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Pcp4l1 contains an auto-inhibitory element that prevents its IQ motif from binding to calmodulin

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Abstract

Purkinje cell protein 4-like 1 (Pcp4l1) is a small neuronal IQ motif protein closely related to the calmodulin binding protein Pcp4/PEP-19. PEP-19 interacts with calmodulin via its IQ motif to inhibit calmodulin-dependent enzymes and we hypothesized Pcp4l1 would have similar properties. Surprisingly, full length Pcp4l1 does not interact with calmodulin in yeast two-hybrid or pulldown experiments yet a synthetic peptide constituting only the IQ motif of Pcp4l1 binds calmodulin and inhibits calmodulin-dependent kinase II. A nine-residue glutamic acid rich sequence in Pcp4l1 confers these unexpected properties. This element lies outside the IQ motif and its deletion or exchange with the homologous region of PEP-19 restores calmodulin binding. Conversion of a single isoleucine (Ile36) within this motif to phenylalanine, the residue present in PEP-19, imparts calmodulin binding onto Pcp4l1. Moreover, only aromatic amino acid substitutions at position 36 in Pcp4l1 allow binding. Thus, despite their sequence similarities PEP-19 and Pcp4l1 have distinct properties with the latter harboring an element that can functionally suppress an IQ motif. We speculate Pcp4l1 may be a latent calmodulin inhibitor regulated by post-translational modification and/or co-factor interactions.

Keywords

Calmodulin; Calcium; IQ motif; Pcp4l1; PEP-19

Introduction

Pcp4l1 is a member of the calpacitin family of IQ motif proteins that comprises RC3/neurogranin, Pcp4/PEP-19, GAP-43/neuromodulin, Igloo and SP17 (Gerendasy et al., 1999; Bulfone et al., 2004). Calpacitin IQ motifs interact with the ubiquitous EF-hand calcium sensor calmodulin (CaM) and modulate its calcium binding kinetics (Putkey et al., 2003). Moreover, the IQ motifs of PEP-19 and neurogranin act as inhibitors of the CaM-dependent enzymes neuronal nitric oxide synthase (nNOS) and calmodulin-dependent kinase II (CaMKII) (Slemmon et al., 1996; Johanson et al., 2000). PEP-19, Pcp4l1 and neurogranin are strikingly small proteins (62, 68 and 78 amino acids, respectively) with the IQ motif constituting a large proportion of their sequence, suggesting their primary function may be to regulate calmodulin-dependent processes.

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Several calpacitin proteins including GAP-43, neurogranin, PEP-19 and Pcp4l1 are highly expressed in the central nervous system and genetic studies in mice have established their importance in mammalian neurobiology. GAP-43 knockout mice have disrupted barrel cortex patterning (Maier et al., 1999), whereas mice deficient for neurogranin have altered synaptic plasticity in the hippocampus accompanied by spatial learning defects (Pak et al., 2000). We recently reported that *Pcp4/Pep-19*-null mice display long-term potentiation in place of long-term depression at parallel fiber-Purkinje neuron synapses and have locomotor learning deficits (Wei et al., 2011), a phenotype closely resembling CaMKII knockout mice (Hansel et al., 2006; van Woerden et al., 2009). Understanding the molecular mechanisms underlying these synaptic defects is of particular interest as *Pcp4/Pep-19* resides within the Down syndrome critical region (Hubert and Korenberg, 1997) and may play a role in the neuropathology of the disease (Mouton-Liger et al., 2011; Wei et al., 2011).

While calpacitins act as inhibitors of CaM-dependent enzymes *in vitro* the physiological situation *in vivo* is more complex. Genetic ablation of neurogranin in mice causes decreased basal CaMKII activation (Pak et al., 2000) and conversely *in vivo* overexpression of neurogranin results in increased levels of activated CaMKII (Zhong et al., 2009). Intriguingly, neurogranin may localize CaM to the plasma membrane of dendritic spines where it is poised to respond to changes in Ca2+ (Zhong et al., 2009). Similarly, transgenic overexpression of PEP-19 also leads to increased CaMKII activation (Mouton-Liger et al., 2011). The cellular and molecular basis of these differences is an area of active investigation.

Calpacitins bind preferentially to Ca2+-free CaM (apoCaM) and interact poorly with calcium-bound CaM (Liu and Storm, 1990; Baudier et al., 1991; Bahler and Rhoads, 2002; Kleerekoper and Putkey, 2009; Zhong et al., 2009). Conserved serine residues within the IQ motifs of neurogranin, neuromodulin and PEP-19 are protein kinase C (PKC) substrates and their phosphorylation inhibits CaM binding (Alexander et al., 1987; Baudier et al., 1991; Huang et al., 1993; Dickerson et al., 2006). The phosphorylation state of these IQ motif proteins is controlled by the dynamic balance of phosphorylation by PKC and dephosphorylation by calcineurin and protein phosphatases 1 and 2A, thus coupling calpacitin-CaM interactions to intracellular calcium levels (Liu and Storm, 1989; Apel et al., 1990; Seki et al., 1995; Gerendasy and Sutcliffe, 1997; Huang et al., 2004; Xia and Storm, 2005). Intriguingly, Pcp4l1, while highly homologous to PEP-19 in its IQ motif, lacks a phospho-acceptor amino acid in the appropriate position, suggesting unique mechanisms may regulate its CaM binding properties.

