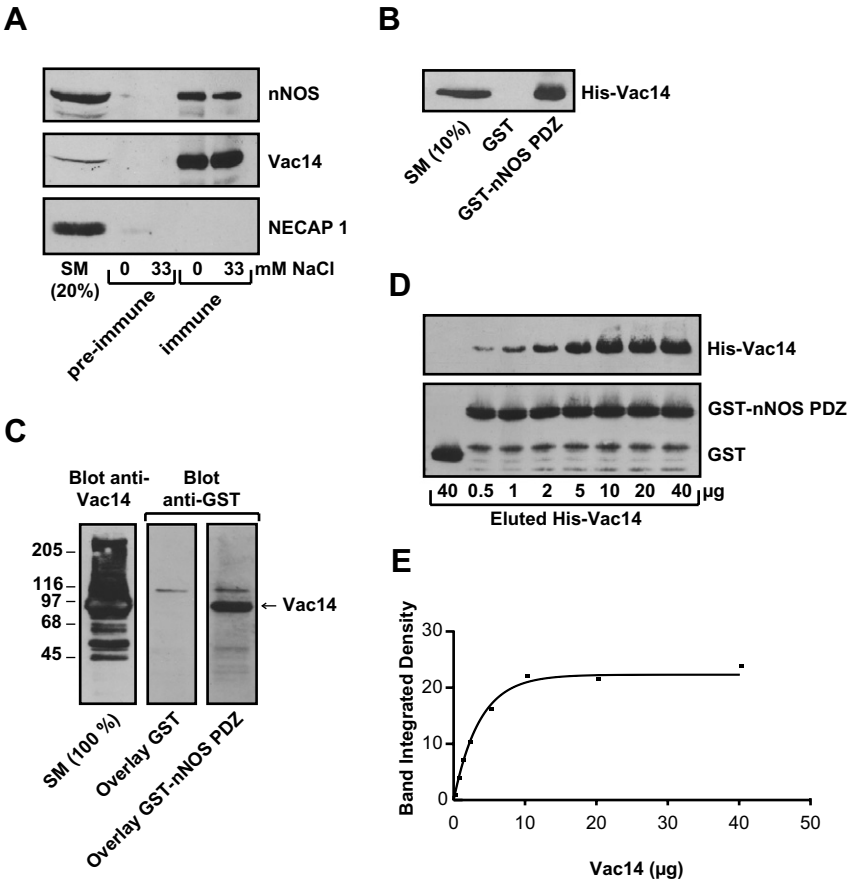

Fig. 2. Vac14 interacts with nNOS. (A) A Triton X-100 soluble rat brain extract (2 mg), with or without 33 mM NaCl was incubated with immune or pre-immune anti-Vac14 sera pre-coupled to protein-A Sepharose. Proteins specifically bound to the beads were blotted with antibodies against nNOS, Vac14 and NECAP 1 as indicated. An aliquot of the brain extract (20%) was loaded as starting material (SM). (B) Purified His-Vac14 was incubated with GST or GST-nNOS PDZ domain coupled to glutathione-Sepharose and proteins specifically bound to the beads were blotted with antibody against the His tag. An aliquot of His-Vac14 (10%) was loaded as starting material (SM). (C) Purified Vac14 was resolved on SDS–PAGE and transferred to nitrocellulose. Membrane strips were blotted with Vac14 antibody or were incubated with soluble GST or GST-nNOS PDZ domain and specifically bound proteins were detected with anti-GST antibody. (D) Purified His-Vac14 was incubated in increasing amounts with GST-nNOS PDZ domain or 40 μg of the protein was incubated with GST (left lane). Proteins specifically bound to glutathione-Sepharose beads were blotted with antibodies against the His tag and GST. (E) Binding curve of the interaction of His-Vac14 with nNOS PDZ domain based on quantification of band intensities from the blot shown in panel D.

