NOTE TO USERS

Binding of Vac14 to Neuronal Nitric Oxide Synthase: Characterisation of a New Internal PDZ-Recognition Motif

Jean-François Lemaire

Department of Neurology & Neurosurgery

McGill University, Montreal

November 2006

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Master of Science in Neurological Science.

Your file Votre référence
ISBN: 978-0-494-32838-5
Our file Notre référence
ISBN: 978-0-494-32838-5

Table of Contents

Table of Contents 2

Abstract 4

Résumé 5

Acknowledgements 6

CHAPTER 1 7

Introduction 8

Literature Review 11

The PDZ Domain: 11

The Nitric Oxide Synthase Family: 12

nNOS: 13

Vac14 and Fab1p/PIKfyve: 15

AMPK and Contraction-Induced GLUT-4 Translocation: 17

The Nitric Oxide Pathway and GLUT-4 Translocation: 18

Insulin-Induced GLUT-4 Translocation: 19

PIKfyve, Phosphoinositides and GLUT-4 Translocation: 20

Figure 1-1. Contraction-Induced GLUT-4 Translocation and nNOS 22

Figure 1-2. Insulin-Induced GLUT-4 Translocation and PIKfyve 23

CHAPTER 2 24

Contribution of Authors 25

Binding of Vac14 to Neuronal Nitric Oxide Synthase: 26

Abstract: 27

Introduction: 28

Material and Methods: 31

Results: 34

Discussion: 39

Acknowledgements: 42

Figure 2-1: Expression pattern of Vac14. 43

Figure 2-2: Vac14 interacts with nNOS. 44

Figure 2-3: Vac14 interacts with the nNOS PDZ domain through an internal motif. 45

Figure 2-4: Characterization of the internal motif responsible for PDZ interactions. 46

Table 2-1: List of sense and anti-sense primers and oligonucleotides used for cloning 47

Figure 2-5: BLAST alignments of the C-terminal regions of vertebrate orthologs of 5-HT_{2B} receptor and Vac14. 48

CHAPTER 3 ..... **49**

Summary & Conclusion 50

Figure 3-1: BLAST alignments on the vertebrate orthologs of Vac14 and AMPK γ3 subunit, harboring the proposed PDZ binding consensus motif. 53

Figure 3-2. Regulation of GLUT-4 Trafficking by Insulin and Contraction 54

Abbreviations **55**

Bibliography **57**

Appendix **64**

Research Compliance Certificates: 64

Abstract

Protein interactions mediated by PDZ domains involve specific modes of recognition. The most common type depends on the recognition of a classical peptide motif found at the very carboxyl end of the PDZ domain binding protein. Although less common, other modes of recognition have been described involving internal sequences. The best characterized involves an internal β-finger structure that mimics the carboxyl terminus of a protein. This work describes a new PDZ domain recognition sequence, that is found internally but which does not adopt a β-finger conformation. The sequence mediates the previously uncharacterized interaction between the neuronal nitric oxide synthase (nNOS) and the PtdIns(3)*P* 5-kinase PIKfyve-activator Vac14. Mutational analyses reveal essential residues within the motif allowing for the description of a new type of PDZ domain interaction.

Résumé

Les intéractions protéiques impliquant les domaines PDZ sont régies par des modes de reconnaissances de domaines PDZ très spécifiques. Le mode le plus courant nécessite la présence d'un groupement carboxyle libre, et par conséquent dépend d'un motif de reconnaissance situé à l'extrémité carboxyle de la protéine liant le domaine PDZ. D'autres modes de reconnaissances moins courants, ceux-ci impliquant des séquences de reconnaissance internes, ont également été décrits. Un des modes les mieux caractérisés nécessite la présence d'une structure interne adoptant la conformation dite "β-finger" et ainsi simulant une extrémité carboxyle. La présente étude décrit une nouvelle séquence de reconnaissance de domaines PDZ, ne se situant pas à l'extrémité C-terminale et n'adoptant pas de conformation "β-finger", responsable de l'interaction jusqu'à présent inconnue entre l'oxyde nitrique synthase de type neuronal (nNOS) et Vac14, protéine connue comme activatrice de la PtdIns(3)*P* 5-kinase PIKfyve. Des analyses mutationnelles révélant les acides aminés composant le motif et trouvés essentiaux à l'interaction, ont permis la description d'un nouveau type d'interactions protéiques impliquant les domaines PDZ.

Acknowledgements

First, I would like to thank my supervisor, Dr. Peter McPherson for his advice, his goal-focussed guidance and for his financial support. I would also like to thank my committee, Drs. Martin Latterich and Jean-François Cloutier for their helpful comments and recommendations.

Special thanks to Dr. Brigitte Ritter for her valuable comments in the preparation of this document and for technical recommendations, as well as to Drs. Valérie Legendre-Guillemin, Viviane Poupon and Sébastien Thomas for their kind support, encouragement and technical recommendations. Thanks to the past and present members of the McPherson and Barker laboratories for beneficial discussions.

To my parents, family and friends, especially Joannie Roy, Alain Long, and Jonathan Grégoire, thank you all for your priceless encouragement and moral support, which have guided me during the rougher times.

CHAPTER 1

Introduction & Literature Review

Introduction

Clathrin-mediated membrane budding occurs at both the plasma membrane and the trans-Golgi network (TGN) [1]. This cellular process is responsible for the internalization of receptors, nutrients and others proteins, as well as for trafficking cargo molecules from the TGN to the endosomal/lysosomal system [1; 2; 3; 4]. A large number of proteins are involved in the machinery underlying the formation of clathrin-coated pits (CCPs) and vesicles (CCVs). Our laboratory mainly focuses on understanding the functional implications of these proteins as well as characterizing novel components of the machinery and studying their interactions with other members of the system. A better knowledge of the clathrin-mediated membrane budding machinery leads directly to a higher understanding of the regulation of cellular trafficking events.

A proteomic analysis of liver CCV preparations conducted in our laboratory led to the identification of several novel proteins presumably involved in the clathrin-mediated membrane budding machinery [5]. One of them was the mammalian homolog of the yeast Vac14 protein (Vac14p), known to be involved in the hyperosmotic stress response and to control phosphatidylinositol 3,5-biphosphate (PtdIns(3,5)P_2) synthesis by interacting with and activating the PtdIns3P 5-kinase Fab1p [6; 7]. Shortly after our laboratory identification of Vac14 [5], a study describing the initial characterisation of mammalian Vac14 appeared, which demonstrated that the mammalian protein had similar cellular properties as the yeast protein [8]. Indeed, Vac14 binds to and activates PIKfyve, the mammalian ortholog of Fab1p, also leading to PtdIns(3,5)P_2 production [8].

We sought to test for a potential role for Vac14 in clathrin trafficking. We developed a Vac14 antibody, which revealed that the protein is present but not enriched on CCVs. In an effort to identify potential clathrin related binding partners for Vac14, we performed mass spectrometric analysis of proteins co-immunoprecipitated with Vac14. Interestingly, a binding partner identified in this screen was the neuronal nitric oxide synthase (nNOS). The work presented herein examines this novel association between Vac14 and nNOS. nNOS is a Class III PDZ domain-containing protein that has been exhaustively studied over the past years. PDZ domains are protein-protein interaction modules that mediate the formation of protein complexes in a multitude of cellular processes. Interestingly, the direct interaction between Vac14 and nNOS is mediated through the PDZ domain of nNOS.

The most common modes of PDZ recognition are mediated through four to five residue long peptide motifs at the C-terminus of interacting partners or through PDZ/PDZ dimerization. Internal modes of PDZ binding have also been described, which mainly involve β-finger structures. Several classical C-terminal motifs have been described to bind nNOS PDZ domain, but no internal peptide motifs have been identified to date. Significantly, this study demonstrates two regions within Vac14 involved in the association with the PDZ domain of nNOS: an internal motif and a C-terminal motif. Interestingly these two regions have distinct roles in promoting the binding, the internal being crucial for binding, whereas the C-terminal region corresponds to a canonical Class II motif which is neither crucial nor sufficient, but enhances the interaction.

The work presented in this thesis describes 1) the novel Vac14/nNOS direct association, 2) the involvement of dual motifs in PDZ interactions, as well as 3) the characterisation of an internal mode of recognition targeting the nNOS PDZ domain which is composed of 4) a novel internal motif; and suggests potential implications of this new interaction in a biological context such as the translocation of the glucose transporter GLUT-4. Finally, this study proposes a new consensus recognition motif targeting the PDZ domain of nNOS.

Literature Review

The PDZ Domain:

A large variety of proteins contain protein interaction modules. PDZ domains are one of the most common modules mediating protein-protein interactions [9], with over 343 PDZ-containing proteins found in human [10]. PDZ-bearing proteins are involved in the formation of protein scaffolds and signalling networks. Good examples are the post-synaptic density protein PSD-95, the septate junction protein *Drosophila* Discs-large Dlg and the epithelial tight junction protein ZO-1, from which the acronym PDZ originates [9]. Canonical PDZ domains contain ~90 residues [11], which fold into a structure in which the peptide ligand binds in a hydrophobic cleft [12]. Being present in one or manifold copies, PDZ domains also occur in combination with other signaling domains (like SH3, PTB, WW) [9]. PDZ domains also occur commonly in proteins involved in apical and basolateral protein segregation, observed in polarized epithelial and neuronal cells [13; 14; 15]. In addition, the PDZ network acts to efficiently retain membrane proteins at the cell surface [13].

Although PDZ-PDZ dimerizations can occur [16; 17], PDZ domains classically mediate their protein interactions through specific recognition motifs composed of the last four to five residues at the C-terminal end of the interacting protein [18]. Exhaustive studies on the binding specificity of PDZ domains have led to a classification system for PDZ domains [9; 12; 19; 20; 21; 22]. Based on their preferred C-terminal peptide binding motifs, PDZ domains are sorted into four classes: 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]* [9], where * indicates the free carboxyl group at the C-terminus, Φ represents hydrophobic residues, Ψ represents aromatic residues and x is any amino acid.

PDZ domains can also be recognized by internal sequences; however this mode of PDZ recognition is by far less common. The best described example involves an internal β-hairpin "finger-like" structure in which only one strand of the hairpin mediates the interaction with the PDZ domain by mimicking a canonical C-terminus motif [23]. More details about this type of interaction are provided in the section below.

Besides β-fingers, there are 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 [24] and Idax [25] and the internal sequence -H-R-E-M-A-V- that mediates the Pals1–Par-6 interaction [26]. Nevertheless, the β-finger style of PDZ interaction remains a standard for internal modes of recognition mediating various protein associations involving nNOS [23; 27; 28] and the ET_A endothelin receptor [29].

The Nitric Oxide Synthase Family:

Nitric oxide synthase (NOS) proteins are the enzymes responsible for the oxidation of L-arginine into L-citrulline and nitric oxide (NO) and simultaneously require five bound cofactors: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), haem, tetrahydrobiopterin (BH_4) and Ca^{2+}-calmodulin (CaM) [30]. Major efforts have been put together to map the functional repercussions of the resulting NO production. It is now known that the NO is a free radical involved in numerous physiological processes

including vasodilatation, neurotransmission, leukocyte adhesion, formation of memory, pro- and anti-apoptotic signalling capabilities and GLUT-4 translocation [31; 32; 33] There are three distinct genes encoding for NOS isoenzymes in mammals: neuronal NOS (nNOS or NOS-1), endothelial NOS (eNOS or NOS-2) and inducible NOS (iNOS or NOS-3) [34]. Although each isoform is conserved throughout species, the homology is only 51-57% between human isoforms [30]. Originally distinguished by their expression pattern, in which nNOS and eNOS were considered constitutively expressed, and iNOS expressed upon an induction signal, it is now clear that all three can be induced, although by different stimuli, and all can be constitutively expressed in some cells or tissues [30]. However, a major characteristic that distinguishes the neuronal isoform is the presence of an additional N-terminal 230-residues section containing a Class III PDZ domain [12].

nNOS:

The nNOS gene is post-transcriptionally regulated, mainly through alternative mRNA splicing, leading to the generation of 5 splice variants: the full length protein (nNOSα), the shorter variants nNOSβ, nNOSγ, and nNOS-2 and the elongated variant nNOSμ [30; 35]. Whereas nNOSβ and nNOSγ lack the PDZ domain, nNOS-2 has an internal deletion but still harbours the PDZ domain. The nNOSμ splice variant is the predominant isoform in skeletal muscles and is identical to the full length sequence with the exception of a 34-residues insert in the FMN-binding domain [30]. Although the two splice variants harbouring the PDZ domain can anchor to the sarcoplasmic reticulum, whereas the two others cannot, the biological significance of the nNOS splice variants remains poorly understood [30; 35].