The developmental expression pattern of *Pcp4l1* suggests it performs an important function in the mammalian central nervous system (Bulfone et al., 2004). During early brain development *Pcp4l1* is localized to the midbrain-hindbrain boundary in a pattern that partially overlaps with *Wnt1*, *Pax2* and *Fgf8* expression domains (Bulfone et al., 2004). In the adult brain *Pcp4l1* is detected in the choroid plexus, olfactory bulb and the striatum where it marks medium spiny neurons (Bulfone et al., 2004; Arlotta et al., 2008). However, to date, no studies have addressed the biochemical properties of Pcp4l1. Here we used yeast two-hybrid (Y2H) to compare the binding of PEP-19, neurogranin and Pcp4l1 to a panel of EF hand proteins. PEP-19 and neurogranin interact specifically with CaM, but surprisingly Pcp4l1 did not bind any of the EF-hand proteins. However, using peptide binding competition and enzyme inhibition assays we demonstrate the Pcp4l1 IQ motif is competent to bind CaM with properties similar to the PEP-19 IQ motif. Inhibition of CaM binding is mediated by a glutamate rich sequence upstream of the IQ motif. Critically, when an isoleucine in this sequence is substituted with phenylalanine, Pcp4l1 can bind CaM. These results suggest Pcp4l1 may function as a latent CaM binding protein that becomes activated by post-translational modification or additional protein interactions.

MATERIALS AND METHODS

DNA constructs

Expression constructs were PCR amplified from mouse brain cDNA (Platinum Pfx DNA polymerase, Invitrogen, Carlsbad, CA, USA). For Y2H constructs, PCR products were digested with EcoRI-SpeI or NotI-SpeI as indicated and cloned into the pSD10a (VP16 fusion) and Y.LexA (LexA fusion) galactose inducible expression plasmids (Dalton and Triesman, 1992). Site directed mutagenesis was performed using standard PCR based methods (Sambrook and Russell, 2001). Primers used to clone Y2H constructs are as follows (restriction endonuclease sites used for cloning are underlined):

Pep-19 For: GTCAGAATTCAGTGAGAGACAAAGTGCTGGAGCG

Pep-19 Rev: GTCAACTAGTCTAGGACTGTGATCCTGCCTTTTTC

Pep-19-ΔIQ Rev: GTCAACTAGTCTATGCACGCTCTGTCTCTGGTG

Pcp4l1 For: GTCAGAATTCAGCGAGCTTAACACCAAAACAC

Pcp4l1 Rev: GTCAACTAGTTCAGGAGCTGGAATCCTTTTTCCTC

Nrgn For: GTCAGAATTCGACTGCTGCACGGAGAGCGCCTGCTCC

Nrgn Rev: GTCAACTAGTCTAGTCTCCGCTGGGGCCGCCGCC

CaM For: GTCAGCGGCCGCGGCTGACCAACTGACTGAAGAGCAG

CaM Rev: GTCAACTAGTCTACTTCGCTGTCATCATTTGTAC

L/S-CaBP-1 For: GTCAGAATTCGGCAACTGCGTCAAGTCGCCACTAAG

L/S-CaBP-1 Rev: GTCAACTAGTTCAGCGAGACATCATCCGGACAAAC

CnB For: GTCAGCGGCCGCGGGAAATGAGGCGAGTTACCCTTTG

CnB Rev: GTCAACTAGTTCACACATCCACCACCATCTTTTTG

Cetn2 For: GTCAGAATTCGCCTCTAATTTTAAGAAGACAACC

Cetn2 Rev: GTCAACTAGTTTAATAGAGGCTGGTCTTTTTCATG

Calml4 For: GTCAGAATTCGCCAAGTTCCTTTCCCAGGAC

Calml4 Rev: GTCAACTAGTTTAGTAGTCTCGCACAGGAATGG

Caln1 For: GTCAGCGGCCGCGCCGTTCCACCATGTAACCGCTG

Caln1 Rev: GTCAACTAGTCTACTCCATGCCGCTCCGCAGGATC

Pcp4l1-Δ11 For: GATCGAATTCGCCAACCAGGCATCTGACCCTGAG

Pcp4l1-Δ21 For: GATCGAATTCGGGAAGCCTGGCAGCATCAAGAAG

Pcp4l1-Δ29 For: GATCGAATTCGCCGAGGAGGAGGAAGAAATTGAC

Pcp4l1-Δ36 For: GATCGAATTCGACATTGACCTGACAGCGCCAGAGACAG

Pcp4l1 IQ For: GATCGATATCGACCTGACAGCGCCAGAGACAGAG

Pcp4l1 (KKVQEEF) Rev:

GATCGATATCAAATTCTTCTTGGACCTTCTTGATGCTGCCAGGCTTCC

Pcp4l1 I36X For: GAAGAAXXXGACATTGACCTGACAGCGCG

Pcp4l1 I36X Rev: GGCGCTGTCAGGTCAATGTCXXXTTCTTC

Mammalian expression constructs were cloned into SalI-BglII sites of the pCMV-Myc vector (Clontech, Madison, WI, USA). Primers used to amplify coding sequences are as follows (restriction endonuclease sites used for cloning are underlined):

Pep-19 For: GATCGTCGACCAGTGAGAGACAAAGTGCCGGAGC

Pep-19 Rev: GATCAGATCTCTAGGACTGTGATCCTGCCTTTTTC

Nrgn For: GATCGTCGACCGACTGCTGCACGGAGAGCGCCTG

Nrgn Rev: GATCAGATCTCTAGTCTCCGCTGGGGCCGCC

Pcp4l1 For: GATCGTCGACCAGCGAGCTTAACACCAAAACACC

Pcp4l1 Rev: GATCAGATCTTCAGGAGCTGGAATCCTTTTTCCTC

Yeast-two-hybrid (Y2H)

Y2H was performed as described (Pang et al., 2000; Bao et al., 2005; Dalton and Triesman, 1992). Briefly, S260 yeast were co-transformed with LexA and VP16 fusion constructs and plated onto glucose selective plates lacking tryptophan and uracil (Trp-, Ura-). Colonies were streaked onto Hybond-N filters (GE Healthcare, Amersham, UK), switched to galactose (Trp-, Ura-) to induce expression and assayed for lacZ activity using 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal). Constructs were tested for autoactivation of the *lacZ* reporter by transformation with LexA and VP16 control vectors. Constructs were tested as both LexA and VP16 fusions unless otherwise stated. Colonies that developed blue color within 1 hour of addition of X-gal were designated positive interactions. Colonies that became blue between 2-4 hours were designated weak interactions.

COS-7 transfection and CaM Sepharose pulldown

The procedure to assess calpacitin binding to CaM followed published protocols (Li and Sacks, 2003; Zhong et al., 2009). African green monkey kidney COS-7 cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Invitrogen) in 5% CO_2 at 37°C. Cells were transfected in 6cm plates at 80% confluency with 3μg of plasmid using 15μl Fugene 6 reagent (Roche, Indianapolis, IN, USA). 48 hours post-transfection, cells were lysed by incubation in TX-buffer (50 mM Tris-HCl, pH 6.9, 0.5% Triton X-100, 1x Roche Complete Protease Inhibitor Cocktail) for 30 minutes at 4°C. Lysates were cleared by centrifugation at 20,000xg for 15 minutes at 4°C. 300μl cleared lysate was diluted 1:1 with TX-buffer containing 10mM $CaCl_2$ or 10mM EGTA. Diluted lysate was then incubated with 100μl calmodulin-Sepharose (GE Healthcare) overnight at 4°C on a rotator. Beads were washed 3 times with 1ml of TX-buffer containing 5mM $CaCl_2$ or 5mM EGTA, re-suspended in 100μl 2xSDS sample buffer and heated to 95°C for 10 minutes. Samples (10μl) were analyzed by SDS-PAGE and western blotting.

SDS-PAGE and western blotting

Protein samples were separated by SDS-PAGE through 4-20% Tris-Tricine gels (Bio-Rad, Hercules, CA, USA). Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) by wet-tank transfer in 10 mM CAPS, pH 11.0, 20% methanol. Blots were blocked for 1 hour in PBSTM (10mM sodium phosphate, pH 7.4, 150mM sodium chloride, 0.1% Tween, 5% non-fat dry milk), and then incubated overnight at 4°C with anti-Myc (0.8 g/ml; clone 9E10; Roche) diluted in PBSTM. Blots were washed with PBST (PBS, 0.1% Tween) and then incubated with anti-mouse IgG-horseradish peroxidase (1:10,000; Roche) diluted in PBSTM at room temperature for 1 hour. Protein bands were visualized using the ECL Plus reagent (GE Healthcare).

Synthetic peptides

PEP-19 (APETERAAVAIQSQFRKFQKKKAGS) and Pcp4l1 IQ (APETEKAALAIQGKFRRFQKRKKDS) peptides were synthesized and purified by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital.

