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Novel functions of CCM1 delimit the relationship of PTB/PH domains

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Abstract

Background: Three NPXY motifs and one FERM domain in CCM1 makes it a versatile scaffold protein for tethering the signaling components together within the CCM signaling complex (CSC). The cellular role of CCM1 protein remains inadequately expounded. Both phosphotyrosine binding (PTB) and pleckstrin homology (PH) domains were recognized as structurally related but functionally distinct domains.

Methods: By utilizing molecular cloning, protein binding assays and RT-qPCR to identify novel cellular partners of CCM1 and its cellular expression patterns; by screening candidate PTB/PH proteins and subsequently structurally simulation in combining with current X-ray crystallography and NMR data to defined the essential structure of PTB/PH domain for NPXY-binding and the relationship among PTB, PH and FERM domain(s).

Results: We identified a group of 28 novel cellular partners of CCM1, all of which contain either PTB or PH domain(s), and developed a novel classification system for these PTB/PH proteins based on their relationship with different NPXY motifs of CCM1. Our results demonstrated that CCM1 has a wide spectrum of binding to different PTB/PH proteins and perpetuates their specificity to interact with certain PTB/PH domains through selective combination of three NPXY motifs. We also demonstrated that CCM1 can be assembled into oligomers through intermolecular interaction between its F3 lobe in FERM domain and one of the three NPXY motifs. Despite being embedded in FERM domain as F3 lobe, F3 module acts as a fully functional PH domain to interact with NPXY motif. The most salient feature of the study was that both PTB and PH domains are structurally and functionally comparable, suggesting that PTB domain is likely evolved from PH domain with polymorphic structural additions at its N-terminus.

Conclusions: A new β 1A-strand of the PTB domain was discovered and new minimum structural requirement of PTB/PH domain for NPXY motif-binding was determined. Based on our data, a novel theory of structure, function and relationship of PTB, PH and FERM domains has

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbapap.2017.07.002>.

Transparency document

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been proposed, which extends the importance of the NPXY-PTB/PH interaction on the CSC signaling and/or other cell receptors with great potential pointing to new therapeutic strategies.

General significance: The study provides new insight into the structural characteristics of PTB/PH domains, essential structural elements of PTB/PH domain required for NPXY motif-binding, and function and relationship among PTB, PH and FERM domains.

Keywords

CCM1; CSC; PTB and PH domains; FERM domain; NPXY motifs; Protein folding; Tertiary structure

1. Introduction

Cerebral cavernous malformations (CCMs) are characterized by distended intracranial capillary cavities amidst a thin layer of endothelial cells (EC) that lack support of intervening parenchyma and predispose to intracranial hemorrhage [1,2]. Till date, the mutation in the three CCM genes, including *KRIT1* (*CCM1*) [3–7]; *MGC4607/Malcavernin* (*CCM2*) [7–9]; and *PDCD10* (*CCM3*) [7,10] are identified as the main causes of CCMs. At least one of these genes is disrupted in most CCM cases in humans [2]. The three CCM proteins interact to form a core complex [11–15] which further interacts with other proteins [16–18] to form a complex referred to here as the CCM signaling complex (CSC).

Within the CSC, the first cellular partner of CCM1 was identified as integrin cytoplasmic domain associated protein-1-alpha (ICAP1a), suggesting a role in mediating cellular/extracellular matrix (ECM) signaling [11,16,18,19]. CCM1, as a key component of the CSC [11,15,16,20,21], influences the angiogenic activity of vascular ECs by regulating β 1-integrin-mediated signaling cascades [20]. An enlarged heart [22], dilated axial primitive vessels [23,24] and intra- and pericardiac blood stasis are all different consequences of obstructed microvasculature [25].

Despite scrupulous investigation, the detailed molecular role of CCM1 within the CSC during angiogenesis remains elusive. We have previously reported that there are three distinct NPXY motifs in CCM1, the first NPXY motif (*Residues 191–194*) of CCM1 specifically binds to ICAP1a [16], while the second and third NPXY motifs (*Residues 231–34 and 250–53*, respectively) of CCM1 are critically important for the specific interaction with CCM2 [15]. Although there have been some reports indicating that a single modular phosphotyrosine-binding (PTB) domain can recognize a diverse array of NPXY motifs [26,27], we noticed that the different NPXY motifs within the CCM1 protein interact specifically with different PTB domains based upon position and/or composition of flanking sequences. We hypothesized that each of the three NPXY motifs of CCM1 may have a unique spectrum of interaction with PTB domains. To validate this hypothesis, we identified 28 candidate gene products that contain PTB/PH domain(s) and explored the possibility of them being the new members in the CSC (Suppl. Tables 1, 2).