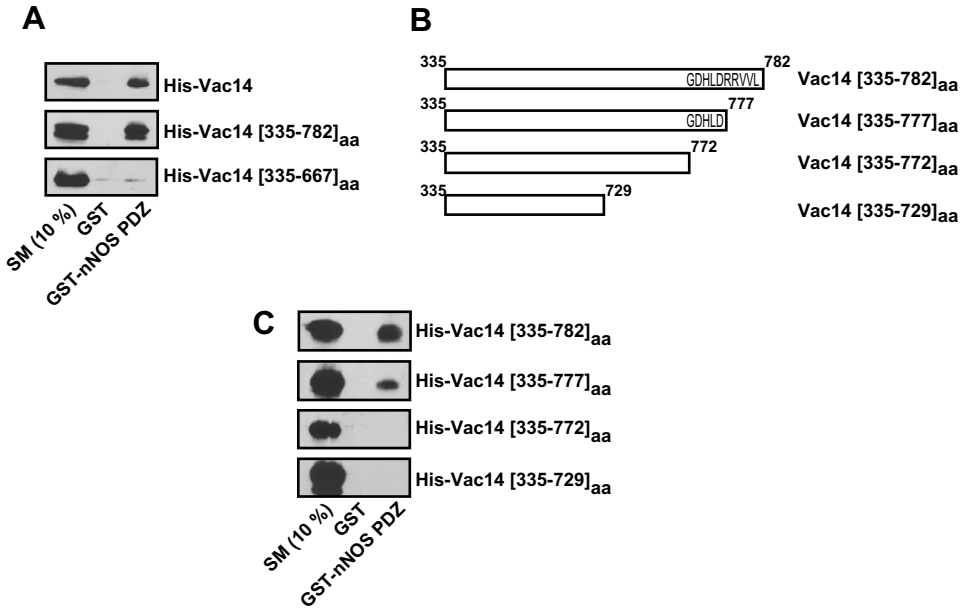

Fig. 3. Vac14 interacts with the nNOS PDZ domain through an internal motif. (A and C) Purified His-Vac14 and His-Vac14 deletion constructs were incubated with GST alone and GST-nNOS PDZ domain coupled to glutathione-Sepharose. Proteins specifically bound to the beads were blotted with antibody against the His tag. An aliquot of each His-tagged protein (10%) was loaded as starting material (SM). (B) Schematic representation of Vac14 deletion constructs with the C-terminal most 10 amino acids indicated.

PDZ binds with comparable levels to full-length Vac14 and a Vac14 N-terminal deletion construct $[335–782]_{aa}$. In contrast, a Vac14 variant encoding residues 335–667 failed to bind, narrowing down the site of interaction to the last 115 amino acids (Fig. 3A). The last five residues of Vac14 (-R-R-V-V-L*) do not fit the Class III PDZ domain consensus motif -[D/E/K/R]-x-Φ*, but match the Class II consensus motif -Φ-x-Φ*, which is not expected to recognize the Class III nNOS PDZ domain. Interestingly, Vac14 contains an internal sequence -G-D-H-L-D- immediately upstream of the -R-R-V-V-L* stretch, which is similar to the C-terminal consensus motif -G-[D/E]-x-V* previously described to bind to the nNOS PDZ domain [4] (Fig. 3B). In order to elucidate the contribution of these potential peptide motifs to nNOS PDZ domain binding, we tested a series of C-terminal Vac14 deletion constructs (Fig. 3B and C). Binding is not abolished by deletion of the last five amino acids, but is abolished with deletions of the last 53 or last 10 residues (Fig. 3C). The loss of binding seen for Vac14 $[335–772]_{aa}$ when compared to Vac14 $[335–777]_{aa}$ reveals the sequence -G-D-H-L-D- from amino acids 773 to 777 as a functional internal binding motif (Fig. 3C). The decrease in binding for Vac14 $[335–777]_{aa}$ compared to $[335–782]_{aa}$ (Fig. 3C) raises two possibilities: the Vac14/nNOS PDZ interaction is mediated (1) by internal and C-terminal motifs contributing equally to binding or (2) by binding primarily to an internal motif with secondary contributions from a C-terminal motif.