CaMKII inhibition assay

CaMKII inhibition was measured using a CaM Kinase II assay kit (Millipore/Upstate; cat# 17-135) following the manufacturer's protocol, except that various concentrations of synthetic PEP-19 or Pcp4L1 IQ motif peptides were included in the reactions. Briefly, triplicate reactions containing 10μCi [γ-^{32}P]ATP (Perkin Elmer, Waltham, MA, USA), 100 M synthetic substrate peptide (Autocamtide 2: KKALRRQETVDAL), and 25ng purified CaMKII (Millipore; cat# 14-723) as well as PEP-19 or Pcp4L1 IQ motif peptides were incubated at 30°C for 10 minutes. Reactions were terminated by spotting onto P81 phosphocellulose paper, followed by washing three times with 0.75% phosphoric acid and once with acetone. Phosphorylation of the substrate peptide was measured by scintillation spectrometry. To determine background binding to P81 paper, control reactions were performed containing all components except CaMKII.

RESULTS

Full length Pcp4l1 does not interact with calmodulin in yeast-two-hybrid or pulldown experiments

The small IQ motif proteins PEP-19 and neurogranin bind the EF-hand calcium sensor CaM (Baudier et al., 1991; Slemmon et al., 1996). However, interaction between PEP-19 and other EF-hand proteins has not been reported. We initially performed yeast-two-hybrid (Y2H) analysis to test for interaction of PEP-19, neurogranin and Pcp4l1 with a panel of EF-hand proteins selected from brain microarray data (Rong et al., 2004). PEP-19 and neurogranin (but not a mutant PEP-19 lacking its IQ motif) gave a strong positive signal when co-expressed with CaM but not the other EF-hand proteins (Fig. 1A). Surprisingly, despite high sequence conservation with PEP-19, Pcp4l1 did not interact with any EF-hand protein, including CaM (Fig. 1A).

CaM-Sepharose pulldown assays have been used to demonstrate differential binding of IQ motif proteins, such as neurogranin, under high and low calcium conditions (Zhong et al., 2009; Zhong et. 2011; Kalek et al., 2012). To validate our Y2H results, PEP-19, Pcp4l1 and neurogranin were expressed in mammalian cells and subjected to CaM-Sepharose pulldown. Consistent with previous studies, PEP-19 (Slemmon et al., 1996) and neurogranin (Gerendasy, et al., 1994; Zhong et al., 2009) bind CaM in low calcium conditions but associate weakly in high calcium (Fig. 1B). Pcp4l1, by contrast, interacts poorly with CaM in both low and high calcium conditions (Fig. 1B).

The Pcp4l1 IQ motif is competent to bind calmodulin and inhibits CaMKII

PEP-19 and Pcp4l1 are most similar in their C-terminal IQ motifs, but contain unique N-terminal regions (Bulfone et al., 2004; Dickerson et al., 2006) (Fig. 2A, 3A). Therefore, sequence differences outside the IQ motif could contribute to the distinct characteristics observed in Y2H and CaM-Sepharose pulldown. Indeed, short synthetic peptides constituting the PEP-19 or Pcp4l1 IQ motifs but lacking N-terminal sequences behave similarly in two independent CaM binding assays. PEP-19 and Pcp4l1 IQ peptides compete with full-length PEP-19 binding to CaM-Sepharose in a dose-dependent manner (Fig. 2B, C). PEP-19 and neurogranin inhibit the CaM-dependent enzymes nNOS and CaMKII (Martzen and Slemmon, 1995; Slemmon et al., 1996; Johanson et al., 2000). We found the Pcp4l1 IQ peptide also inhibits CaMKII activity in a manner comparable to the PEP-19 IQ peptide (Fig. 2D). Thus, the Pcp4l1 IQ motif is competent to bind CaM and acts as a camstatin (Slemmon et al., 1996).

An inhibitory motif within Pcp4l1 suppresses binding to CaM

The foregoing experiments indicate that the Pcp4l1 IQ motif binds CaM, whereas the full-length protein cannot, implying the N-terminal region contains inhibitory sequences. Serial N-terminal truncations of Pcp4l1 were tested for CaM binding in Y2H (Fig. 3B). Deletion of 36 amino acids (Δ36) including a lysine/glutamic acid rich (KE) motif from Pcp4l1 restores CaM binding (Fig. 3B). Informatively, whereas Δ36 binds CaM Δ29 does not, suggesting that critical sequences reside within the KE motif (Fig. 3B). Domain swap experiments were performed to test if CaM binding could be imparted by replacing the entire N-terminal region or just the inhibitory KE motif sequences in Pcp4l1 with the equivalent sequences from PEP-19. Exchanging the N-terminal region of Pcp4l1 with that of PEP-19 restores CaM binding in Y2H and CaM-Sepharose pulldown assays (Fig. 3C, D). Moreover, specifically replacing the Pcp4l1 KE motif (KKAEEEEEI) with the PEP-19 KE motif (KKVQEEF) confers CaM binding properties onto Pcp4l1 (Fig. 3C, D). Thus, despite the KE motif being well outside of the IQ domain it is capable of suppressing CaM binding.