Protein interactions mediated through the nNOS PDZ domain have been exhaustively studied over the past years, including binding to syntrophin [23], PSD-95/93 [27; 28; 36], and CAPON [37]. Whereas CAPON illustrates the canonical carboxyl-terminal peptide binding mode to Class III PDZ domains, the PDZ-containing proteins syntrophin and PSD-95/93 display interactions with nNOS that are now defined as classical examples of the internal PDZ recognition mode using β-finger structures [23; 27; 28]. In fact, nNOS harbours an internal β-finger structure, C-terminal of its PDZ domain, which allows nNOS to associate with the other PDZ-containing proteins and brings the PDZ domains of each protein in close proximity [23; 27; 28]. The PDZ-PDZ interactions are therefore mediated through the internal nNOS β-finger structure, which mimics a typical C-terminal PDZ recognition motif and brings the PDZ domains in a head-to-tail fashion [23; 27; 28]. This gives nNOS the potential for coordinated C-terminal canonical interactions with other partners through its PDZ domain [23].

Given the physiological importance of nNOS in numerous cellular processes such as neurotransmitter release, stress response, cell survival, muscle contraction and GLUT4 translocation, major efforts have been made to identify novel nNOS PDZ domain-binding partners and to define the peptide motifs mediating the interactions. Indeed, studies including screens for billions of C-terminal peptides using peptide display [21] or two-hybrid technologies [18] were performed. In all cases, peptide library screens revealed preferences of the nNOS PDZ domain for peptides ending with -D-x-V* and more specifically -G-[D/E]-x-V* [18].

Vac14 and Fab1p/PIKfyve:

vac14 is an evolutionarily conserved gene present as single-copy in all sequenced eukaryotic genomes [38]. First identified based on its gene mutation causing defects in vacuole inheritance, acidification, and membrane morphology [39], the gene product Vac14p was then shown to control phosphatidylinositol 3,5-biphosphate (PtdIns(3,5)P_2) synthesis by activating the PtdIns3P 5-kinase Fab1p [6; 7]. This lipid kinase transforms PtdIns3P into PtdIns(3,5)P_2, which is a non-abundant phospholipid whose levels increase in response to osmotic stress [6; 40]. PtdIns(3,5)P_2 is known to be essential for retrograde trafficking between the vacuole/lysosome and the late endosomes as well as for the delivery of cargo into the vacuole via multivesicular bodies (MVB) [41; 42]. A recent study describes Fab1p and the clathrin adaptor AP-1 as required, linked players in trafficking of cargo to the vacuole lumen [43]. Vac14p activity is also required, through PtdIns(3,5)P_2 synthesis, for the polyphosphatase Phm5p and the carboxypeptidase S protein transport into MVB [7]. Localization and activation of the specific PtdIns(3,5)P_2 5-phosphatase Fig4p, which functions both in PtdIns(3,5)P_2 synthesis (activates Fab1p) and turnover (phosphatase activity) [44], depends on its physical association with Vac14p [45]. Indeed, in the absence of Vac14p, Fig4p no longer localizes to the limiting membrane of the vacuole [45] and no longer mediates PtdIns(3,5)P_2 turnover after an hyperosmotic shock [44]. Thus, Vac14p controls PtdIns(3,5) P_2 levels in two different ways, synthesis and turnover. PIKfyve, the mammalian ortholog of Fab1p, when active, is known to associate with and to promote the membrane attachment of the Rab9 effector p40, involved in the transport from late endosomes to TGN [46].

In mammalian cells, PIKfyve, the mammalian ortholog of Fab1p, is also positively regulated by the mammalian ortholog of Vac14p (Vac14) [8]. In addition, PIKfyve can rescue the vacuolar defects in the *Δfab1* yeast strain [47]. Indeed, the Vac14/PIKfyve pathway in mammalian cells shares many similarities other than structural homology, with the yeast Vac14p/Fab1p pathway. They both control MVB (mammals) or vacuole (yeast) size and morphology and in both cases, gene inactivation or protein knockdown (either Fab1p, Vac14p, Vac14 or PIKfyve) lead to similar phenotypes: dilated MVBs or swollen vacuoles [6; 7; 8]. Moreover, both pathways regulate sorting and trafficking of specific proteins to lysosomes [6; 7; 8]. Moreover, PIKfyve was recently described to regulate endosome-to-TGN retrograde transport [48]. With the exception of 3T3-L1 adipocytes [49], the mammalian pathway is otherwise not sensitive to osmoregulation, whereas an 18- to 28-fold increased of PtdIns(3,5)P_2 levels is observed under osmotic stress in yeast [6; 8]. In addition to the production of PtdIns(3,5)P_2, PIKfyve but not Fab1p is also capable of PtdIns(5)*P* production in mammalian cells upon Vac14 activation [8; 47]. This phosphoinositide can also be produced by myotubularin family members of PtdIns 3-phosphatases where PtdIns(5)*P* acts in a positive feedback loop in activating myotubularins [50]. Insulin causes PtdIns(5)*P* increased production which is involved in actin remodelling and GLUT-4 dynamics [8; 51].

The yeast Vac14p proteins harbour at least one HEAT-like repeat and six dileucine motifs, both involved in membrane trafficking events, and a VHS domain that serves to bind acidic-cluster dileucine motifs [7]. Although HEAT-like repeats and dileucine motifs could be recognized by search programs within the human ortholog, no conserved domains are detected including the VHS domain.

AMPK and Contraction-Induced GLUT-4 Translocation:

The 5'AMP-activated protein kinase (AMPK) is a ubiquitous metabolic sensor composed of one catalytic α subunit and two regulatory β and γ subunits [52; 53]. The α and β subunits are encoded by 2 genes (α1 and α2 or β1 and β2), while the γ subunit is encoded by 3 genes (γ1, γ2 and γ3) [54]. All three subunits are necessary to achieve full enzymatic activity of this heterotrimer protein [55]. AMPK is activated by increases in adenosine monophosphate (AMP)/adenosine triphosphate (ATP) cellular ratio. Numerous metabolic stresses are also responsible for AMPK activation including hyperosmotic and oxidative stresses, hypoxia and ischemia [54]. Exercise and glucose deprivation are also strong AMPK activators [55]. More specifically, AMPK is a key player in contraction-induced translocation of the glucose transporter 4 (GLUT-4) from intracellular compartments to the plasma membrane (Fig. 1-1). Indeed, AMPK pharmacological activation by the cell-permeable adenosine analog 5-aminoimidazole-4-carboxamide riboside (AICAR) increases GLUT-4 translocation without affecting total cellular content of GLUT-4 [33].

Muscle contraction leads to a decrease in creatine phosphate and increases AMP levels, which leads to the allosteric activation of both AMPK and its kinase AMPKK (Fig. 1-1) [56]. Moreover, the activated AMPKK leads to the phosphorylation and activation of AMPK through covalent modification of α subunits [57]. Whereas ATP allows the dephosphorylation and therefore the inactivation of AMPK by protein phosphatase, the latter action on AMPK is inhibited in AMP-bound AMPK [54]. The pharmacologic activation of AMPK by AICAR leads to increases in phosphorylation of the Rab GTPase-

activating protein (GAP) AS160 [54]. This Akt/protein kinase B (PKB) substrate was already described to be involved in the insulin-induced Akt/PKB-dependent GLUT-4 translocation (Fig. 1-2) [58]. In fact, phosphorylation by Akt/PKB, which inhibits AS160 GAP activity, leads to the elevation of the GTP-bound form of a yet unidentified Rab protein [58; 59; 60]. The AMPK-mediated phosphorylation of AS160 is dependent on AMPK α2 catalytic and γ3 regulatory subunits [61]. However, it is interesting to note that in response to muscle contraction, the α2, but not the γ3 subunit-containing heterotrimers are essential for AS160 phosphorylation [54].

AMPK also directly phosphorylates and activates NOS (eNOS Ser^{1177} and nNOSμ Ser^{1451}) leading to NO production [62]. This coupled phosphorylation is thought to protect the muscle from metabolic stress, like ischemia given that NO is a strong inhibitor of lipid oxidation [62; 63]. It is notable that the nNOSμ phosphorylation site is not located in the isoform-specific insert and is therefore also present in the full length nNOS isoform (nNOSα). Moreover, both eNOS and nNOSμ were detected in rat skeletal muscle, whereas only the nNOSμ isoform was detected in the human tissue [62]. Interestingly, eNOS but not nNOSμ is also phosphorylated by Akt/PKB and the protein kinase A (PKA) [62; 64; 65].

The Nitric Oxide Pathway and GLUT-4 Translocation:

One of the major downstream effecters of NO production is the activation of soluble guanylate cyclase leading to the formation of cyclic guanosine monophosphate (cGMP). cGMP is a second messenger functioning in numerous signaling cascades involving cGMP-dependent protein-kinases. The exogenous addition of the cell-permeable cGMP

analog bromo-cGMP on heart muscle increases glucose uptake [33]. Furthermore, the NO pathway was also shown to be involved in stimulated GLUT-4 translocation where AICAR-stimulated muscle glucose transport and uptake is partly inhibited by the N-nitro-L-arginine methyl ester (L-NAME) inhibition of NOS enzymatic activity [66; 67]. These observations propose NOS as part of the downstream effectors of AMPK-induced GLUT-4 translocation (Fig. 1-1) [33].

Insulin-Induced GLUT-4 Translocation:

GLUT-4 translocation from intracellular compartments to the plasma membrane can also be induced by circulating insulin molecules binding to the transmembrane insulin receptor (IR) (Fig. 1-2). Although insulin-dependent glucose uptake is mainly observed in muscle and adipose cells, insulin can cause GLUT-4 translocation in other cellular types such as neurons [68]. Upon insulin binding to its extracellular subunits, the IR undergoes autophosphorylation of the transmembrane subunits and activation of their intrinsic kinase activity towards the insulin receptor substrates 1 and 2 (IRS1/2) (Fig. 1-2) [56]. Once phosphorylated, the IRS1 or IRS2 recruits and activates the phosphatidylinositol 3-kinase (PI 3-kinase) for the production of PtdIns(3,4,5)P_3 from the plasma membrane localized phosphoinositide PtdIns(4,5)P_2 [56]. The PtdIns(3,4,5)P_3 then activates the 3'-phosphoinositide-dependent kinases (PDK1/2) which in turns phosphorylate and activate Akt/PKB and atypical protein kinase C-zeta/lambda (PKC-ζ/λ) (Fig. 1-2) [56]. Both proteins were proposed to be downstream mediators of the insulin-induced GLUT-4 translocation [69; 70; 71]. However, the precise position of these kinases in the GLUT-4 trafficking pathway is still unknown but must transduce

through downstream phosphorylation of a key substrate localized close to the GLUT-4-containing vesicles [72].