To examine these possibilities, we tested the binding of nNOS to a series of Vac14 mutants within the context of Vac14 $[768–782]_{aa}$, containing both motifs and Vac14 $[768–777]_{aa}$, containing the internal motif only (Fig. 4A). The wild-type sequences interact with nNOS in pull-down assays (Fig. 4B–D) and the reduction in binding for the short construct verifies the contribution of both motifs to the interaction (Fig. 4B). Similar results are seen when the C-terminal motif is mutated by conversion of L782 to glycine or by the mutation of residues 780–782 to alanine, further validating the importance of the internal motif (Fig. 4B). We next performed an alanine scan for the internal motif. Interestingly, exchange of D777 to alanine abolishes the interaction within the context of $[768–782]_{aa}$, revealing that the internal motif is crucial for interaction (Fig. 4C). Moreover, the loss of interaction shows that the C-terminal motif alone is not sufficient to promote binding and thus appears to enhance the interaction mediated by the internal motif. In the context of the $[768–777]_{aa}$ peptide, alanine mutation of G773, D774, L776, and D777 reduces or abolishes binding to nNOS (Fig. 4D). Similar results were obtained for alanine mutations in the context of $[768–782]_{aa}$ harbouring a triple alanine mutation of residues 780–782 (data not shown). Together, these data reveal the internal sequence G-D-H-L-D in Vac14 as a binding motif for the nNOS PDZ domain. Due to the similarity of this motif to the C-terminal consensus motif -G-[D/E]-x-V*, we propose -G-[D/E]-x-Φ-[D/E/*] as a general recognition motif for the nNOS PDZ domain.

In order to test whether this novel motif could function in a context other than Vac14, we performed a database search of the vertebrate proteome with the consensus motif -G-[D/E]-x-Φ-[D/E]-. Of the multiple entries found, the 5-HT_{2B} receptor was of particular interest since it was already described to bind the nNOS PDZ domain. Indeed, the 5-HT_{2B} receptor binds the nNOS PDZ domain through a Class I recognition motif located at the C-terminus of the protein [30]. Supplementary Fig. 1A illustrates an alignment of 5-HT_{2B} receptor sequences from various species and reveals that the potential internal motif is found just N-terminal of the C-terminal motif, similar to the arrangement in Vac14. To determine if the internal motif is functional in nNOS interaction, we generated wild-type and mutant constructs in the context of the 5-HT_{2B} receptor