The foregoing domain swap studies establish that the KE motif from PEP-19 and Pcp4l1 can confer gain or loss of CaM binding function, respectively. This afforded an experimental platform to perform further mutation studies using hybrid proteins to define critical amino acids that either support or inhibit CaM binding. A notable distinction between the KE motifs of Pcp4l1 and PEP-19 is the presence of five glutamate residues in the former compared to two in the latter (Fig. 4A). Therefore, we mutated the three additional glutamate residues in Pcp4l1 either singly or in combination to alanine or the potential homologous residues in PEP-19 (Fig 4A). However, none of the mutations alleviated the inhibitory potential of the Pcp4l1 KE motif, indicating these three glutamate residues are not sufficient to suppress binding in the context of PEP-19 (Fig. 4A). We next analyzed mutations of the PEP-19 KE in the context of a domain swap into Pcp4l1. Remarkably, changing phenylalanine 34 of PEP-19 to isoleucine (the homologous residue in Pcp4l1) or alanine attenuates or eliminates CaM binding, respectively (Fig. 4B). This implicates the corresponding Pcp4l1 isoleucine (Ile36) as a potential modifier of CaM binding. Therefore, we tested multiple mutants of Ile36 in the context of full length Pcp4l1. Substitution with phenylalanine, tryptophan and to a lesser extent tyrosine converted Pcp4l1 into a CaM binding protein (Fig. 4C).

DISCUSSION

Despite their high sequence homology, full length PEP-19 and Pcp4l1 have disparate interactions with CaM. PEP-19 binds preferentially to calcium-free CaM, whereas Pcp4l1 exhibits little or no binding in either calcium poor or replete conditions. By contrast,

synthetic peptides that contain the minimal IQ motifs of PEP-19 or Pcp4l1 have indistinguishable CaM binding properties, indicating that amino acids beyond the IQ motif must impair CaM binding in Pcp4l1. A nine amino acid region in Pcp4l1 confers this property. Elimination of these residues, or their exchange with the homologous amino acids from PEP-19 restores CaM binding.

A single isoleucine residue (Ile36) in the inhibitory region of Pcp4l1 is essential to mediate the inhibitory effect on CaM binding as its mutation to the homologous residue in PEP-19 (phenylalanine) restores CaM binding. The importance of Ile36 is further underscored by the findings that when the permissive PEP-19 KE motif is inserted into Pcp4l1 mutation of phenylalanine to isoleucine or alanine, inhibits or eliminates binding respectively. We also investigated the specificity of the requirement for isoleucine at position 36 in Pcp4l1. Of the amino acids tested phenylalanine, tryptophan and to lesser degree tyrosine rescue CaM binding when substituted for Ile36 in Pcp4l1. Thus, the presence of an aromatic amino acid at position 36 in Pcp4l1 restores CaM binding.

Although Pcp4l1 Ile36 plays an essential role in mediating the inhibitory effect on CaM binding, substituting the homologous Phe30 in the PEP-19 KE motif with an isoleucine only leads to a decrease in CaM binding. This implies that other residues within the KE region of Pcp4l1 must contribute to the inhibition of CaM binding, at least in the context of hybrid proteins with PEP-19. Additionally, neurogranin, a strong CaM binder in our assays, contains a leucine at the homologous position, however this substitution is insufficient to permit binding in the context of Pcp4l1. These results also imply that Pcp4l1 likely exhibits structural features not evident in PEP-19.

Like neurogranin (Cui et al., 2003; Ran et al., 2003) structural analysis of PEP-19 by NMR and circular dichroism reveals that it is disordered in solution although it has a propensity to helix formation (Dickerson, 2004; Dickerson et al., 2006; Kleerekoper and Putkey, 2009). At this time there are no solved structures of PEP-19 bound to CaM. However, NMR structural information relevant to our findings has appeared (Dickerson, 2004; Kleerekoper and Putkey, 2009). In a ^{1}H-^{15}N heteronuclear nuclear Overhauser effect (NOE) study, the Apo-CaM interacting region in PEP-19 was shown to reside between residues 30 and 59 (Dickerson, 2004). In two heteronuclear single-quantum correlation (HSQC) spectroscopy studies, the interaction regions between PEP-19 and Apo-CaM minimally spanned residues 30-59 (Dickerson, 2004) and 25-61 (Kleerekoper and Putkey, 2009). An acidic region in PEP-19 (EEFDIDMDAPETE) that lies adjacent to, and partially overlaps with the region identified here contributes to PEP-19 function (Putkey et al., 2008). This region is dispensable for CaM binding, but is essential for modulating the Ca2+ binding kinetics of the C-domain of CaM (Putkey et al., 2008). Thus, the C-terminal half of PEP-19, including its IQ motif acquires structure when bound to CaM. Moreover, Phe30 and the KE motif are at or near the N-terminal boundary of the CaM interacting region of PEP-19. One can speculate that unlike the other small neuronal IQ motif proteins, Pcp4l1 might have some limited structure in solution that is mediated by the KE motif and blocks its interaction with CaM. The introduction of a bulky aromatic residue may disrupt this structure, permitting the IQ motif to bind to CaM. Alternatively, Pcp4l1 may be unstructured but there may be some other factor that overcomes the lack of an aromatic amino acid at position 36 to reinstate CaM binding, otherwise it would be difficult to understand the biological relevance of a protein that has a competent IQ motif but cannot bind CaM.