PTB domain is the second largest family of “phosphotyrosine-binding” modules. Understanding PTB function is fundamental to define the pivotal cellular signaling

processes. Pleckstrin homology (PH) domain is a structurally conserved and membrane-binding module. Structural studies suggested that PTB domain belongs to PH superfold family in which proteins with or without some degree of sequence homology but share similar tertiary folding [28–30]. A common PH core fold consists of a partially open β barrel with two nearly orthogonal antiparallel β sheets of 4 and 3 β strands capped by a C-terminal α helix [29,30] while PTB domain contains a relatively closed β barrel with two similar orthogonal antiparallel β sheets but capped by α helix at both ends [28]. In a recent mutation screening study, two mutations potentially associated with CCM2 phenotypes were found inside core fold of CCM2 PTB domain [7]. It was predicted by structural computation that one missense mutation located in C-terminal α helix (cys194gly) could cause the misfolding of PTB domain, while another mutation in the first β sheet (val120ile) could alter the intramolecular hydrogen bonds inside core fold, indicating the importance of PTB domain in the pathogenesis of CCM [7]. We identified a group of 28 novel cellular partners of CCM1, all containing PTB or PH domains, by screening candidate proteins and subsequently defined the relationship between different NPXY motifs of CCM1 and PTB/PH domain(s). Our results defined new partnerships of PTB/PH domains with NPXY motifs, and established a novel definition of PTB, PH and FERM (protein 4.1, ezrin, radixin, moesin) domains.

2. Materials and methods

2.1. Plasmid construction

Candidate PTB/PH domains and full-length PTB-containing proteins (Suppl. Table 1) accompanied by full-length CCM1 (FLHK) and NPXY-containing fragments of CCM1, K2 (Residues 1–207), K5 (Residues 208–245), K8 (Residues 240–306) and K12 (Residues 208–306), were amplified with Platinum Pfx50 DNA Polymerase (Invitrogen) [15]. For yeast two-hybrid analysis, both GAL4 binding domain and activation domain fusion constructs were assembled by cloning amplified fragments into pGBKT7 and pGADT7 vectors, respectively (BD Clontech) [15,16]. For in vitro protein binding assays, PTB domain and NPXY motif fusion constructs were assembled by cloning amplified fragments into pcDNA3.1/V5/HIS-TOPO and pcDNA4/HisMax-TOPO vectors (Invitrogen) for mammalian, and pGEX-4 T-1 (GE), pBAD-TOPO (Invitrogen) and pCOLDII (TaKaRa) vectors for bacterial expression systems.

2.2. Yeast two-hybrid analysis and in vitro co-immunoprecipitation

Yeast two-hybrid analysis was performed with a standard liquid phase β -galactosidase assay using *o*-nitrophenyl-D-galactoside (ONPG) (BD Clontech). β -galactosidase activity identifies the degree of interaction between pGBKT7 and pGADT7 constructs calculated as Miller units. Binding of the NPXY motif- PTB domain was determined by a β -galactosidase liquid assay in selective (–His/–Ade) medium. To minimize intergroup measurement variation, the measurement of each transformant was normalized and converted to relative β -galactosidase activity (RBGA = Miller units of each transformant/max [Miller units of controls in the same group]). Results for each experiment reflect the performance of three independent assays for each of three independent transformants, each of which is an average of triplicates.

2.3. Protein preparation and modeling of tertiary structures

In vitro co-immunoprecipitation (Co-IP) was performed as previously described [15,16]. The proteins were expressed in BL 21 cells for GST-tagged (GE) and HIS-tagged (TaKaRa) constructs and LMG194 cells for HIS-tagged constructs (Invitrogen) while potential target proteins were expressed with TNT® Quick Coupled Transcription/Translation and *E. coli* S30 Extract Systems (Promega) with S35-labeled methionine. The expressions were confirmed by Western blots. Standard in vitro pull-down assays were performed with MagneGST Glutathione Particles (Promega) for GST-tagged proteins and dynabeads (Invitrogen) for HIS-tagged proteins. Anti-GST and Anti-HIS antibodies (Santa Cruz) were also used in Western Blot and pull-down assays. In competition-binding assays, GST tagged PTB/PH domains were mixed with initial 100 μ L Icap1a PTB domain and increasing volumes of Icap1 a PTB domain were titrated.

Recombinantly expressed proteins were purified with chromatography, GST-tagged PTB/PH domains were purified with GSTrap HP column (GE), while HIS-tagged NPXY motifs were purified using HiTrap TALON column (GE), followed by size-exclusion column, S100-HR (GE).