PIKfyve, Phosphoinositides and GLUT-4 Translocation:

Several studies have suggested that PIKfyve and AS160 are Akt/PKB substrates involved in insulin-induced GLUT-4 translocation [72; 73]. Indeed, PIKfyve co-immunoprecipitates both p85 and p110 subunits of Akt/PKB and vice versa [74] and was shown to be phosphorylated in response to insulin and in a PI 3-kinase-dependent manner [75]. Interestingly, PIKfyve is present on a highly motile subpopulation of GLUT-4 vesicles that traffic from the cell periphery to the perinuclear region [75]. The phosphoinositide 5-kinase activity of PIKfyve enables the protein to produce PtdIns(3,5)P_2, responsible for endomembrane homeostasis [76] and PtdIns(5)P, found to be important in GLUT-4 trafficking and actin dynamics [51; 75; 76]. Indeed, overexpression of PIKFyve or microinjection of PtdIns(5)P, but not other phosphatidylinotides, decreases the length and number of F-actin stress fibers to levels comparable of those seen in response to insulin [51]. Microinjection of PtdIns(5)P partially mimics the insulin-induced effect on GLUT-4 translocation in 3T3-L1 adipocytes [51]. Interestingly, PtdIns(5)P was recently described to promote PI 3-kinase activation and Akt/PKB phosphorylation in host cells infected with the facultative intracellular parasite *Shigella flexneri* in order to control massive rearrangements of the host cytoskeleton [77]. Whether PtdIns(5)P could, to a certain extent, activate the PI 3-kinase-Akt/PKB pathway in response to insulin remains to be addressed. Another recent study also describes the endogenous PtdIns(3)P, produced from the hydrolysis of PtdIns(3,5)P_2 by the 72-kDa inositol polyphosphate 5-phosphatase (72-5ptase), as being

able of promoting GLUT-4 translocation [78]. Whether this phosphatase acts in concert with Vac14 to control PtdIns(3,5)P_2 synthesis and turnover, like the Vac14/Fig4p complex in yeast [44], still requires investigations.

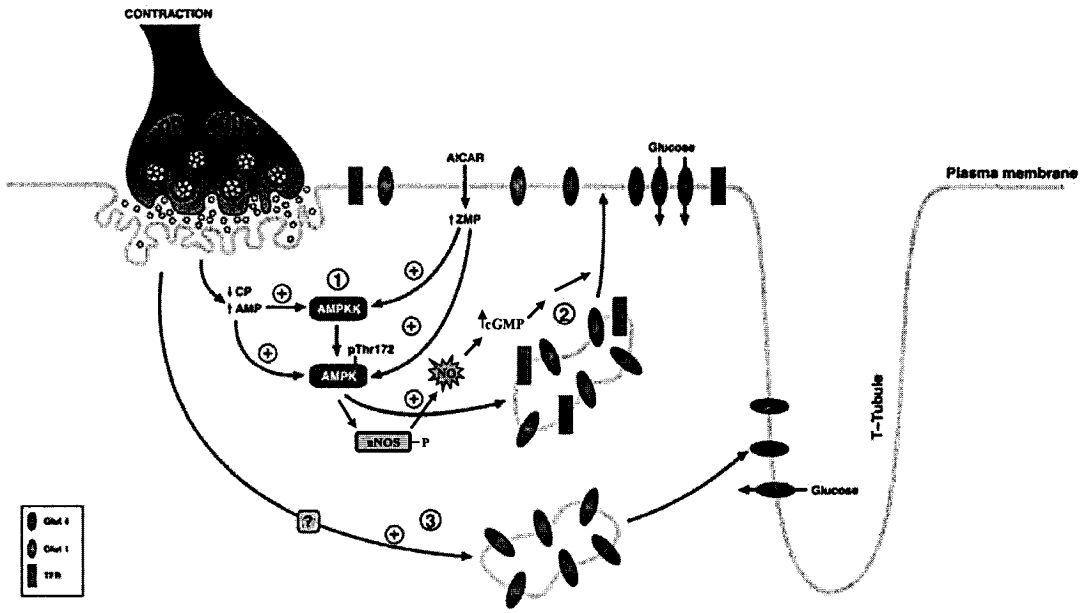

Figure 1-1. Contraction-Induced GLUT-4 Translocation and nNOS

1) Muscle contraction reduces creatine phosphate and increases AMP levels, leading to the activation of AMPK through allosteric activation. Furthermore, AMPK is covalently activated by AMPK kinase (AMPKK). AMPKK, like AMPK, is also allosterically activated by AMP and the synthetic drug AICAR. 2) Activation of AMPK by either contraction or AICAR leads to the translocation of GLUT4 to the plasma membrane, but not to the T-tubules, possibly via phosphorylation and activation of NOS, which in turn leads to increased NO production and elevation of cGMP levels. 3) Contraction, but not AICAR treatment, further promotes GLUT4 translocation to the T-tubules by an unknown signaling mechanism.

(modified from [56])

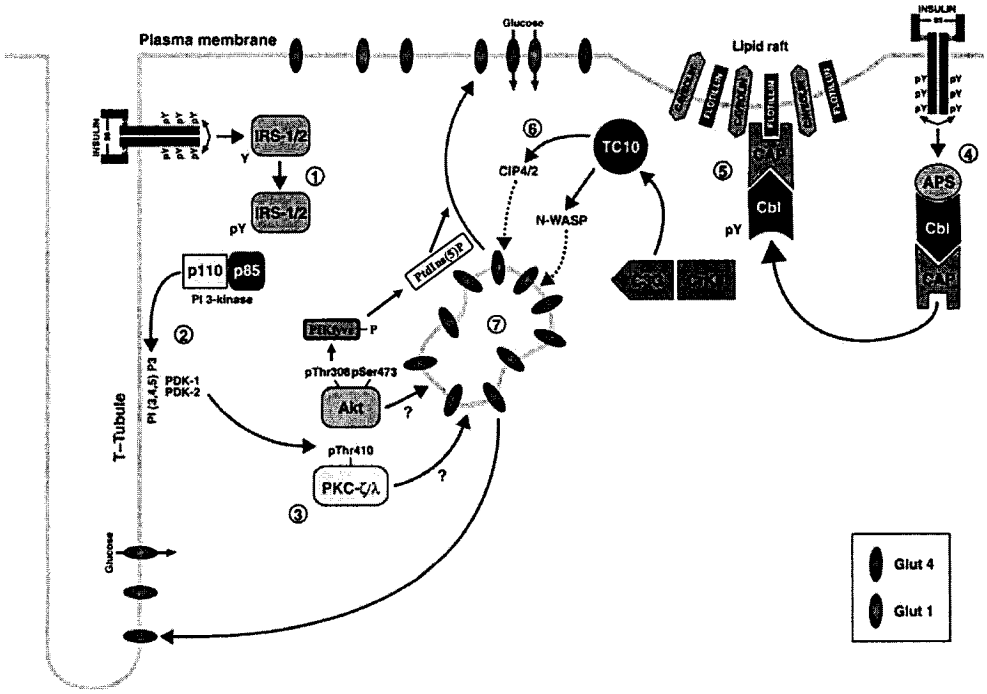

Figure 1-2. Insulin-Induced GLUT-4 Translocation and PIKfyve

1) Once insulin binds to its receptor, it leads to the IR autophosphorylation and activation of its intrinsic kinase activity towards IRS-1/2. 2) Tyrosine-phosphorylated IRS proteins recruit and lead to the activation of PI 3-kinase. 3) PI 3-kinase mediated production of $PI(3,4,5)P_3$ which activates the 3'-phosphoinositide-dependent protein kinases (PDK1/2) that in turn phosphorylates Akt and PKC-zeta/lamda. Akt phosphorylates PIKfyve leading to an increased production in PtdIns(5)*P*, a proposed mediator of GLUT-4 translocation. 4-6) Insulin receptor signaling also results in another phosphorylation and activation cascade involving the Cbl/CAP complex recruitment and translocation to lipid rafts and interaction with flotillin subsequently leading to activation of TC10 and GLUT-4 translocation. 7) The convergence of the two signaling pathways is necessary for optimal GLUT4 translocation to the cell surface.

(Modified from [56])

CHAPTER 2

Binding of Vac14 to Neuronal Nitric Oxide Synthase: Characterisation of a New Internal PDZ-Recognition Motif

FEBS Letters (in press)

Contribution of Authors

All the work here, including the writing of the manuscript, was carried out by Jean-François Lemaire under the supervision of Dr. Peter McPherson.

Binding of Vac14 to Neuronal Nitric Oxide Synthase: Characterisation of a New Internal PDZ-Recognition Motif

Jean-François Lemaire and Peter S. McPherson

Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, QC H3A 2B4, Canada

Address Correspondence to:
Dr. Peter S. McPherson
Department of Neurology and Neurosurgery
Montreal Neurological Institute
McGill University
3801 University St.
Montréal, Québec
Canada
H3A 2B4
peter.mcpherson@mcgill.ca
(P) 01 (514) 398-7355
(F) 01 (514) 398-8106

Keywords:

Internal motif; PDZ; nNOS; Vac14; PIKfyve, 5-HT_{2B} receptor

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.

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 post-synaptic 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 [9]. Although PDZ/PDZ domain interactions can occur [16; 17], 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 [18]. 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]* [9], 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 [12]. 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* [21] and more specifically -G-[D/E]-x-V* [18]. 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 [23; 27; 28]. Besides β-fingers, there are only a few examples of internal motifs of PDZ recognition. These include binding of the Dishevelled PDZ domain to internal -K-T-x-x-x-[W/I]- motifs in Frizzled [24] and Idax [25] and the internal sequence -H-R-E-M-A-V- that mediates the Pals1–Par-6 interaction [26].

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 [38; 39]. 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 PtdIns3P 5-kinase Fab1p, and its mammalian ortholog PIKfyve [6; 8; 39; 49]. 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 [41; 79]. Intringuinly, we have identified Vac14 in a screen for vesicle trafficking proteins [5]. Upon activation with Vac14, PIKfyve but not Fab1p, also produces PtdIns(5)P, which has implications in actin remodeling and GLUT4 dynamics [47; 51].

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 domain.

Material and Methods:

Antibodies:

A polyclonal antibody against Vac14 was raised in rabbits and affinity purified against a peptide of human Vac14 ($_{762}$HLEVRHQRSGRGDHLDRRVVL$_{782}$) using a previously described technique [80]. Polyclonal sera against glutathione-S-transferase (GST) and NECAP 1 were previously described [81; 82]. Monoclonal antibodies against synaptophysin and FLAG were from Sigma-Aldrich, nNOS and His6 monoclonal antibodies were from BD Transduction Laboratories and Qiagen, respectively.

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 (Suppl. 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 $[768\text{-}782]_{aa}$ and $[768\text{-}777]_{aa}$ and 5-HT_{2B} receptor $[464\text{-}479]_{aa}$ were generated by subcloning annealed complementary oligos into pGEX-4T1 (Suppl. Table 1). All constructs were confirmed by sequencing.

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 x *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 x g for 5 min. Subcellular fractionation of brain extracts was performed as previously described [83]. 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.

Binding Studies:

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 [84]. 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 [85].

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 figure 2-3A and 2-3C, 1% Triton X-100 was replaced with 1% Tween-20. Protein amounts shown in figure 2-2D were quantified using ImageJ (National Institutes of Health, USA).

Overlay Assays:

The GST tag from the GST-Vac14 full-length ($[1\text{-}782]_{aa}$) 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 $[1\text{-}100]_{aa}$ (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 [81].

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 mock or FLAG-Vac14-transfected cells (Fig. 2-1A). 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. 2-1B). For brain, Vac14 is detected in all regions of adult brain tested and is also present in embryonic brain (Fig. 2-1C). 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. 2-1D). The synaptic vesicle protein synaptophysin shows the expected fractionation profile (Fig. 2-1D).

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. 2-2A). In contrast, the abundant brain protein NECAP 1 [86] does not co-immunoprecipitate with Vac14 (Fig. 2-2A).