We considered the possibility that CaM is not the true in vivo binding partner for Pcp4l1. CaM is the prototype of a superfamily of proteins involved in calcium sensing and signaling that are characterized by the presence of calcium-binding EF hand domains (reviewed in Burgoyne and Weiss, 2001; Burgoyne et al., 2004; Mikhaylova et al., 2011). As many of the

CaM-like proteins are enriched in the nervous system (Burgoyne et al., 2004; Mikhaylova et al., 2011) they could in principle be the targets for Pcp4l1 and potentially other IQ motif proteins. However, when we assessed this possibility with representative members of the various subfamilies of CAM-related proteins that are expressed in brain no binding was detected with Pcp4l1 (or PEP-19 and neurogranin). The proteins assessed were Cabp1 (caldendrin), Calml4 (calmodulin-like 4), Caln1 (calneuron 1), Cetn2 (centrin 2) and CnB (calcineurin subunit B). While this is not an exhaustive analysis of CaM-related proteins it is consistent with studies demonstrating that IQ motifs can specifically discriminate between EF-hand proteins (Rhoads and Friedberg, 1997).

A remaining possibility is that the inhibitory region in Pcp4l1 can be suppressed in vivo by posttranslational modification, such as phosphorylation or proteolytic cleavage, through changes in the intracellular milieu such as pH or through binding to ancillary proteins. A scenario already exists for neurogranin, neuromodulin and PEP-19 where phosphorylation of a serine residue in the core of the IQ motif by PKC leads to reduced CaM binding (Alexander et al., 1987; Baudier et al., 1991; Huang et al., 1993; Dickerson et al., 2006). Pcp4l1 has no potential phosphorylation sites within its IQ motif, an observation that originally spurred us to assess its properties. However, a potential phosphorylase kinase (PhK) phosphorylation site (R/K, X, X, S, I/V/F, R) (Pearson and Kemp, 1991; Songyang et al., 1996; Lowe et al., 1997) resides just 2 amino acids upstream of the Pcp4l1 KE motif (K, P, G, S, I, K). Intriguingly, calmodulin is a regulatory subunit of PhK and confers calcium sensitivity (Cohen et al., 1978). Moreover, PhK and PKC phosphorylate the same serines in neurogranin and GAP-43 to inhibit CaM binding (Paudel et al., 1993), whereas PEP-19 lacks a PhK consensus motif. It will be important in future studies to determine whether Pcp4l1 is a bona fide PhK substrate and if phosphorylation influences its CaM binding activities.

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Abbreviations

CaM	calmodulin
CaMKII	calmodulin-dependent kinase II
Pcp4l1	Purkinje cell protein 4-like 1
PhK	phosphorylase kinase
PKC	protein kinase C
Y2H	yeast two-hybrid

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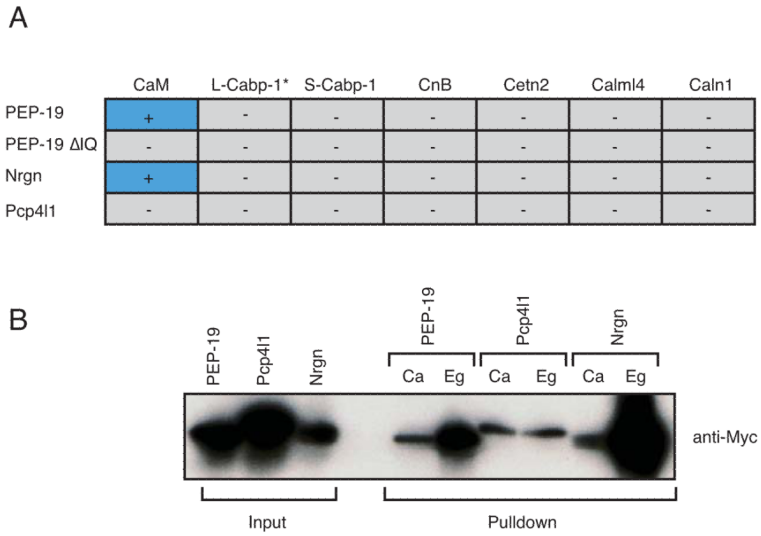