The molecular modeling of tertiary structure of PTB/PH domains was established by Iterative Threading ASSEmbly Refinement (I-TASSER), a long-time top-ranked hierarchical approach listed in CASP (Critical Assessment of Techniques for Protein Structure Prediction) [31,32]. RasMol (version 2.7.5) was used for the structure visualization.

2.4. Label-free measurement of molecular interactions

The binding interaction between PTB/PH domains and NPXY motifs were quantified by three label-free and real-time detection platforms; Bio-Layer Interferometry (BLI), Surface Plasmon Resonance (SPR) and Isothermal Titration Calorimetry (ITC). BLI binding scouting experiments were carried out on Octet RED96 platform (ForteBio) at single analyte concentration (1 μ M). SPR binding experiments were performed on Biacore 3000 (GE) at 25 °C. The capture antibody goat anti- GST antibody was directly immobilized by amine coupling (EDC/NHS) at 5000 RU (Response units) on 4 flow cells of the CM5 (GE). The unoccupied sites were blocked with 1 M ethanol amine. Among four chamber cells, GST alone on flow cell 1 as blank for reference subtraction and GST-tagged PTB/PH proteins on flow-cell 2, 3 and 4 were captured. The analytes were flowed over the chip at variable concentrations. Full kinetic analyses were initiated at high concentration followed by 2 fold serial dilutions. Binding of analyte to the ligand was monitored in real time. The flow rate used for capturing the ligand is 5 μ L/min. The flow rate for kinetics analysis is 30 μ L/min. Equilibrium K_D values were determined from the observed on rate (k_a) and off rate (k_d). Chi square (χ^2) analysis was carried out to determine the accuracy of the analysis using the BIAAnalysis software. Similarly, ITC analysis were performed in ITC200 (Malvern) at 15 °C. Purified target and bait proteins were eluted in 1 $\times$ PBS buffer. ITC data fitting were performed in the Origin software.

Dynamic light scattering (DLS) provides a measure of protein hydrodynamic diameter and permits an assessment of protein-protein self-association. Protein samples in 1 $\times$ PBS were

centrifuged for 10 min at 1200 rpm to sediment any possible protein precipitate. A 10 μ L aliquot of supernatant for each sample was applied to a separate well of a Corning 384-well glass bottom plate, and 5 μ L of mineral oil was then applied on top of each sample to prevent solvent evaporation at higher temperatures. The DLS profiles at 25 °C for samples with and without mineral oil showed no differences. The sample plate was centrifuged for 15 min at 1600 rpm to remove bubbles from the sample wells. The DLS data were acquired on a DynaPro PlateReader II (Wyatt Technology Corporation, Santa Barbara, CA), with a 5 s acquisition time, 128 acquisitions per experiment, and auto-attenuation of the laser power. The data were recorded from 25 °C to 75 °C, in five-degree increments with an incubation period of 15 min in between each temperature point. The resulting autocorrelation functions were exported to DYNALS (Alongo, Ltd.) for multi-exponential analysis. Apparent melting temperatures were obtained by estimating the temperature midpoint of the coupled oligomerization and unfolding reactions.

2.5. Multiplex lipid binding assay

HPLC purified PTB domains were added to FlowPIP Buffer 1 (1 $\times$ PBS, pH 7.4, 1% BSA, 1% GS, 0.09% sodium azide) with FlowPIP Bead Mix at volume of 250 μ L, incubated at 4 °C overnight with gentle agitation; then FlowPIP Bead Mix were washed three times with 250 μ L FlowPIP Buffer 2 (1 $\times$ PBS, pH 7.4, 1% BSA, 1% GS, 0.05% Tween 20, 0.09% sodium azide), followed by the addition of Alexa Fluor 488 anti- GST IgG (*Molecular Probes*) in 250 μ L of FlowPIP Buffer 1 (final concentration of 2 μ g/mL), incubate for 1 h at RT with gentle agitation, FlowPIP Bead Mix were washed three times with 250 μ L FlowPIP Buffer 2; after the final wash, the FlowPIP Bead Mix were resolved into 300 μ L 1 $\times$ PBS buffer for binding assays on BD Accuri C6 flow cytometer according to manufacturer's instructions (Echelon Biosciences).

2.6. Quantitative real-time PCR

Real-time PCR (qPCR) assays were used to quantify the relative expression level of *CCM1* in different tissues and cell lines using Power SYBR Green Master Mix with a primer-set for CCM1 (KRIT01F1: TGCATGCTGGTATGGAAAAG/KRIT01R1:CGTTTCTGGGTGGTTTAGGA) in a ViiA-7 Real-Time PCR System (Applied Biosystems). TissueScan™ Real-Time PCR panels (HMRT100, 103, CSRT502) and human β -actin control primers (Origene) were used to determine the relative expression levels of *CCM1* in multiple human tissues. Human brain tissues and cell-line qPCR plates were prepared using an epMotion 5075 automated liquid handling systems (Eppendorf). The qPCR data were analyzed with DataAssist (ABI) and Rest 2009 software (Qiagen). The relative expression level (2- Δ CT) was calculated from all samples and normalized to reference gene (β -actin). All experiments were performed with triplicates.