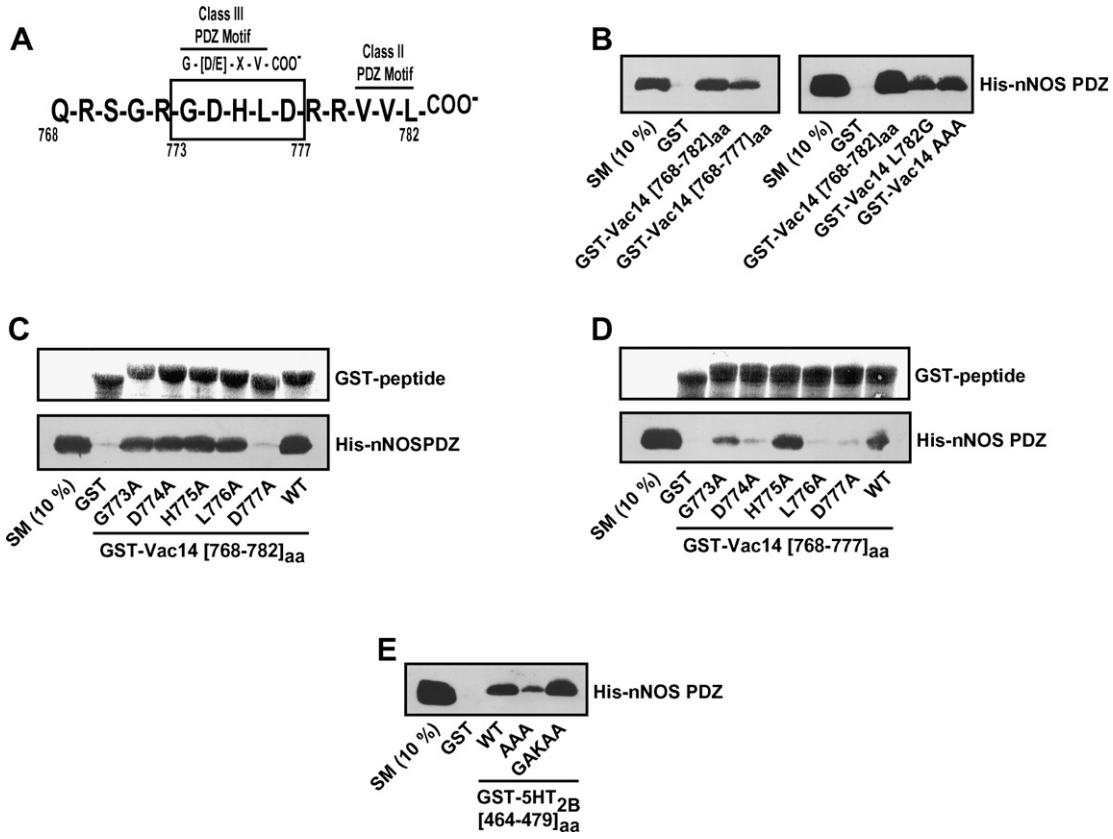

Fig. 4. Characterization of the internal motif responsible for PDZ interactions. (A) Amino acids 768–782 of human Vac14 with consensus motifs for PDZ domain interactions indicated. (B–D) Purified His-nNOS PDZ domain was incubated with GST alone or GST-Vac14 fusion proteins coupled to glutathione-Sepharose. Proteins specifically bound to the beads were blotted with antibody against the His tag. An aliquot of the His-nNOS PDZ domain (10%) was loaded as starting material (SM). For B, GST-Vac14 L782G and GST-Vac14 AAA represent GST-Vac14 [768–782] with L782 mutated to glycine or residues 780–782 mutated to alanine, respectively. For C and D, point mutations introduced into the fusion proteins are indicated and Ponceau S stained transfers indicating the levels of GST/GST-peptide are included. (E) Purified His-nNOS PDZ domain was incubated with GST alone or GST-5HT$_{2B}$ receptor [464–479]$_{aa}$ fusion proteins coupled to glutathione-Sepharose. Proteins specifically bound to the beads were blotted with anti-His antibody. An aliquot of the His-nNOS PDZ domain (10%) was loaded as starting material (SM). WT, wild-type; AAA, mutation of residues 477–479 to alanine; GAKAA, mutation of residues 470, 472 and 473 to alanine, all prepared in the context of 5-HT$_{2B}$ receptor [464–479]$_{aa}$.

[464–479]$_{aa}$ rat sequence, which contains both motifs. In agreement with a previous study [30], the wild-type peptide (GST-5HT$_{2B}$ WT) binds the nNOS PDZ domain (Fig. 4E). Binding is reduced following mutation of the last three residues (GST-5HT$_{2B}$ AAA) but is maintained with mutation of the internal motif (GST-5HT$_{2B}$ GAKAA), confirming the involvement of the canonical C-terminal motif (Fig. 4E). Interestingly, the triple mutation of the C-terminal motif does not fully disrupt binding, indicating an involvement of the internal motif of the 5-HT$_{2B}$ receptor in its association with the PDZ domain of nNOS (Fig. 4E). These data further support the involvement of the internal motif in nNOS PDZ domain interactions.