Fig. 1. Full length Pcp4l1 does not interact with calmodulin

(A) Summary of Y2H experiments. Positive (blue) and negative (grey) lacZ reporter expression is indicated. PEP-19, PEP-19 ΔIQ, neurogranin (Nrgn) and Pcp4l1 were screened against the neurally expressed EF hand proteins calmodulin (CaM), calcium binding protein-1 long isoform (L-Cabp-1), calcium binding protein-1 short isoform (S-Cabp-1), calcineurin B (CnB), centrin 2 (Cetn2), calmodulin-like 4 (Calml4) and calneuron 1 (Caln1). Constructs were screened as both LexA and VP16 fusions. *LexA-L-Cabp-1 strongly activates lacZ reporter expression when co-transformed with the VP16 control vector, making it impossible to unambiguously assess IQ motif protein interactions with this protein. The only positive interactions detected were between CaM and PEP-19 or neurogranin. (B) CaM-Sepharose pulldown of Myc-tagged PEP-19, Pcp4l1 and Nrgn performed in 5mM $CaCl_2$ (Ca) or 5mM EGTA (Eg). Both PEP-19 and neurogranin bind to CaM-Sepharose in calcium-poor (Eg) conditions whereas Pcp4l1 binds weakly or not at all to CaM under both calcium-poor (Eg) and calcium-replete (Ca) conditions. Protein input for the pulldown assays are shown at left.

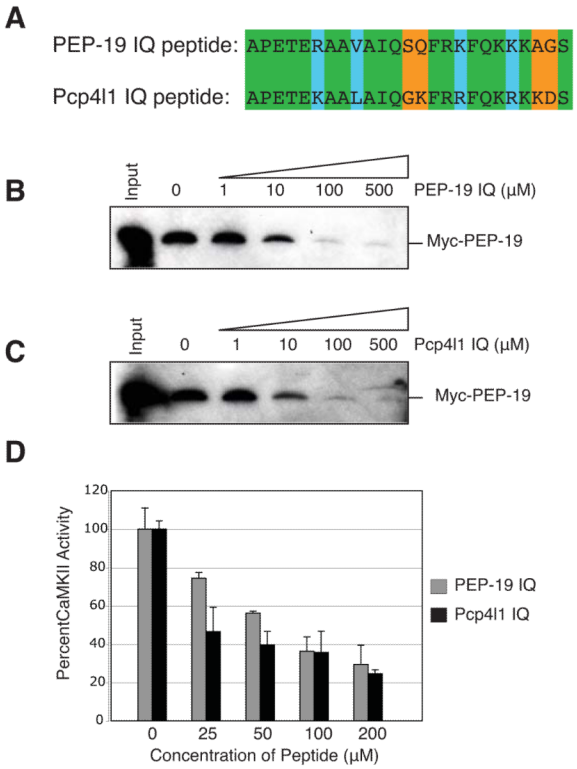

Fig. 2. The Pcp4l1 IQ motif is competent to bind calmodulin

(A) Amino acid sequence of the synthetic PEP-19 and Pcp4l1 IQ motif peptides used in panels B-D. Identical residues are highlighted in green, conservative differences in blue and non-conservative differences in orange. (B, C) Peptide-binding competition assays. CaM-Sepharose pulldown of Myc-PEP-19 performed with increasing concentrations of PEP-19 (B) or Pcp4l1 (C) IQ motif peptides. Both synthetic IQ motif peptides compete PEP-19 binding to CaM-Sepharose. Protein input for the pulldown assays is shown at left. (D) CaM-dependent kinase II (CaMKII) inhibition assay. Kinase reactions were carried out for 10 minutes in the presence of increasing concentrations of PEP-19 or Pcp4l1 IQ motif peptides. Both IQ motif peptides inhibited CaMKII activity in a dose-dependent manner. Results are the mean and SEM of 3 determinations.