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. 2-2B), which was confirmed in overlay assays (Fig. 2-2C). When testing a range of His-Vac14 concentrations for nNOS binding, we found saturation at approximately 20 μg (0.23 μM) (Fig. 2-2D and 2-2E).

The detection of the 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. 2-3A-C). GST-nNOS PDZ binds with comparable levels to full-length Vac14 and a Vac14 N-terminal deletion construct $[335\text{-}782]_{aa}$. 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. 2-3A). 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 [18] (Fig. 2-3B). 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. 2-3B and 2-3C). 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. 2-3C). The loss of binding seen for Vac14 $[335\text{-}772]_{aa}$ when compared to Vac14 $[335\text{-}777]_{aa}$ reveals the sequence -G-D-H-L-D- from amino acids 773 to 777 as a functional internal binding motif (Fig. 2-3C). The decrease in binding for Vac14 $[335\text{-}777]_{aa}$ compared to $[335\text{-}782]_{aa}$ (Fig. 2-3C) 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 $[768\text{-}782]_{aa}$, containing both motifs and Vac14 $[768\text{-}777]_{aa}$, containing the internal motif only (Fig. 2-4A). The wild-type sequences interact with nNOS in pull-down assays (Fig. 2-4B-D) and the reduction in binding for the short construct verifies the contribution of both motifs to the interaction (Fig. 2-4B). 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. 2-4B). We next performed an alanine scan for the internal motif. Interestingly, exchange of D777 to alanine abolishes the interaction within the context of $[768\text{-}782]_{aa}$, revealing that the internal motif is crucial for interaction (Fig. 2-4C). 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 $[768\text{-}777]_{aa}$ peptide, alanine mutation of G773, D774, L776,

and D777 reduces or abolishes binding to nNOS (Fig. 2-4D). Similar results were obtained for alanine mutations in the context of $[768\text{-}782]_{aa}$ 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 [87]. Figure 2-5A 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 $[464\text{-}479]_{aa}$ rat sequence, which contains both motifs. In agreement with a previous study [87], the wild-type peptide (GST-$5HT_{2B}$ WT) binds the nNOS PDZ domain (Fig. 2-4E). 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. 2-4E). 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. 2-4E). These data further support the involvement of the internal motif in nNOS PDZ domain interactions.

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 [18]. 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 [23]. 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 [86]. In the NECAP proteins, the motif is found at the C-terminus and the free carboxyl group is critical for α-ear binding [82]. 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 [82]. 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 (Fig. 2-5B). Even the less stringent consensus motif -G-[D/E]-x-Φ-[D/E/*] is, with exception of *X. 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 (Fig. 2-5A). 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 (Fig. 2-5). Interestingly, 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 editing 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.

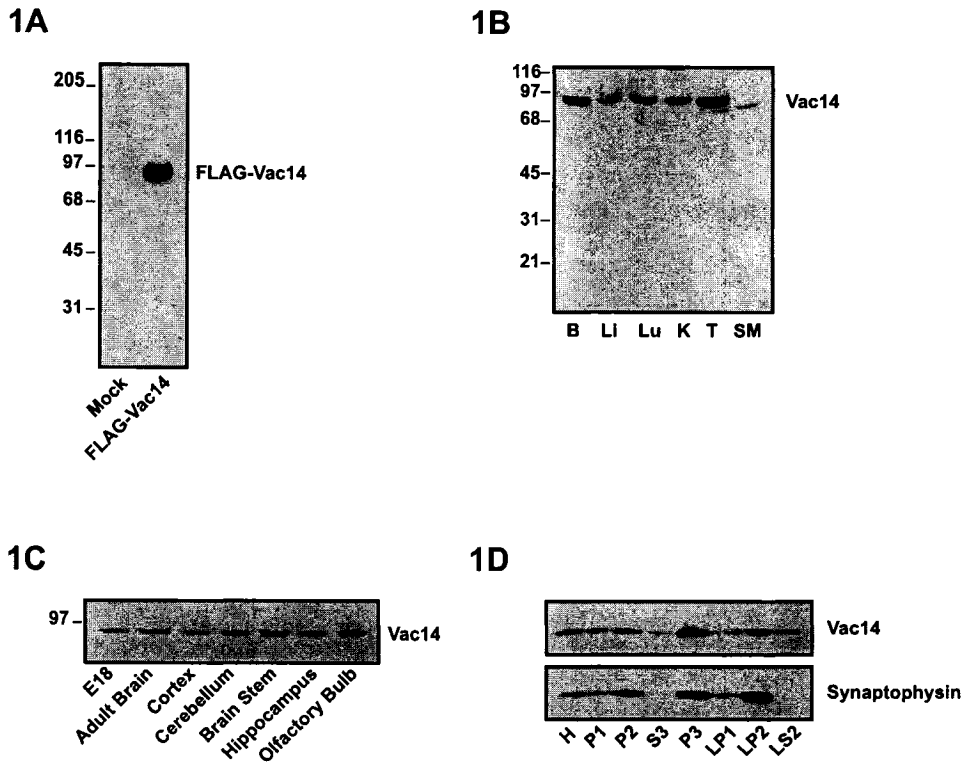

Figure 1. Lemaire and McPherson

Figure 2-1: Expression pattern of Vac14.

A) Lysates (10 μg/lane) from FLAG and FLAG-Vac14 tansfected 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).

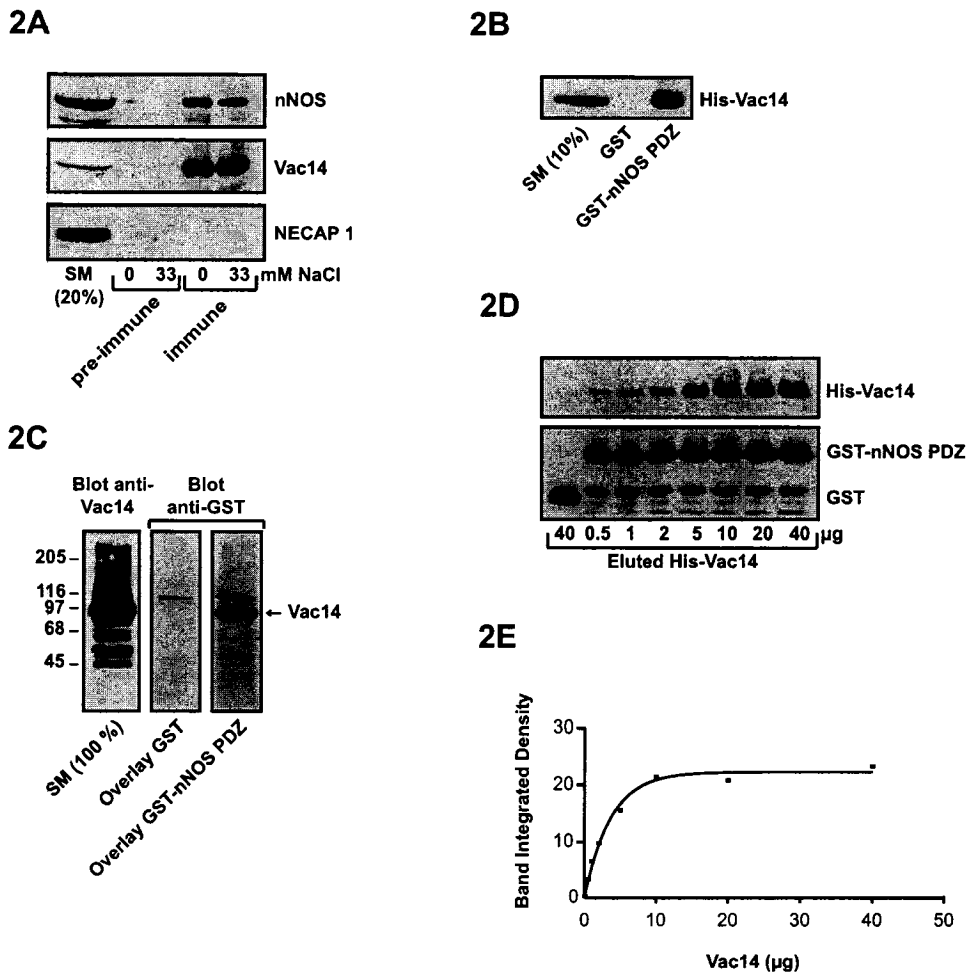

Figure 2. Lemaire and McPherson

Figure 2-2: 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 anti-His tag antibody. An aliquot of His-Vac14 (10%) was loaded as starting material (SM). C) Purified His-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.

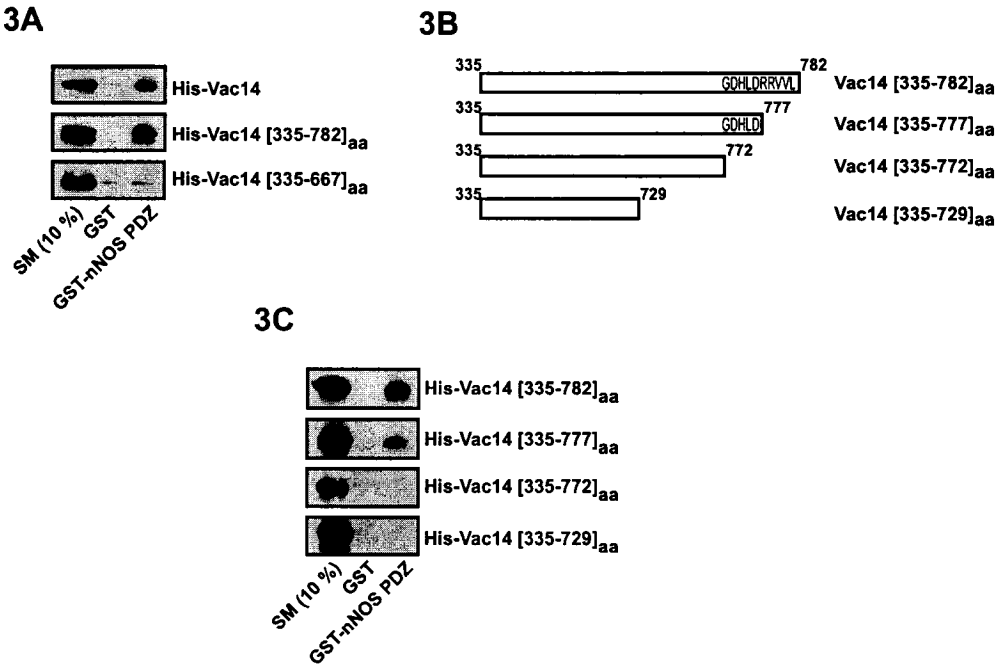

Figure 3. Lemaire and McPherson

Figure 2-3: 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 anti-His antibody. 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.