2.7. Statistical analysis

For multiple comparisons in yeast two-hybrid analysis, one-way analysis of variance (ANOVA) was used to detect the differences in the mean values among the groups. All pairwise multiple comparison procedures (Tukey's *t*-test) were used to test the difference between each group. In qPCR analysis, pairwise *t*-tests were performed for all paired samples (normal/tumor) using SPSS 11.5 and graphed by Sigmaplot 12.0.

3. Results

3.1. Diverse NPXY motifs enable CCM1 to interact with multiple PTB-containing proteins

Based on our previous findings [15,16,20], we hypothesized that among three NPXY motifs of CCM1 each would possess its own uniquespectrum of interaction with PTB domains. To test this hypothesis, we examined the potential interaction of a few candidate PTB-containing proteins (Suppl. Table 1) with CCM1 NPXY motifs using yeast two-hybrid analysis. Our data indicate that selected PTB domains specifically interact with the second and third NPXY motifs of CCM1 i.e. similar to CCM2, which leads to the inference that CCM1 can potentially interact with many PTB-containing proteins via its diversified NPXY motifs (Fig. 1A). Further, compound mutant forms of the second (N231A/F234A) and third (N250A/F253A) NPXY motifs were examined for interaction with PTB domains in two hybrid system. Our data demonstrate that the compound mutation in either NPXY motif impairs its ability to bind to PTB domain (Fig. 1B).

3.2. Identification of new CCM1 cellular partners

To further corroborate these findings, we used co-immunoprecipitation (Co-IP) to screen a total of 27 different candidate PTB domains or combination (Suppl. Table 1). Among these PTB domains, many of them were found to bind either a specific NPXY motif, combination of these NPXY motifs, and/or full-length CCM1 (Fig. 2A, B). These PTB domain-NPXY motif interactions were further validated by the interaction between full-length PTB-containing proteins (Suppl. Table 2) with full-length CCM1 (Fig. 2C). This indicates that full-length CCM1 protein can potentially bind many of the PTB-containing proteins. The tertiary folds of these PTB domains were calculated by I-TASSER, a program proven to accurately predict the tertiary folds of PTB domains [33] (Suppl. Fig. 1). These structural topologies corroborated well with published X-ray crystallography [34–39] and nuclear magnetic resonance (NMR) data [26,27,39–46].

3.3. Determination of the dissociation constants for NPXY-PTB interactions

Knowledge of the kinetics of NPXY motif and PTB interactions is vital for understanding the relationship between CCM1 and PTB-containing proteins. To further quantify putative interactions between the NPXY motifs of CCM1 and various PTB domains, we measured binding affinity between NPXY motifs of CCM1 and candidate PTB domains, using three major technologies of label-free protein binding analysis; BLI, SPR and ITC analysis. Using the 1st and 3rd NPXY motifs (K2, K8) as analytes, the candidate PTB domains were screened with a high-throughput platform, BLI, followed by SPR scouting. Our preliminary screening generated ample positive binding data (Table 1), further supporting our yeast two-hybrid and Co-IP data (Figs. 1, 2, 3). Using the same probes, binding experiments were performed on both SPR and ITC platforms. The binding affinity data corroborated with our preliminary screening data (Table 1), further validating the interactions between the 1st and 3rd NPXY motifs of CCM1 and the candidate PTB domains (Figs. 1, 2, 3, Suppl. Table 1).