4. Discussion

We have identified a sequence in Vac14, -G-D-H-L-D- that mediates interactions with the PDZ domain of nNOS and which is similar to a motif, -G-[D/E]-x-V* (where * equals the free carboxyl group) identified from peptide library screens to mediate nNOS PDZ domain interactions [4]. Interestingly, the motif in Vac14 is unique in that it is internal, unlike canonical PDZ domain-binding motifs, which are located at the C-terminus. In the later case, the free carboxyl group is essential for interactions with PDZ domains [7]. For the internal motif in Vac14, the necessity of the second D (D777) for nNOS PDZ domain binding suggests that this residue mimics the free carboxyl group of C-terminal recognition motifs. The ability of acidic residues C-terminal of a protein interaction motif to mimic contributions provided by a C-terminal carboxyl group has been previously described. In particular, we recently identified a novel motif, W-X-X-F-acidic that mediates interactions of endocytic accessory proteins with the α-ear domain of the α-adaptin subunit of the clathrin accessory protein AP-2 [29]. In the NECAP proteins, the motif is found at the C-terminus and the free carboxyl group is critical for α-ear binding [25]. However, in other endocytic proteins, the motif is found internally and is always followed by one or more acidic residues. Mutation of these residues disrupts α-ear interactions [25]. Thus, based on this, the mutational analysis and the similarity of the internal motif to the C-terminal consensus motif -G-[D/E]-x-V*, we propose -G-[D/E]-x-Φ-[D/E/*] as a consensus motif for nNOS PDZ domain interactions.

The internal nNOS binding sequence -G-D-H-L-D- identified in this study is conserved in mammalian orthologs of

Vac14 but is not found in Vac14 from other vertebrates including chicken and bony fish (Supplementary Fig. 1B). Even the less stringent consensus motif -G-[D/E]-x-Φ-[D/E/*] is, with exception of *Xenopus laevis*, only found in mammalian Vac14. It is interesting to note that *X. laevis* has lost the C-terminal motif that enhances Vac14/nNOS interaction. These observations suggest a specialized role for Vac14 in nNOS regulation in mammals.

A search of the vertebrate proteome with the consensus motif -G-[D/E]-x-Φ-[D/E]- revealed a large number of entries. Although the biological significance of these sequences is to date unknown, we have determined that the internal motif found in the 5-HT_{2B} receptor contributes to nNOS PDZ domain interactions. Although the internal motif matches the consensus sequence only in rat, most other mammals possess a sequence similar to this consensus (Supplementary Fig. 1A). Interestingly, the internal consensus motif found in the 5-HT_{2B} receptor is positioned with a similar spacing to the C-terminus as seen in Vac14 (Supplementary Fig. 1). Both Vac14 and the 5-HT_{2B} receptor display nNOS PDZ domain interactions that utilize both the internal and C-terminal motifs. Whereas Vac14/nNOS interaction is primarily mediated through an internal motif, the 5-HT_{2B} receptor/nNOS interaction appears to primarily use the C-terminal motif. It is interesting to note that in the case of Vac14, the Class III internal motif prevails over the Class II C-terminal motif for the critical binding to the nNOS Class III PDZ domain. Further characterisation of this new type of PDZ interaction involving dual motifs is needed to better understand the ability of PDZ domains to contribute to the formation of protein scaffolding complexes.

Thus, nNOS could be regulated by at least two proteins, Vac14 and the 5-HT_{2B} receptor, which use the newly defined consensus motif for interaction with the nNOS PDZ domain in concert with a canonical C-terminal motif.

Acknowledgements: We thank Dr. Phil Marsden (University of Toronto) for his gift of nNOS cDNA. We also thank Elaine de Heuvel, Jacynthe Philie and Lyne Bourbonnière for technical assistance and Drs. Valérie Legendre-Guillemin, Viviane Poupon, Sébastien Thomas and other members of the McPherson laboratory for discussion. A special thanks to Dr. Brigitte Ritter for her significant help in the preparation of this manuscript. This work was supported by Grant MOP62684 from the Canadian Institutes of Health Research to PSM. PSM is a *Fonds de la recherche en santé du Québec* Senior Scholar, a Killam Scholar of the Montreal Neurological Institute and a McGill University William Dawson Scholar.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.11.061.