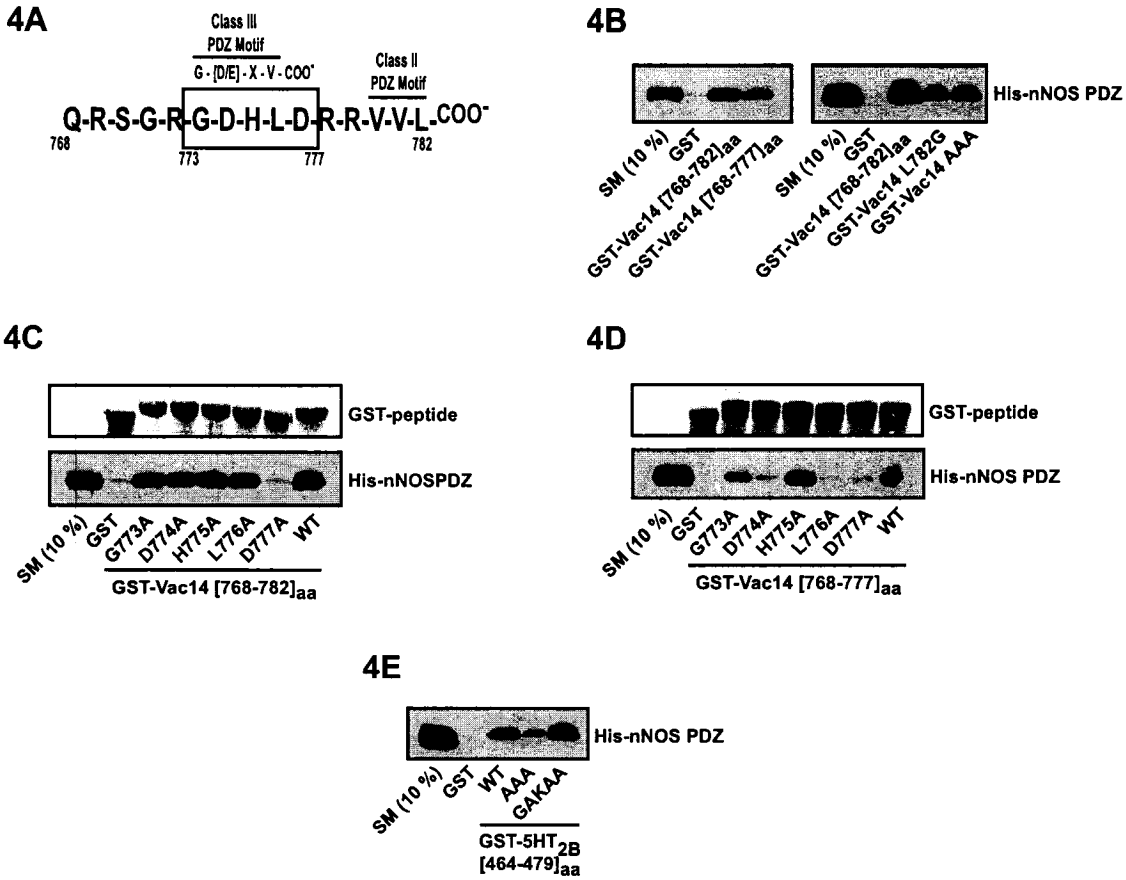

Figure 4. Lemaire and McPherson

Figure 2-4: Characterization of the internal motif responsible for PDZ interactions.

A) Amino acids 768 to 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 $[464\text{-}479]_{aa}$ 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 $[464\text{-}479]_{aa}$.

Table 2-1: List of sense and anti-sense primers and oligonucleotides used for cloning.

Primer's Name	Primer's Orientation	Primer Sequence
hVac14 F1	Foward	5'-GGGAGATCTATGAACCCCGAGAAGGATTTC-3'
hVac14 F335	Foward	5'-GTCAGATCTGAGGACGACGAGCTGGATG-3'
hVac14 R667	Reverse	5'-CAGCTCGAGTCACTTGTCCACCTCTGCGAGG-3'
hVac14 R782	Reverse	5'-CCACTCGAGTCAGAGGACAACCCTCCGG-3'
hVac14 R777	Reverse	5'-CCAAAGCTTTCAGTCCAGGTGGTCCCCACG-3'
hVac14 R772	Reverse	5'-CCAAAGCTTTCAACGCCCGCTCCGCTGGTG-3'
hVac14 R729	Reverse	5'-CCAAAGCTTTCATTCGGTCTGCAGCAGCTCAG-3'
hnNOS F1	Foward	5'-GGGGAATTCATGGAGGATCACATGTTCGG-3'
hnNOS R100	Reverse	5'-GGTGTCGACTCAAGGGCCCCTCAGAATGAGG-3'

Peptide Oligos	Oligo's Orientation	Oligo Sequence
Vac14 768-782	Sense	5'-GATCCCAGCGGAGCGGGCGTGGGGACCACCTGGACCGGAGGGTTGTCCTCTGAG-3'
Vac14 768-782	Anti-Sense	5'-TCGACTCAGAGGACAACCCTCCGGTCCAGGTGGTCCCCACGCCCGCTCCGCTGG-3'
Vac14 768-777	Sense	5'-GATCCCAGCGGAGCGGGCGTGGGGACCACCTGGACTGAG-3'
Vac14 768-777	Anti-Sense	5'-TCGACTCAGTCCAGGTGGTCCCCACGCCCGCTCCGCTGG-3'
Vac14 768-782 G773A	Sense	5'-GATCCCAGCGGAGCGGGCGTGCGGACCACCTGGACCGGAGGGTTGTCCTCTGAG-3'
Vac14 768-782 G773A	Anti-Sense	5'-TCGACTCAGAGGACAACCCTCCGGTCCAGGTGGTCCGCACGCCCGCTCCGCTGG-3'
Vac14 768-782 D774A	Sense	5'-GATCCCAGCGGAGCGGGCGTGGGGCGCACCTGGACCGGAGGGTTGTCCTCTGAG-3'
Vac14 768-782 D774A	Anti-Sense	5'-TCGACTCAGAGGACAACCCTCCGGTCCAGGTGCGCCCCACGCCCGCTCCGCTGG-3'
Vac14 768-782 H775A	Sense	5'-GATCCCAGCGGAGCGGGCGTGGGGACGCGCTGGACCGGAGGGTTGTCCTCTGAG-3'
Vac14 768-782 H775A	Anti-Sense	5'-TCGACTCAGAGGACAACCCTCCGGTCCAGCGCGTCCCCACGCCCGCTCCGCTGG-3'
Vac14 768-782 L776A	Sense	5'-GATCCCAGCGGAGCGGGCGTGGGGACCACGCGGACCGGAGGGTTGTCCTCTGAG-3'
Vac14 768-782 L776A	Anti-Sense	5'-TCGACTCAGAGGACAACCCTCCGGTCCGCGTGGTCCCCACGCCCGCTCCGCTGG-3'
Vac14 768-782 D777A	Sense	5'-GATCCCAGCGGAGCGGGCGTGGGGACCACCTGGCGCGGAGGGTTGTCCTCTGAG-3'
Vac14 768-782 D777A	Anti-Sense	5'-TCGACTCAGAGGACAACCCTCCGCGCCAGGTGGTCCCCACGCCCGCTCCGCTGG-3'
Vac14 768-782 L782G	Sense	5'-GATCCCAGCGGAGCGGGCGTGGGGACCACCTGGACCGGAGGGTTGTCGGCTGAG-3'
Vac14 768-782 L782G	Anti-Sense	5'-TCGACTCAGCCGACAACCCTCCGGTCCAGGTGGTCCCCACGCCCGCTCCGCTGG-3'
Vac14 768-782 AAA	Sense	5'-GATCCCAGCGGAGCGGGCGTGGGGACCACCTGGACCGGAGGGCGGCGGCGTGAG-3'
Vac14 768-782 AAA	Anti-Sense	5'-CCAGCGGAGCGGGCGTGGGGACCACCTGGACCGGAGGGCGGCGGCGTGAGTCGA-3'
Vac14 768-777 G773A	Sense	5'-GATCCCAGCGGAGCGGGCGTGCGGACCACCTGGACTGAG-3'
Vac14 768-777 G773A	Anti-Sense	5'-TCGACTCAGTCCAGGTGGTCCGCACGCCCGCTCCGCTGG-3'
Vac14 768-777 D774A	Sense	5'-GATCCCAGCGGAGCGGGCGTGGGGCGCACCTGGACTGAG-3'
Vac14 768-777 D774A	Anti-Sense	5'-TCGACTCAGTCCAGGTGCGCCCCACGCCCGCTCCGCTGG-3'
Vac14 768-777 H775A	Sense	5'-GATCCCAGCGGAGCGGGCGTGGGGACGCGCTGGACTGAG-3'
Vac14 768-777 H775A	Anti-Sense	5'-TCGACTCAGTCCAGCGCGTCCCCACGCCCGCTCCGCTGG-3'
Vac14 768-777 L776A	Sense	5'-GATCCCAGCGGAGCGGGCGTGGGGACCACGCGGACTGAG-3'
Vac14 768-777 L776A	Anti-Sense	5'-TCGACTCAGTCCGCGTGGTCCCCACGCCCGCTCCGCTGG-3'
Vac14 768-777 D777A	Sense	5'-GATCCCAGCGGAGCGGGCGTGGGGACCACCTGGCGTGAG-3'
Vac14 768-777 D777A	Anti-Sense	5'-TCGACTCACGCCAGGTGGTCCCCACGCCCGCTCCGCTGG-3'
$5HT_{2B}$ 464-479	Sense	5'-GATCCCTGACCGAAAACGATGGCGATAAAGTGGAAGATCAGGTGAGCTATATTTGAG-3'
$5HT_{2B}$ 464-479	Anti-Sense	5'-TCGACTCAAATATAGCTCACCTGATCTTCCACTTTATCGCCATCGTTTTCGGTCAGG-3'
$5HT_{2B}$ 464-479 AAA	Sense	5'-GATCCCTGACCGAAAACGATGGCGATAAAGTGGAAGATCAGGTGGCGGCGGCGTGAG-3'
$5HT_{2B}$ 464-479 AAA	Anti-Sense	5'-TCGACTCACGCCGCCGCCACCTGATCTTCCACTTTATCGCCATCGTTTTCGGTCAGG-3'
$5HT_{2B}$ 464-479 GAKAA	Sense	5'-GATCCCTGACCGAAAACGATGGCGCGAAAGCGGCGGATCAGGTGAGCTATATTTGAG-3'
$5HT_{2B}$ 464-479 GAKAA	Anti-Sense	5'-TCGACTCAAATATAGCTCACCTGATCCGCCGCTTTCGCGCCATCGTTTTCGGTCAGG-3'

Supplementary Table 1. Lemaire and McPherson

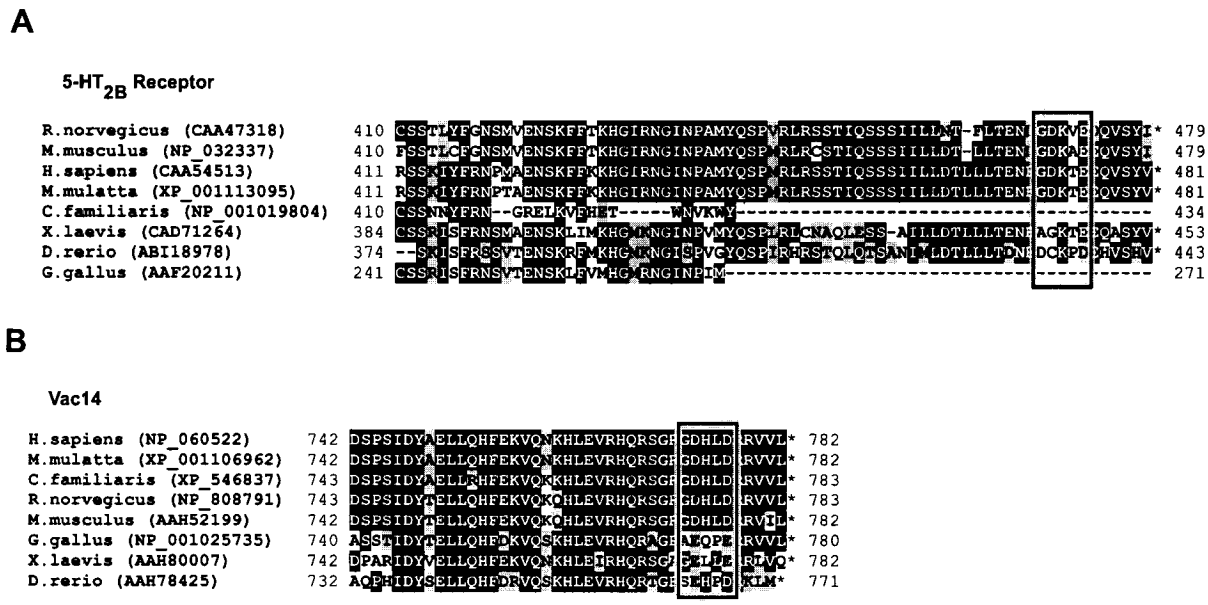

Figure 2-5: BLAST alignments of the C-terminal regions of vertebrate orthologs of 5-HT$_{2B}$ receptor and Vac14.