3.4. NPXY motifs interact with N-terminal truncated PTB domain

There are several identified mammalian Numb protein isoforms [47,48], which could be classified into two groups regarding variation in their PTB domain. One group has a normal PTB domain and another has 11 amino acids insertion in $\alpha 2/\beta 2$ loop within N-terminal PTB domain. Similar variant loop insertions have been observed in other PTB domains, such as an isoform of CAPON with a 5 amino acid insert within $\beta 3/\beta 4$ loop [49] and a 107 amino acids insert in $\alpha 2/\beta 2$ loop in PTB2 of TBC1D4 protein [50]. These variant loop insertions are believed to have impact on binding to phospholipids but not to NPXY motifs. In fact, it was reported that both forms of the PTB domain of Numb protein isoforms bind efficiently to NPXY motif, suggesting the disrupted N-terminal structure of PTB domain does not affect the NPXY binding [51,52]. To test this hypothesis, we removed the N-terminal $\beta 1$ strand from several randomly selected PTB domains (Suppl. Table 1; Suppl. Fig. 2) and found that these truncated PTB domains retain their ability to interact with NPXY motifs (Table 1). By removing N-terminal $\beta 1$ strand, we also discovered a novel $\beta 1A$ strand from where N-terminal $\beta 1$ strand grow out. $\beta 1A$ strand exists in many X-ray crystallography [34–39] or NMR structure [26,27,39–46] data but went un-noticed, probably due to its obscure location within PTB structure (Suppl. Fig. 1B, C). Through our study we found that $\beta 1A$ strand is parallel to $p5$, 6, 7 strands and join them as part of the second β sheet. Then, a portion of N-terminal sequence starting at the insertion site of NUMB PTB domain was deleted to create a short form of PTB domain, NUMB-ss-PTB in which the N-terminal region of PTB domain containing $\alpha 2$ helix and both $\beta 1$ and $\beta 1A$ strands (one β strand on each side of antiparallel β sheets) has been deleted, and measured its potential binding to NPXY motifs of CCM1 (Fig. 4A). Our Co-IP data showed that the truncated PTB domain (NUMB-ss-PTB) binds to the 1st NPXY motif of CCM1, similar to the inserted form of PTB domain (NUMB-L-PTB) (Fig. 3). Actually, the truncated PTB domain (NUMB-ss-PTB) can bind to all three NPXY motifs of CCM1 (Figs. 1, 3; Table 1), demonstrating that this N-terminal region of PTB domain (containing $\alpha 2$ helix, $\beta 1$ strand in the first β sheet and $\beta 1A$ strand in the second β sheet) is not essential for binding to NPXY motif (Fig. 4A).

3.5. NPXY motifs interact with various PH domains

It has been 20 years since the discovery of the binding of the PTB-like domain of Talin to NPXY motifs at β -integrin cytoplasmic tails [53–55]. The PTB-like domain of Talin was further defined as a F3 lobe of FERM domain within the N-terminal head region of Talin [56,57], which is a PH domain as a module embedded in FERM domain [58]. We termed this FERM embedded PH domain as fPH domain (Suppl. Fig. 1A) and observed that the fPH domain of Talin interacts with all three NPXY motifs of CCM1 (Fig. 2, Table 1). Interestingly, the fPH domain within a conserved C-terminal FERM domain in SNX17 (PX-protein sorting nexin 17) also binds to multiple NPXY motifs [59], including the 1st NPXY motif of CCM1 [60]; indicating the fPH domain can function as PTB domain. Like SNX17, CCM1 also has a conserved C-terminal FERM domain, suggesting the fPH domain of CCM1 might also bind to NPXY motif. In fact, our binding data demonstrated that both fPH domains from SNX17 and CCM1 indeed bind to the 1st and 3rd NPXY motifs of CCM1 (Fig. 3, Table 1).

Much data have pointed out that although the fPH domain acts as PTB domain, the fPH domain is structurally a bona fide PH domain (Fig. 4B), raising the possibility of potential interaction between PH domain and NPXY motif. We randomly select two PH domains (AGAP2-PH1 and PLEK-PH2, Suppl. Table1, Fig. 4B) to test this hypothesis and found that both PH domains bind to the 1st and 3rd NPXY motifs of CCM1 (Fig. 3, Table 1). Intriguingly, the tertiary fold of the truncated PTB domain of NUMB (NUMB-ss-PTB) closely resembles that of PH domain. In fact, PH domain is more structurally similar to PTB domain than the truncated PTB domain of NUMB, NUMB-ss-PTB (two orthogonal antiparallel β sheets of 3 β strands each side capped by a C-terminal α helix) (Fig. 4A, B), further supporting our observation that PH domain can bind to NPXY motif like PTB domain does.

The measurement of thermodynamics of both PTB and PH domains showed a small statistically significant difference ($P < 0.01$) of the temperatures midpoint. The slightly lower temperature midpoint of PH domain (mean $T_m = 47.6^\circ\text{C}$) reflects its relatively simpler structure (Fig. 5) with shorter length of peptides (Suppl. Table1). We further investigated whether PTB domain of ICAP1 α , which has been confirmed to strongly interact with the 1st NPXY motif of CCM1 [16], could compete with either truncated PTB domain of NUMB or PH domain of PLEK for interaction with the 1st NPXY motif of CCM1. Our results demonstrated that the interaction between the 1st NPXY motif of CCM1 with either truncated PTB domain of NUMB1 and PH2 of PLEK was inhibited by the ICAP1 α PTB domain (Fig. 6), further validating our finding that the PH domain, or even a simpler version of PH (truncated PTB domain of NUMB1), is indeed capable of interacting with the 1st NPXY motif of CCM1.