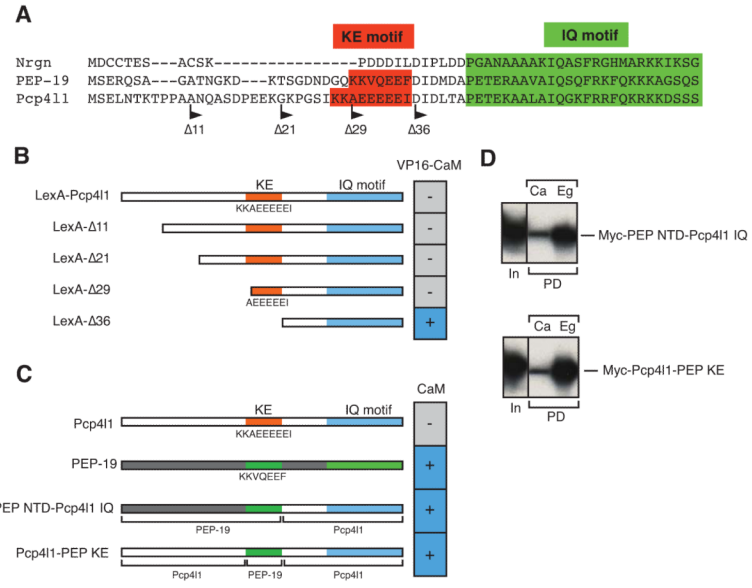

Fig. 3. An inhibitory motif in Pcp4l1 suppresses calmodulin binding

(A) Amino acid alignment of Nrgn, Pcp4l1 and PEP-19. Amino acid sequences were obtained from the National Center for Biotechnology Information (NCBI) and aligned using T-coffee (http://www.ebi.ac.uk/Tools/t-coffee/). C-terminal IQ motifs are highlighted in green and a lysine/glutamate rich (KE) motif present in PEP-19 and Pcp4l1 is highlighted in red. Black arrowheads underneath Pcp4l1 indicate the first amino acid of the truncated constructs. (B) Serial N-terminal truncations of LexA-Pcp4l1 were tested for binding with VP16-CaM in Y2H. Positive (blue) and negative (grey) lacZ reporter expression is indicated. Deletion of 36 amino acids (LexA-Δ36) in Pcp4l1 that includes the KE motif restores CaM binding. (C, D) Domain-swap experiments. (C) Summary of Y2H results for chimeric constructs tested as LexA and VP16 fusions for binding to CaM. A fusion of the N-terminal domain of PEP-19 with the Pcp4l1 IQ motif (PEP NTD-Pcp4l1 IQ) binds to CaM. Similarly replacing the Pcp4l1 KE motif with the PEP-19 KE motif (Pcp4l1-PEP KE) permits CaM binding. (D) CaM-Sepharose pulldown of chimeric constructs in high (Ca) and low (Eg) calcium conditions. Consistent with the Y2H data, Pcp4l1 harboring the KE motif of PEP-19 (Pcp4l1-PEP KE) or the IQ motif of Pcp4l1 with the N-terminal domain of PEP-19 (PEP-NTD-Pcp4l1 IQ) both bound to CaM-Sepaharose under calcium-poor conditions. Just like PEP-19 they interacted weakly or not at all with CaM-Sepharose under calcium-replete conditions. Protein input (In) is shown at left of panel.

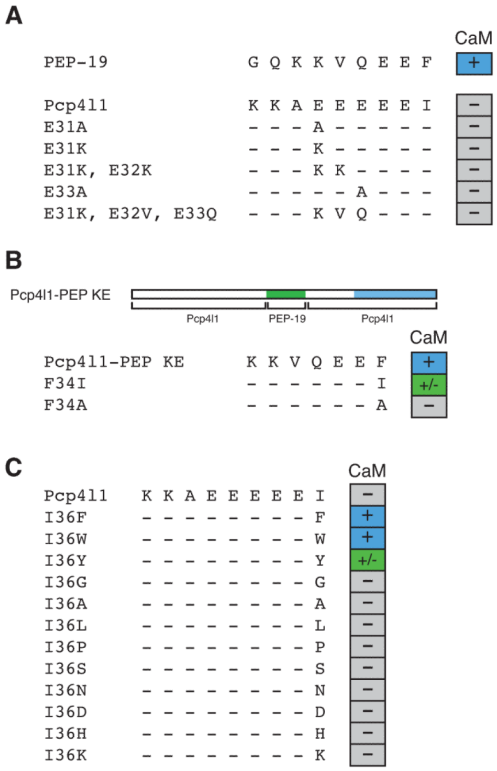

Fig. 4. Mutation analysis indicates a role for isoleucine 36 in Pcp4l1 in suppression of calmodulin binding

(A) Y2H analysis of Pcp4l1 KE motif glutamic acid mutations. Mutations of the Pcp4l1 KE motif are indicated and dashes represent non-mutated residues. The PEP-19 KE motif is shown for comparison. Multiple mutations of glutamic acid residues in Pcp4l1 fail to confer CaM binding on Pcp4l1. (B) Y2H analysis of Pcp4l1-PEP KE hybrid protein mutations. When swapped for the KE motif of Pcp4l1, the KE motif of PEP-19 confers CaM binding (blue +) to Pcp4l1. Phenylalanine 34 of the PEP-19 KE motif is important for conferring CaM binding on Pcp4l1 in the context of a KE domain swap as its mutation to isoleucine (F34I) or alanine (F34A) impairs (green +/−) or eliminates (grey −) CaM binding. (C) Summary of Y2H results for a panel of Pcp4l1 isoleucine 36 mutations. Only phenylalanine (I36F, blue +), tryptophan (I36W, blue +) and tyrosine (I36Y, green +/−) substitutions confer CaM binding on Pcp4l1.