References

[1] Jelen, F., Oleksy, A., Smietana, K. and Otlewski, J. (2003) PDZ domains – common players in the cell signaling. Acta Biochim. Pol. 50, 985–1017.
[2] Utepbergenov, D.I., Fanning, A.S. and Anderson, J.M. (2006) Dimerization of the scaffolding protein ZO-1 through the second PDZ domain. J. Biol. Chem. 281, 24671–24677.
[3] Im, Y.J., Lee, J.H., Park, S.H., Park, S.J., Rho, S.H., Kang, G.B., Kim, E. and Eom, S.H. (2003) Crystal structure of the Shank PDZ-ligand complex reveals a class I PDZ interaction and a novel PDZ–PDZ dimerization. J. Biol. Chem. 278, 48099–48104.
[4] Schepens, J., Cuppen, E., Wieringa, B. and Hendriks, W. (1997) The neuronal nitric oxide synthase PDZ motif binds to -G(D,E)XV* carboxyterminal sequences. FEBS Lett. 409, 53–56.
[5] Tochio, H., Zhang, Q., Mandal, P., Li, M. and Zhang, M. (1999) Solution structure of the extended neuronal nitric oxide synthase PDZ domain complexed with an associated peptide. Nat. Struct. Biol. 6, 417–421.
[6] Stricker, N.L. et al. (1997) PDZ domain of neuronal nitric oxide synthase recognizes novel C-terminal peptide sequences. Nat. Biotechnol. 15, 336–342.
[7] Hillier, B.J., Christopherson, K.S., Prehoda, K.E., Bredt, D.S. and Lim, W.A. (1999) Unexpected modes of PDZ domain scaffolding revealed by structure of nNOS-syntrophin complex. Science 284, 812–815.
[8] Tochio, H., Mok, Y.K., Zhang, Q., Kan, H.M., Bredt, D.S. and Zhang, M. (2000) Formation of nNOS/PSD-95 PDZ dimer requires a preformed beta-finger structure from the nNOS PDZ domain. J. Mol. Biol. 303, 359–370.
[9] Brenman, J.E., Christopherson, K.S., Craven, S.E., McGee, A.W. and Bredt, D.S. (1996) Cloning and characterization of postsynaptic density 93, a nitric oxide synthase interacting protein. J. Neurosci. 16, 7407–7415.
[10] Wong, H.C. et al. (2003) Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. Mol. Cell 12, 1251–1260.
[11] London, T.B., Lee, H.J., Shao, Y. and Zheng, J. (2004) Interaction between the internal motif KTXXXI of Idax and mDvl PDZ domain. Biochem. Biophys. Res. Commun. 322, 326–332.
[12] Penkert, R.R., DiVittorio, H.M. and Prehoda, K.E. (2004) Internal recognition through PDZ domain plasticity in the Par-6-Pals1 complex. Nat. Struct. Mol. Biol. 11, 1122–1127.
[13] Shisheva, A. (2001) PIKfyve: the road to PtdIns 5-P and PtdIns 3,5-P(2). Cell Biol. Int. 25, 1201–1206.
[14] Bonangelino, C.J., Catlett, N.L. and Weisman, L.S. (1997) Vac7p, a novel vacuolar protein, is required for normal vacuole inheritance and morphology. Mol. Cell. Biol. 17, 6847–6858.
[15] Sbrissa, D., Ikonomov, O.C., Strakova, J., Dondapati, R., Mlak, K., Deeb, R., Silver, R. and Shisheva, A. (2004) A mammalian ortholog of Saccharomyces cerevisiae Vac14 that associates with and up-regulates PIKfyve phosphoinositide 5-kinase activity. Mol. Cell. Biol. 24, 10437–10447.
[16] Bonangelino, C.J., Nau, J.J., Duex, J.E., Brinkman, M., Wurmser, A.E., Gary, J.D., Emr, S.D. and Weisman, L.S. (2002) Osmotic stress-induced increase of phosphatidylinositol 3,5-bisphosphate requires Vac14p, an activator of the lipid kinase Fab1p. J. Cell Biol. 156, 1015–1028.
[17] Sbrissa, D. and Shisheva, A. (2005) Acquisition of unprecedented phosphatidylinositol 3,5-bisphosphate rise in hyperosmotically stressed 3T3-L1 adipocytes, mediated by ArPIKfyve-PIKfyve pathway. J. Biol. Chem. 280, 7883–7889.
[18] Shaw, J.D., Hama, H., Sohrabi, F., DeWald, D.B. and Wendland, B. (2003) PtdIns(3,5)P2 is required for delivery of endocytic cargo into the multivesicular body. Traffic 4, 479–490.
[19] Odorizzi, G., Babst, M. and Emr, S.D. (1998) Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. Cell 95, 847–858.
[20] Girard, M., Allaire, P.D., McPherson, P.S. and Blondeau, F. (2005) Non-stoichiometric relationship between clathrin heavy and light chains revealed by quantitative comparative proteomics of clathrin-coated vesicles from brain and liver. Mol. Cell Proteomics 4, 1145–1154.
[21] Sbrissa, D., Ikonomov, O.C., Strakova, J. and Shisheva, A. (2004) Role for a novel signaling intermediate, phosphatidylinositol 5-phosphate, in insulin-regulated F-actin stress fiber breakdown and GLUT4 translocation. Endocrinology 145, 4853–4865.
[22] McEwen, R.K. et al. (1999) Complementation analysis in PtdInsP kinase-deficient yeast mutants demonstrates that Schizosaccharomyces pombe and murine Fab1p homologues are phosphatidylinositol 3-phosphate 5-kinases. J. Biol. Chem. 274, 33905–33912.
[23] Burman, J.L., Wasiak, S., Ritter, B., de Heuvel, E. and McPherson, P.S. (2005) Aftiphilin is a component of the clathrin machinery in neurons. FEBS Lett. 579, 2177–2184.