3.6. PTB domains bind to phosphatidylinositol

PH domains have been found to bind inositol lipids in a pocket formed by the $\beta 1$ – $\beta 4$ sheets and connecting loops while PTB domains are also reported to bind phosphoinositide in a pocket formed by N-terminal $\alpha 2$ helix and connecting loops [61]. However, no systemic studies have been done to consolidate the findings. Since all PH domains we investigated show their strong NPXY binding property, we further explored the potential interaction between PTB domains and membrane lipids using a two-set of Multiplex Lipid Beads (FlowPIPs, A, B; Echelon Biosciences) and our collection of cloned PTB domains. Although no interaction was detected in Multiplex Lipid Beads set-A (data not shown), interactions between PTB domains and 4 types of phosphatidylinositol were found in Multiplex Lipid Beads set-B (Table 2). Interactions with phosphoinositides were found in all 4 PH positive controls, 2 controls from Echelon (GRP1 and PLC Delta) and 2 from us (AGAP2-PH1 and PLEK-PH2). No interaction was detected in our negative control (CCM2–1209) indicating the well functioned FlowPIPs system. Among tested 25 PTB domains, 13 of them (52%) showed phosphoinositide-binding capability, indicating that approximate half of PTB domains examined can bind to major types of membrane phosphatidylinositol like PH domains. Among 13 defined phosphoinositide-binding PTB domains, 7 of them (DAB1, DAB2, SHC1, TBC1D4, NUMB1, LDLRAP1, and APPL1) have been described before, further validated the reliability of our Multiplex system, while the remaining 6 of them are newly discovered phosphoinositide-binding PTB domains. Only

one previously reported phosphoinositide-binding PTB domain, CAPON-PTB [49], could not be confirmed with this system.

3.7. The essential tertiary structure of PTB/PH domains for binding to NPXY motifs

Since both PTB and PH domains can swap roles for binding to either NPXY motif or phosphatidylinositol, both domains must share a common tertiary structural core. We examined the predicted tertiary structure of PTB domain of FRS2 supported by multiple NMR-derived structural data [42,43,62] and found that it only has primitive residues for N-terminal $\alpha 2$ helix; which is structurally very similar to the PH domain with PH superfold core structure (Suppl. Fig. 1A). Further, in the deletion analysis, the tertiary structure of the truncated PTB domain of NUMB (NUMB-ss-PTB) is one β strand less than that of PH domain (Fig. 4), only 3 β strands on each side of the antiparallel β sheets capped by a C-terminal $\alpha 3$ helix. This finding provides a new minimum structural requirement for NPXY motif-binding (Fig. 4).

Therefore, we propose a new structure-based classification of PTB domain into three major types: 1). the primitive N-terminal $\alpha 2$ -helix initiated from a variable loop of $\beta 1/\beta 2$ strands although they still shared an identical β -barrel conformation of seven anti-parallel β -strands with PH domain (Suppl. Fig. 1A); 2). as the primitive $\alpha 2$ -helix developed into a 4-turn α -helical formation, the β -barrel conformation changed to extend an additional $\beta 1A$ -strand before $\beta 1$ -strand, which is not parallel to $\beta 5-7$ β sheet yet; but β -barrel conformation becomes a set of anti-parallel β -strands with 4 β -strands each side (Upper row, Suppl. Fig. 1B); 3) this β -barrel conformation with $\alpha 2$ -helix continuing to grow from 4-turn α -helical formation to 5-turn α -helical formation until its maturity with a 6-turn α -helical formation and newly defined $\beta 1A$ -strand before $\beta 1$ -strand is parallel to $\beta 5-7$ β sheet to become a palindromic β -barrel conformation of eight anti-parallel β -strands with 4 β -strands each side (middle and lower rows, Suppl. Fig. 1B). Interestingly, PTB domains in some dual PTB-containing proteins are all shown structurally matured palindromic β -barrel conformations (Suppl. Fig. 1C).