Alignment of (A) serotonin (5-HT) receptor 2B and (B) Vac14 orthologs from the indicated species. The internal PDZ-recognition motifs are boxed.

CHAPTER 3

Summary & Conclusion

Summary & Conclusion

The present study characterizes a novel protein association between Vac14 and nNOS. This direct interaction is mediated through the PDZ domain of nNOS, which recognizes two peptide motifs in Vac14; one internal and one C-terminal. Although the most common modes of PDZ recognition are through C-terminal peptide motifs, the Vac14/nNOS binding entails the critical involvement of the internal motif. Binding to the second C-terminal motif probably only helps to stabilize the interaction with the internal motif. Mutational analysis revealed the essential residues involved in the interaction. These results also strengthen the notion of a crucial involvement of acidic residues flanking an internal binding site, as previously described in other protein-protein interactions like the ones involving -W-x-x-F- acidic motifs in endocytic proteins [82; 86]. Finally, this study led us to propose a new nNOS PDZ recognition consensus motif -G-[D/E]-x-Φ-[D/E/*], that could be found either as an internal sequence or at the very C-terminus.

Additional experiments have confirmed the general use of the consensus motif -G-[D/E]-x-Φ-[D/E]- in mediating nNOS PDZ interaction, as shown in the nNOS PDZ interaction with the 5-HT_{2B} receptor (Fig. 2-4E). This is particularly interesting considering the search for vertebrate proteins harbouring the consensus motif -G-[D/E]-x-Φ-[D/E]- that has revealed other functionally interesting candidates for nNOS binding. One of them is the gamma-3 subunit of the 5'-AMP-activated protein kinase (AMPK). The putative AMPK/nNOS PDZ domain interaction is of high interest since AMPK is involved in

contraction-induced GLUT-4 translocation [62] and was previously described as phosphorylating the muscle-specific splice isoform of nNOS (nNOSμ) [33; 62]. AMPK phosphorylation of nNOS leads to nitric oxide production, and this pathway is involved in glucose uptake and GLUT-4 translocation in heart muscle [33; 67; 88]. Although little information is available regarding the interaction with nNOS, it is notable that AMPK lacks a C-terminal PDZ domain-binding motif. Instead, it contains an internal sequence that matches the Vac14-derived consensus sequence and like in Vac14, this sequence is present only in mammals (Fig. 3-1). As Vac14 also interacts with PIKfyve, which regulates GLUT-4 transport upon direct phosphorylation by Akt/PKB [75], Vac14 could regulate GLUT-4 transport in a multi-protein complex together with PIKfyve, AMPK, and nNOS.

Specific functional implications of the Vac14/nNOS interaction could simply be that Vac14 regulates nNOS enzymatic activity. Similarly, Vac14 could be important to bring nNOS to the right place at the right time with or without regulating nNOS activity. To a larger magnitude, the present study suggests the presence of a multi-protein complex composed of nNOS, Vac14 and PIKfyve and possibly AMPK, which harbours the proposed consensus PDZ-binding motif in its γ3 subunit. This multi-protein complex could potentially have major implications in the translocation of the glucose transporter GLUT-4 both upon insulin and contraction stimulation (Fig. 3-2). Indeed, few studies have suggested PIKfyve and AS160 as candidate Akt/PKB substrates in insulin-induced GLUT-4 translocation [72; 73]. Interestingly, recent studies described AS160 as a downstream target of AMPK in contraction-induced glucose uptake [58; 59; 60; 61],

linking both the insulin- and the contraction-induced pathways together in GLUT-4 translocation. Moreover, the dispensability of the γ3 subunit-containing AMPK heterotrimers in muscle contraction-induced AS160 phosphorylation suggests other AMPK cellular implications. Indeed, considering the AMPK γ3 subunit being the only one harbouring the proposed nNOS PDZ recognition consensus motif -G-[D/E]-x-Φ-[D/E/*], it must account for a distinct cellular implication possibly mediated through nNOS downstream pathways and/or through association with the conceivable PIKfyve/Vac14/nNOS multi-protein complex (Fig. 3-2). This way, AMPK could regulate GLUT-4 translocation through at least two independent routes.

The description of the novel Vac14/nNOS interaction, in concert with the involvement of dual motifs mediating PDZ interactions, led us to the characterisation of a novel and internal type of PDZ recognition. These findings, in concert with the proposed nNOS PDZ recognition consensus motif -G-[D/E]-x-Φ-[D/E/*] and the resulting potential functional implications, will certainly be of broad interest for the various fields of biological sciences.

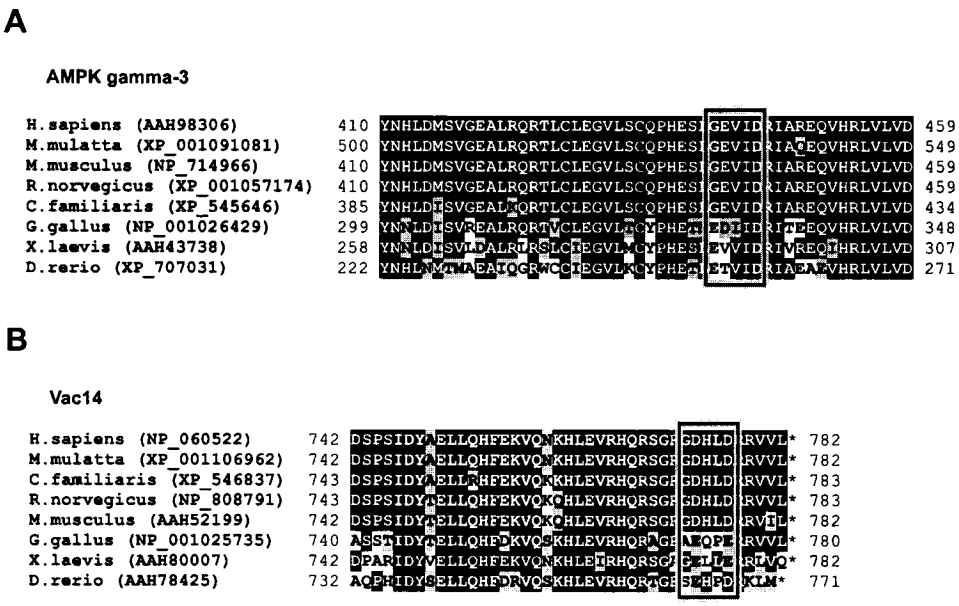

Figure 3-1: BLAST alignments of the vertebrate orthologs of the AMPK γ3 subunit and Vac14, harboring the proposed PDZ binding consensus motif.

A) Alignment of AMPK γ3 subunit orthologs. B) Alignment of Vac14 orthologs. In all cases, the putative internal PDZ-recognition motif is boxed.

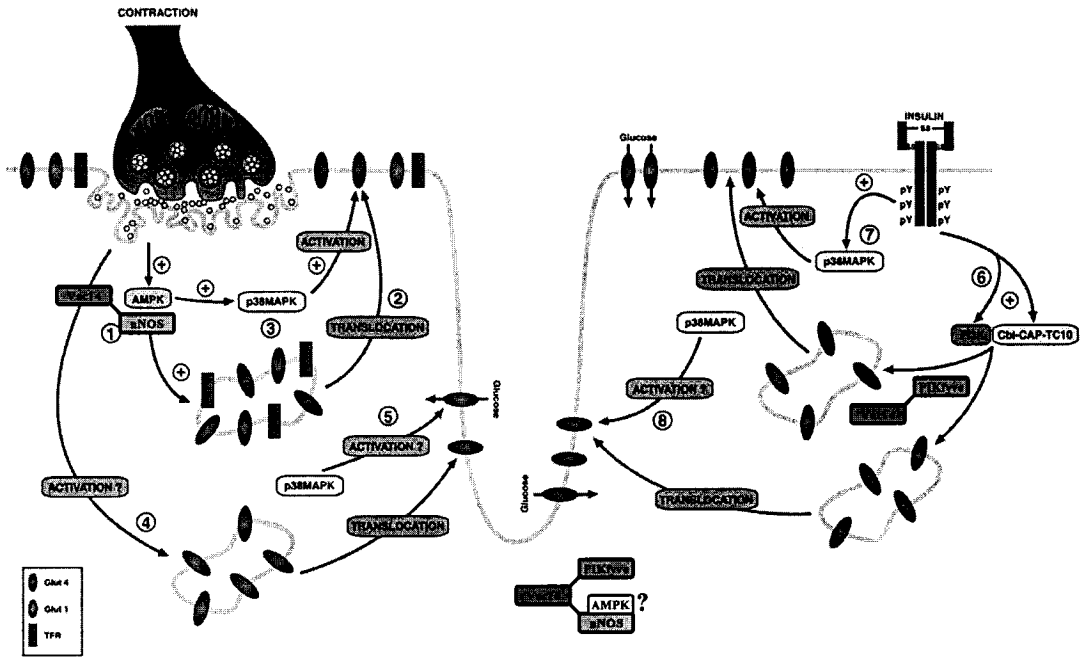

Figure 3-2. Regulation of GLUT-4 Trafficking by Insulin and Contraction

Vac14 could regulate both PIKfyve and nNOS activity/localization in a multi-protein complex possibly composed of, or interacting with AMPK through its plausible nNOS PDZ internal recognition motif.

(Modified from [56])

Abbreviations

5-HT:	5-hydroxy tryptamine or serotonin
5-HT_{2B}:	serotonin receptor 2B
72-5ptase:	72 kDa inositol polyphosphate 5-phosphatase
Φ:	hydrophobic amino acid residue
*:	terminal free carboxyl group (COO^-)
A:	alanine
aa:	amino acid
ACC:	acetyl-coenzyme A carboxylase
AICAR:	5'-aminoimidazole-4-carboxamide riboside
AMP:	adenosine monophosphate
ATP:	adenosine triphosphate
AMPK:	5'AMP-activated protein kinase
AMPKK:	5'AMP-activated protein kinase kinase
BH_4:	tetrahydrobiopterin
BSA:	bovine serum albumin
C:	cysteine
CaM:	calcium-calmodulin
CCPs:	clathrin coated pits
CCVs:	clathrin coated vesicles
cDNA:	complementary deoxyribonucleic acid
cGMP:	cyclic guanosine monophosphate
CPT1:	carnitine palmitoyltransferase 1
D:	aspartic acid
DNA:	deoxyribonucleic acid
DTT:	dithiothreitol
E:	glutamic acid
eNOS:	endothelial nitric oxide synthase
F:	phenylalanine
FAD:	flavin adenine dinucleotide
Fig.:	figure
FMN:	flavin mononucleotide
G:	glycine
GAP:	GTPase-activating protein
GLUT-4:	glucose transporter isoform 4
GST:	glutathione S-transferase
GTP:	guanosine triphosphate
H:	histidine
HEK:	human embryonic kidney
I:	isoleucine
iNOS:	inducible nitric oxide synthase
IR:	insulin receptor
IRS1/2:	insulin receptor substrate 1 and 2
K:	lysine
L:	leucine

L-NAME:	N-nitro-L-arginine methylesther
M:	methionine
mRNA:	messenger ribonucleic acid
MS:	mass spectrometry
MVB:	multivesicular bodies
N:	asparagine
NaCl:	sodium chloride
nNOS:	neuronal nitric oxide synthase
NO:	nitric oxide
NOS:	nitric oxide synthase
P:	proline
PDK1/2:	3'-phosphoinositide-dependent kinase 1 and 2
PI:	phosphatidylinositol
PIs:	protease inhibitors
PI 3-kinase:	phosphatidylinositol 3-kinase
PKA:	protein kinase A
PKB:	protein kinase B or Akt
PKC:	protein kinase C
PtdIns:	phosphatidylinositol
PtdIns(3)*P*:	phosphatidylinositol 3-phosphate
PtdIns(5)*P*:	phosphatidylinositol 5-phosphate
PtdIns(3,5)P_2:	phosphatidylinositol 3,5-biphosphate
PtdIns(3,4,5)P_3:	phosphatidylinositol 3,4,5-triphosphate
PtdIns(4,5)P_2:	phosphatidylinositol 4,5-biphosphate
PtdIns(3)*P* 5-kinase:	phosphatidylinositol 3-phosphate 5-kinase
Q:	glutamine
R:	arginine
S:	serine
SM:	starting material
Suppl.:	supplementary
T:	threonine
TBS:	Tris-buffered saline
TGN:	trans-Golgi network
V:	valine
Vac14:	mammalian vacuolar protein 14
Vac14p:	yeast vacuolar protein 14
W:	tryptophan
Y:	tyrosine
x:	any amino acid residue

Bibliography

[1] P.S. McPherson, B.K. Kay, and N.K. Hussain, Signaling on the endocytic pathway. Traffic 2 (2001) 375-84.