[24] McPherson, P.S., Czernik, A.J., Chilcote, T.J., Onofri, F., Benfenati, F., Greengard, P., Schlessinger, J. and De Camilli, P. (1994) Interaction of Grb2 via its Src homology 3 domains with synaptic proteins including synapsin I. Proc. Natl. Acad. Sci. USA 91, 6486–6490.
[25] Ritter, B., Denisov, A.Y., Philie, J., Deprez, C., Tung, E.C., Gehring, K. and McPherson, P.S. (2004) Two WXXF-based motifs in NECAPs define the specificity of accessory protein binding to AP-1 and AP-2. EMBO J. 23, 3701–3710.
[26] de Heuvel, E., Bell, A.W., Ramjaun, A.R., Wong, K., Sossin, W.S. and McPherson, P.S. (1997) Identification of the major synaptojanin-binding proteins in brain. J. Biol. Chem. 272, 8710–8716.
[27] Wasiak, S. et al. (2002) Enthoprotin: a novel clathrin-associated protein identified through subcellular proteomics. J. Cell Biol. 158, 855–862.
[28] Blondeau, F. et al. (2004) Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling. Proc. Natl. Acad. Sci. USA 101, 3833–3838.
[29] Ritter, B., Philie, J., Girard, M., Tung, E.C., Blondeau, F. and McPherson, P.S. (2003) Identification of a family of endocytic proteins that define a new alpha-adaptin ear-binding motif. EMBO Rep. 4, 1089–1095.
[30] Manivet, P., Mouillet-Richard, S., Callebert, J., Nebigil, C.G., Maroteaux, L., Hosoda, S., Kellermann, O. and Launay, J.M. (2000) PDZ-dependent activation of nitric-oxide synthases by the serotonin 2B receptor. J. Biol. Chem. 275, 9324–9331.