3.8. Intermolecular interactions of NPXY motif - fPH domain mediate CCM1 protein oligomerization

It has been proposed that the fPH domain of CCM1 undergoes intramolecular interaction with its 3rd NPXY motif for self-inhibition [63], which contradicts our yeast-two hybrid data that CCM1 binds itself inter-molecularly [15]. To understand this discrepancy, we revisited the interaction between the fPH domain of CCM1 and its 1st and 3rd NPXY motifs. Our Co-IP and protein binding data supported the strong binding between fPH domain of CCM1 and its 1st and 3rd NPXY motifs (Fig. 3, Table 1). Potential intermolecular interactions of CCM1 can be insinuated as both 1st and 3rd NPXY motifs showed strong binding to full length CCM1 protein (Table 1). Protein electrophoresis was performed under both native and denaturing conditions and multiple retarded bands were found above the band of the full-length CCM1 in native condition (Fig. 7A), further supporting the intermolecular interactions of CCM1. However, there is no lower band observed in native lane, opposing the possibility of existence of intramolecular folding since the mobility of the globular forms of intramolecular binding/folded CCM1 protein should be

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faster than that of unfolded CCM1. Upon measuring the existing forms of CCM1 protein in physiological conditions with DLS we found that there are two major populations of CCM1 protein in physiological condition, the most abundant one accounting to nearly 80% of CCM1 protein is the population around monomeric CCM1 proteins (peak 1, might contain some dimers or trimers and so on, as shown in PAGE gel), followed by a population of oligomers (peak 2, approximate 40mers in size, accounting for nearly 20%) and some even larger soluble oligomers. In our study, 128 acquisitions per run provided high-quality autocorrelation functions and sufficient signal-to-noise to generate stable and reproducible results. There is no large precipitated aggregation observed, suggesting that large oligomers are not randomly constituted aggregations (Fig. 7B). Both PAGE and DLS data further validated our previous findings that CCM1 proteins can assemble into a mesh like network through inter-molecular interaction.

3.9. Tissue and cellular distribution of CCM1

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Prompted by the wide binding spectrum of CCM1 to new PTB-containing protein partners, we examined the expression levels of *CCM1* in different human tissues in detail by screening multi-tissue panels with qPCR. Our data shows that despite varying expression levels in different tissues, *CCM1* is nearly ubiquitously expressed in all major tissues screened (Suppl. Fig. 3A), further enhanced our preliminary observations [5].

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Since the CCM phenotype occurs mainly in CNS and the *CCM1* gene is highly expressed unabridged throughout the whole brain [5], we examined the relative expression level of *CCM1* in different areas of the human brain by qPCR. CCM lesions in the brain occur more often in the supratentorial (~79%) than infratentorial (~21%) area [64,65]. The common infratentorial locations of CCM are the pons and the cerebellum, in which relative expression levels of *CCM1* are higher than average. However, the most common supratentorial locations of CCM lesions are in the frontal and temporal lobes. While the relative expression level of *CCM1* in the frontal lobe is slightly higher than average, the relative expression level of *CCM1* in the temporal lobe is much lower than average, suggesting the possible roles of CCM1 in other cellular and neuronal functions (Suppl. Fig. 3B).

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Further, we investigated the relative expression of the *CCM1* gene in homogenous cell populations from different human cell lines by qPCR. Our data demonstrated that among various cell lines, the relative expression level of the *CCM1* is significantly lower than average in the three endothelial cell lines tested; the *CCM1* is reported to be highly up-regulated in the endothelium (Suppl. Fig. 3C). However, the relative expression level of *CCM1* gene in all cancer cell lines tested (except HeLa cells) was much higher than average, suggesting a potential role of CCM1 in angiogenesis and/or tumorigenesis in the development of cancer.

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To validate our in vitro findings that tumor cell lines express higher levels of *CCM1* than vascular endothelial cell lines (Suppl. Fig. 3C), we explored expression in human tumors. Screening human tumor panels by qPCR, we found that while endometrial tumors have significantly decreased expression level of the *CCM1* ($T = 2.296$, $P < 0.05$), both liver and

testis tumors have significantly ($T = 2.101$, $P < 0.05$) or very significantly ($T = 2.811$, $P < 0.01$) increased expression levels of the *CCM1* compared to their normal controls (Fig. 8).

4. Discussion

Despite intensive investigation, the molecular role of PTB-binding protein, CCM1, within the CSC during angiogenesis remains largely unknown. Our results show that CCM1 protein has a diversified binding ability to the different PTB-containing proteins and encompasses the specificity to interact with certain PTB domains through its own diversified NPXY motifs. CCM1 seems to be involved in a wide range of molecular and cellular events through its specific interaction with many cellular partners. Our data were further supported by a recent report that CCM1 also interact with another PTB-containing protein, ANKS1B, to modulate endothelial permeability [66]. Our findings further exalt the understanding of the cellular dynamics of CSC signaling cascades and elucidate downstream signaling pathways involving novel CSC partners. These results help define the underlined mechanism of angiogenesis and pathogenesis of other human conditions, such as cancer, which may revolutionize the concepts of CSC signaling and lead to new therapeutic strategies. Recently, significant advances in our understanding of the role of CCM1 in cellular signaling have been made and more efforts in the discovery of new cellular partners and the structure and function of these newly defined CCM1 associated proteins are still in progress [67,68]. Our results show that the CCM1 protein has a wide spectrum of binding ability to different PTB-containing proteins and specificity of interacting only with certain PTB/PH domains via its own different NPXY motifs, in addition to its ubiquitous expression pattern, indicating the potential important role of CCM1 in many unknown signaling pathways. Although the sequences flanking these PTB/PH domains and NPXY motifs may be important in the specificity of NPXY motif binding, the detailed binding affinity of major types of PTB/PH domains with three NPXY motifs of CCM1 constitutes a major discovery in PTB/PH domain-NPXY motif interactions and their mediated signaling. These observations can have a major impact on future studies of the cellular functions of PTB/PH-containing proteins and NPXY motif-containing proteins, their interactions, and the related PTB/PH-NPXY complex-mediated cellular signaling.