[2] F.M. Brodsky, C.Y. Chen, C. Knuehl, M.C. Towler, and D.E. Wakeham, Biological basket weaving: formation and function of clathrin-coated vesicles. Annu Rev Cell Dev Biol 17 (2001) 517-68.

[3] S.D. Conner, and S.L. Schmid, Regulated portals of entry into the cell. Nature 422 (2003) 37-44.

[4] I. Hinners, and S.A. Tooze, Changing directions: clathrin-mediated transport between the Golgi and endosomes. J Cell Sci 116 (2003) 763-71.

[5] M. Girard, P.D. Allaire, P.S. McPherson, and F. Blondeau, 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 (2005) 1145-54.

[6] C.J. Bonangelino, J.J. Nau, J.E. Duex, M. Brinkman, A.E. Wurmser, J.D. Gary, S.D. Emr, and L.S. Weisman, Osmotic stress-induced increase of phosphatidylinositol 3,5-bisphosphate requires Vac14p, an activator of the lipid kinase Fab1p. J Cell Biol 156 (2002) 1015-28.

[7] S.K. Dove, R.K. McEwen, A. Mayes, D.C. Hughes, J.D. Beggs, and R.H. Michell, Vac14 controls PtdIns(3,5)P(2) synthesis and Fab1-dependent protein trafficking to the multivesicular body. Curr Biol 12 (2002) 885-93.

[8] D. Sbrissa, O.C. Ikonomov, J. Strakova, R. Dondapati, K. Mlak, R. Deeb, R. Silver, and A. Shisheva, A mammalian ortholog of Saccharomyces cerevisiae Vac14 that associates with and up-regulates PIKfyve phosphoinositide 5-kinase activity. Mol Cell Biol 24 (2004) 10437-47.

[9] F. Jelen, A. Oleksy, K. Smietana, and J. Otlewski, PDZ domains - common players in the cell signaling. Acta Biochim Pol 50 (2003) 985-1017.

[10] P. Zimmermann, The prevalence and significance of PDZ domain-phosphoinositide interactions. Biochim Biophys Acta 1761 (2006) 947-56.

[11] C.P. Ponting, C. Phillips, K.E. Davies, and D.J. Blake, PDZ domains: targeting signalling molecules to sub-membranous sites. Bioessays 19 (1997) 469-79.

[12] H. Tochio, Q. Zhang, P. Mandal, M. Li, and M. Zhang, Solution structure of the extended neuronal nitric oxide synthase PDZ domain complexed with an associated peptide. Nat Struct Biol 6 (1999) 417-21.

[13] B. Brone, and J. Eggermont, PDZ proteins retain and regulate membrane transporters in polarized epithelial cell membranes. Am J Physiol Cell Physiol 288 (2005) C20-9.

[14] Y. Altschuler, C. Hodson, and S.L. Milgram, The apical compartment: trafficking pathways, regulators and scaffolding proteins. Curr Opin Cell Biol 15 (2003) 423-9.

[15] C. Rongo, Disparate cell types use a shared complex of PDZ proteins for polarized protein localization. Cytokine Growth Factor Rev 12 (2001) 349-59.

[16] D.I. Utepbergenov, A.S. Fanning, and J.M. Anderson, Dimerization of the Scaffolding Protein ZO-1 through the Second PDZ Domain. J Biol Chem 281 (2006) 24671-7.
[17] Y.J. Im, J.H. Lee, S.H. Park, S.J. Park, S.H. Rho, G.B. Kang, E. Kim, and S.H. Eom, Crystal structure of the Shank PDZ-ligand complex reveals a class I PDZ interaction and a novel PDZ-PDZ dimerization. J Biol Chem 278 (2003) 48099-104.
[18] J. Schepens, E. Cuppen, B. Wieringa, and W. Hendriks, The neuronal nitric oxide synthase PDZ motif binds to -G(D,E)XV* carboxyterminal sequences. FEBS Lett 409 (1997) 53-6.
[19] D.A. Doyle, A. Lee, J. Lewis, E. Kim, M. Sheng, and R. MacKinnon, Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. Cell 85 (1996) 1067-76.
[20] Z. Songyang, A.S. Fanning, C. Fu, J. Xu, S.M. Marfatia, A.H. Chishti, A. Crompton, A.C. Chan, J.M. Anderson, and L.C. Cantley, Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. Science 275 (1997) 73-7.
[21] N.L. Stricker, K.S. Christopherson, B.A. Yi, P.J. Schatz, R.W. Raab, G. Dawes, D.E. Bassett, Jr., D.S. Bredt, and M. Li, PDZ domain of neuronal nitric oxide synthase recognizes novel C-terminal peptide sequences. Nat Biotechnol 15 (1997) 336-42.
[22] S. Schneider, M. Buchert, O. Georgiev, B. Catimel, M. Halford, S.A. Stacker, T. Baechi, K. Moelling, and C.M. Hovens, Mutagenesis and selection of PDZ domains that bind new protein targets. Nat Biotechnol 17 (1999) 170-5.
[23] B.J. Hillier, K.S. Christopherson, K.E. Prehoda, D.S. Bredt, and W.A. Lim, Unexpected modes of PDZ domain scaffolding revealed by structure of nNOS-syntrophin complex. Science 284 (1999) 812-5.
[24] H.C. Wong, A. Bourdelas, A. Krauss, H.J. Lee, Y. Shao, D. Wu, M. Mlodzik, D.L. Shi, and J. Zheng, Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. Mol Cell 12 (2003) 1251-60.
[25] T.B. London, H.J. Lee, Y. Shao, and J. Zheng, Interaction between the internal motif KTXXXI of Idax and mDvl PDZ domain. Biochem Biophys Res Commun 322 (2004) 326-32.
[26] R.R. Penkert, H.M. DiVittorio, and K.E. Prehoda, Internal recognition through PDZ domain plasticity in the Par-6-Pals1 complex. Nat Struct Mol Biol 11 (2004) 1122-7.
[27] H. Tochio, Y.K. Mok, Q. Zhang, H.M. Kan, D.S. Bredt, and M. Zhang, Formation of nNOS/PSD-95 PDZ dimer requires a preformed beta-finger structure from the nNOS PDZ domain. J Mol Biol 303 (2000) 359-70.
[28] J.E. Brenman, K.S. Christopherson, S.E. Craven, A.W. McGee, and D.S. Bredt, Cloning and characterization of postsynaptic density 93, a nitric oxide synthase interacting protein. J Neurosci 16 (1996) 7407-15.
[29] J.D. Paasche, T. Attramadal, K. Kristiansen, M.P. Oksvold, H.K. Johansen, H.S. Huitfeldt, S.G. Dahl, and H. Attramadal, Subtype-specific sorting of the ETA endothelin receptor by a novel endocytic recycling signal for G protein-coupled receptors. Mol Pharmacol 67 (2005) 1581-90.
[30] W.K. Alderton, C.E. Cooper, and R.G. Knowles, Nitric oxide synthases: structure, function and inhibition. Biochem J 357 (2001) 593-615.

[31] M.A. Haidara, H.Z. Yassin, M. Rateb, H. Ammar, and M.A. Zorkani, Role of oxidative stress in development of cardiovascular complications in diabetes mellitus. Curr Vasc Pharmacol 4 (2006) 215-27.
[32] B. Brune, The intimate relation between nitric oxide and superoxide in apoptosis and cell survival. Antioxid Redox Signal 7 (2005) 497-507.
[33] J. Li, X. Hu, P. Selvakumar, R.R. Russell, 3rd, S.W. Cushman, G.D. Holman, and L.H. Young, Role of the nitric oxide pathway in AMPK-mediated glucose uptake and GLUT4 translocation in heart muscle. Am J Physiol Endocrinol Metab 287 (2004) E834-41.
[34] R.G. Knowles, and S. Moncada, Nitric oxide synthases in mammals. Biochem J 298 (Pt 2) (1994) 249-58.
[35] P.J. Kuhlencordt, S. Hotten, J. Schodel, S. Rutzel, K. Hu, J. Widder, A. Marx, P.L. Huang, and G. Ertl, Atheroprotective effects of neuronal nitric oxide synthase in apolipoprotein e knockout mice. Arterioscler Thromb Vasc Biol 26 (2006) 1539-44.
[36] P. Wang, Q. Zhang, H. Tochio, J.S. Fan, and M. Zhang, Formation of a native-like beta-hairpin finger structure of a peptide from the extended PDZ domain of neuronal nitric oxide synthase in aqueous solution. Eur J Biochem 267 (2000) 3116-22.
[37] S.R. Jaffrey, A.M. Snowman, M.J. Eliasson, N.A. Cohen, and S.H. Snyder, CAPON: a protein associated with neuronal nitric oxide synthase that regulates its interactions with PSD95. Neuron 20 (1998) 115-24.
[38] A. Shisheva, PIKfyve: the road to PtdIns 5-P and PtdIns 3,5-P(2). Cell Biol Int 25 (2001) 1201-6.
[39] C.J. Bonangelino, N.L. Catlett, and L.S. Weisman, Vac7p, a novel vacuolar protein, is required for normal vacuole inheritance and morphology. Mol Cell Biol 17 (1997) 6847-58.
[40] S.K. Dove, F.T. Cooke, M.R. Douglas, L.G. Sayers, P.J. Parker, and R.H. Michell, Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. Nature 390 (1997) 187-92.
[41] J.D. Shaw, H. Hama, F. Sohrabi, D.B. DeWald, and B. Wendland, PtdIns(3,5)P2 is required for delivery of endocytic cargo into the multivesicular body. Traffic 4 (2003) 479-90.
[42] G. Odorizzi, M. Babst, and S.D. Emr, Phosphoinositide signaling and the regulation of membrane trafficking in yeast. Trends Biochem Sci 25 (2000) 229-35.
[43] J.P. Phelan, S.H. Millson, P.J. Parker, P.W. Piper, and F.T. Cooke, Fab1p and AP-1 are required for trafficking of endogenously ubiquitylated cargoes to the vacuole lumen in S. cerevisiae. J Cell Sci (2006).
[44] J.E. Duex, F. Tang, and L.S. Weisman, The Vac14p-Fig4p complex acts independently of Vac7p and couples PI3,5P2 synthesis and turnover. J Cell Biol 172 (2006) 693-704.
[45] S.A. Rudge, D.M. Anderson, and S.D. Emr, Vacuole size control: regulation of PtdIns(3,5)P2 levels by the vacuole-associated Vac14-Fig4 complex, a PtdIns(3,5)P2-specific phosphatase. Mol Biol Cell 15 (2004) 24-36.

[46] O.C. Ikonomov, D. Sbrissa, K. Mlak, R. Deeb, J. Fligger, A. Soans, R.L. Finley, Jr., and A. Shisheva, Active PIKfyve associates with and promotes the membrane attachment of the late endosome-to-trans-Golgi network transport factor Rab9 effector p40. J Biol Chem 278 (2003) 50863-71.
[47] R.K. McEwen, S.K. Dove, F.T. Cooke, G.F. Painter, A.B. Holmes, A. Shisheva, Y. Ohya, P.J. Parker, and R.H. Michell, 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 (1999) 33905-12.
[48] A.C. Rutherford, C. Traer, T. Wassmer, K. Pattni, M.V. Bujny, J.G. Carlton, H. Stenmark, and P.J. Cullen, The mammalian phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) regulates endosome-to-TGN retrograde transport. J Cell Sci 119 (2006) 3944-57.
[49] D. Sbrissa, and A. Shisheva, Acquisition of unprecedented phosphatidylinositol 3,5-bisphosphate rise in hyperosmotically stressed 3T3-L1 adipocytes, mediated by ArPIKfyve-PIKfyve pathway. J Biol Chem 280 (2005) 7883-9.
[50] M.J. Clague, and O. Lorenzo, The myotubularin family of lipid phosphatases. Traffic 6 (2005) 1063-9.
[51] D. Sbrissa, O.C. Ikonomov, J. Strakova, and A. Shisheva, Role for a novel signaling intermediate, phosphatidylinositol 5-phosphate, in insulin-regulated F-actin stress fiber breakdown and GLUT4 translocation. Endocrinology 145 (2004) 4853-65.
[52] S.L. McGee, and M. Hargreaves, Exercise and skeletal muscle glucose transporter 4 expression: molecular mechanisms. Clin Exp Pharmacol Physiol 33 (2006) 395-9.
[53] D.G. Hardie, and D. Carling, The AMP-activated protein kinase--fuel gauge of the mammalian cell? Eur J Biochem 246 (1997) 259-73.
[54] Y.C. Long, and J.R. Zierath, AMP-activated protein kinase signaling in metabolic regulation. J Clin Invest 116 (2006) 1776-83.
[55] W.G. Aschenbach, K. Sakamoto, and L.J. Goodyear, 5' adenosine monophosphate-activated protein kinase, metabolism and exercise. Sports Med 34 (2004) 91-103.
[56] F. Tremblay, M.J. Dubois, and A. Marette, Regulation of GLUT4 traffic and function by insulin and contraction in skeletal muscle. Front Biosci 8 (2003) d1072-84.
[57] S.C. Stein, A. Woods, N.A. Jones, M.D. Davison, and D. Carling, The regulation of AMP-activated protein kinase by phosphorylation. Biochem J 345 Pt 3 (2000) 437-43.
[58] H. Sano, S. Kane, E. Sano, C.P. Miinea, J.M. Asara, W.S. Lane, C.W. Garner, and G.E. Lienhard, Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. J Biol Chem 278 (2003) 14599-602.
[59] S. Kane, H. Sano, S.C. Liu, J.M. Asara, W.S. Lane, C.C. Garner, and G.E. Lienhard, A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. J Biol Chem 277 (2002) 22115-8.
[60] C.P. Miinea, H. Sano, S. Kane, E. Sano, M. Fukuda, J. Peranen, W.S. Lane, and G.E. Lienhard, AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. Biochem J 391 (2005) 87-93.

[61] J.T. Treebak, S. Glund, A. Deshmukh, D.K. Klein, Y.C. Long, T.E. Jensen, S.B. Jorgensen, B. Viollet, L. Andersson, D. Neumann, T. Wallimann, E.A. Richter, A.V. Chibalin, J.R. Zierath, and J.F. Wojtaszewski, AMPK-mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits. Diabetes 55 (2006) 2051-8.
[62] Z.P. Chen, G.K. McConell, B.J. Michell, R.J. Snow, B.J. Canny, and B.E. Kemp, AMPK signaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation. Am J Physiol Endocrinol Metab 279 (2000) E1202-6.
[63] V.B. O'Donnell, P.H. Chumley, N. Hogg, A. Bloodsworth, V.M. Darley-Usmar, and B.A. Freeman, Nitric oxide inhibition of lipid peroxidation: kinetics of reaction with lipid peroxyl radicals and comparison with alpha-tocopherol. Biochemistry 36 (1997) 15216-23.
[64] E. Butt, M. Bernhardt, A. Smolenski, P. Kotsonis, L.G. Frohlich, A. Sickmann, H.E. Meyer, S.M. Lohmann, and H.H. Schmidt, Endothelial nitric-oxide synthase (type III) is activated and becomes calcium independent upon phosphorylation by cyclic nucleotide-dependent protein kinases. J Biol Chem 275 (2000) 5179-87.
[65] D. Fulton, J.P. Gratton, T.J. McCabe, J. Fontana, Y. Fujio, K. Walsh, T.F. Franke, A. Papapetropoulos, and W.C. Sessa, Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 399 (1999) 597-601.
[66] J. Shearer, P.T. Fueger, B. Vorndick, D.P. Bracy, J.N. Rottman, J.A. Clanton, and D.H. Wasserman, AMP kinase-induced skeletal muscle glucose but not long-chain fatty acid uptake is dependent on nitric oxide. Diabetes 53 (2004) 1429-35.
[67] L.G. Fryer, E. Hajduch, F. Rencurel, I.P. Salt, H.S. Hundal, D.G. Hardie, and D. Carling, Activation of glucose transport by AMP-activated protein kinase via stimulation of nitric oxide synthase. Diabetes 49 (2000) 1978-85.
[68] Y. Benomar, N. Naour, A. Aubourg, V. Bailleux, A. Gertler, J. Djiane, M. Guerre-Millo, and M. Taouis, Insulin and leptin induce Glut4 plasma membrane translocation and glucose uptake in a human neuronal cell line by a phosphatidylinositol 3-kinase- dependent mechanism. Endocrinology 147 (2006) 2550-6.
[69] Q. Wang, R. Somwar, P.J. Bilan, Z. Liu, J. Jin, J.R. Woodgett, and A. Klip, Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. Mol Cell Biol 19 (1999) 4008-18.
[70] E. Hajduch, D.R. Alessi, B.A. Hemmings, and H.S. Hundal, Constitutive activation of protein kinase B alpha by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells. Diabetes 47 (1998) 1006-13.
[71] G. Bandyopadhyay, Y. Kanoh, M.P. Sajan, M.L. Standaert, and R.V. Farese, Effects of adenoviral gene transfer of wild-type, constitutively active, and kinase-defective protein kinase C-lambda on insulin-stimulated glucose transport in L6 myotubes. Endocrinology 141 (2000) 4120-7.
[72] G.I. Welsh, I. Hers, D.C. Berwick, G. Dell, M. Wherlock, R. Birkin, S. Leney, and J.M. Tavare, Role of protein kinase B in insulin-regulated glucose uptake. Biochem Soc Trans 33 (2005) 346-9.
[73] R.T. Watson, and J.E. Pessin, Bridging the GAP between insulin signaling and GLUT4 translocation. Trends Biochem Sci 31 (2006) 215-22.

[74] D. Sbrissa, O. Ikonomov, and A. Shisheva, Selective insulin-induced activation of class I(A) phosphoinositide 3-kinase in PIKfyve immune complexes from 3T3-L1 adipocytes. Mol Cell Endocrinol 181 (2001) 35-46.
[75] D.C. Berwick, G.C. Dell, G.I. Welsh, K.J. Heesom, I. Hers, L.M. Fletcher, F.T. Cooke, and J.M. Tavare, Protein kinase B phosphorylation of PIKfyve regulates the trafficking of GLUT4 vesicles. J Cell Sci 117 (2004) 5985-93.
[76] O.C. Ikonomov, D. Sbrissa, K. Mlak, M. Kanzaki, J. Pessin, and A. Shisheva, Functional dissection of lipid and protein kinase signals of PIKfyve reveals the role of PtdIns 3,5-P2 production for endomembrane integrity. J Biol Chem 277 (2002) 9206-11.
[77] C. Pendaries, H. Tronchere, L. Arbibe, J. Mounier, O. Gozani, L. Cantley, M.J. Fry, F. Gaits-Iacovoni, P.J. Sansonetti, and B. Payrastre, PtdIns5P activates the host cell PI3-kinase/Akt pathway during Shigella flexneri infection. Embo J 25 (2006) 1024-34.
[78] A.M. Kong, K.A. Horan, A. Sriratana, C.G. Bailey, L.J. Collyer, H.H. Nandurkar, A. Shisheva, M.J. Layton, J.E. Rasko, T. Rowe, and C.A. Mitchell, Phosphatidylinositol 3-phosphate [PtdIns3P] is generated at the plasma membrane by an inositol polyphosphate 5-phosphatase: endogenous PtdIns3P can promote GLUT4 translocation to the plasma membrane. Mol Cell Biol 26 (2006) 6065-81.
[79] G. Odorizzi, M. Babst, and S.D. Emr, Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. Cell 95 (1998) 847-58.
[80] J.L. Burman, S. Wasiak, B. Ritter, E. de Heuvel, and P.S. McPherson, Aftiphilin is a component of the clathrin machinery in neurons. FEBS Lett 579 (2005) 2177-84.
[81] P.S. McPherson, A.J. Czernik, T.J. Chilcote, F. Onofri, F. Benfenati, P. Greengard, J. Schlessinger, and P. De Camilli, Interaction of Grb2 via its Src homology 3 domains with synaptic proteins including synapsin I. Proc Natl Acad Sci U S A 91 (1994) 6486-90.
[82] B. Ritter, A.Y. Denisov, J. Philie, C. Deprez, E.C. Tung, K. Gehring, and P.S. McPherson, Two WXXF-based motifs in NECAPs define the specificity of accessory protein binding to AP-1 and AP-2. Embo J 23 (2004) 3701-10.
[83] E. de Heuvel, A.W. Bell, A.R. Ramjaun, K. Wong, W.S. Sossin, and P.S. McPherson, Identification of the major synaptojanin-binding proteins in brain. J Biol Chem 272 (1997) 8710-6.
[84] S. Wasiak, V. Legendre-Guillemin, R. Puertollano, F. Blondeau, M. Girard, E. de Heuvel, D. Boismenu, A.W. Bell, J.S. Bonifacino, and P.S. McPherson, Enthoprotin: a novel clathrin-associated protein identified through subcellular proteomics. J Cell Biol 158 (2002) 855-62.
[85] F. Blondeau, B. Ritter, P.D. Allaire, S. Wasiak, M. Girard, N.K. Hussain, A. Angers, V. Legendre-Guillemin, L. Roy, D. Boismenu, R.E. Kearney, A.W. Bell, J.J. Bergeron, and P.S. McPherson, Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling. Proc Natl Acad Sci U S A 101 (2004) 3833-8.
[86] B. Ritter, J. Philie, M. Girard, E.C. Tung, F. Blondeau, and P.S. McPherson, Identification of a family of endocytic proteins that define a new alpha-adaptin ear-binding motif. EMBO Rep 4 (2003) 1089-95.

[87] P. Manivet, S. Mouillet-Richard, J. Callebert, C.G. Nebigil, L. Maroteaux, S. Hosoda, O. Kellermann, and J.M. Launay, PDZ-dependent activation of nitric-oxide synthases by the serotonin 2B receptor. J Biol Chem 275 (2000) 9324-31.
[88] T.W. Balon, and J.L. Nadler, Nitric oxide mediates skeletal glucose transport. Am J Physiol 270 (1996) E1058-9.

Appendix

Research Compliance Certificates:

- Animal Care Procotol found attached