4.1. The interaction between NPXY motif and PTB/PH domain is specific

Our data demonstrate that the three separate NPXY motifs within CCM1 are distinguished by their interaction with different PTB/PH domains, such that each NPXY motif is functionally distinct in possessing unique PTB/PH-interacting partners. Initially, we had some doubts about identifying so many new cellular partners of CCM2 protein once, however, various label-free binding affinity assays eliminated possibility of “sticky” pull-down artifacts in our Co-IP experiments, in addition to the fact that various PTB domains share a common tertiary folding but with less or no sequence homology [28–30]. Previous evidences that flanking sequences of both NPXY motifs [69] and PTB domains [70] do influence their interaction (usually negative), considering that our PTB constructs only contain PTB domain by removing all flanking sequences, make our results plausible. Scaffold proteins assembles signaling proteins into functional modules through various protein-protein interactions (PPIs), and PTB domain is a modular PPI domain usually within

scaffold proteins [71], therefore, PTB domain should be prone to bind its potential targets unless there is an inhibitory element or limited availability of binding partner. We previously proposed that CCM1 acts as a molecular shuttle that transports ICAP1a in or out of the nucleus and mediates recruitment of ICAP1 α to focal adhesions adjacent to the cytoplasmic membrane [11,20,72]. In light of new data presented here, we propose CCM1 acts more like a mobile scaffold that modulates the specificity of signaling responses within the CSC by regulating the assembly of distinct multi-molecular complexes in different cellular compartments.

4.2. A new β 1A-strand of the PTB domain was discovered

The additional β 1A-strand we defined in this report is different from the previous described β 8 strand which is downstream from C-terminal α 3 helix and positioned next to β 5-strand [43]. The newly described β 1A-strand is downstream from N-terminal α 2 helix and upstream to β 1-strand; and parallel and next to β 7-strand to form the second β sheet (Suppl Fig. 1).

4.3. A new minimum structural requirement of PTB/PH domain for NPXY motif-binding

Current structural requirement of PTB for NPXY motif-binding is the pocket formed by the C-terminal α 1 helix and a β sheet formed by β 1– β 4 strands. During studying the PTB domains within the isoforms of NUMB protein, by removing N-terminal portion of NUMB PTB domain to generate the truncated form of NUMB (NUMB-ss-PTB), we further defined the new minimum structural requirement of PTB/PH domains for NPXY motif-binding, a pocket formed by the C-terminal α 1 helix and a β sheet formed by β 2– β 4 strands, reducing one β strands (β 1 strand).

4.4. Novel insight on the relationship between PTB-NPXY interactions

The relationship between NPXY motif and PTB domain has been known to be fairly static, as the presence of a PTB domain for binding a NPXY motif is fairly coercive [28]. A previous effort to define the relationship between NPXY motif and PTB domain has been done using a derived NPXY Peptide Array, NXXY [73]. Even though physiologically less relevant, the data from their study showed a wide-spectrum of binding capacity between various PTB domains and NXXY motif. In this experiment, we used multiple NPXY motifs from a single molecule, C-CM1, and demonstrated that the interaction of PTB-NPXY is a dynamic event. Each PTB domain can interact with one or more NPXY motifs or a combination of these motifs. Consequently, we have measured the binding affinities of the majority of the PTB domain with three NPXY motifs of CCM1. This new information apparently contradicts the previous classification system in which the PTB domain has been categorized into two major groups, phosphotyrosine-dependent (Shc-like, IRS-like) and phosphotyrosine-independent (Dab-like), which was entirely based on the binding preference of phosphotyrosine [28]. Ironically, phosphotyrosine-independent group is the most predominant form (over 75%). Hence, a reevaluation of this classification system needed to be performed.

4.5. PTB is a subtype of the PH domain with a new classification

Although it is in agreement that both PTB and PH domains are involved in recruitment to the intracellular membrane and share a similar tertiary structure [28–30], it is long believed that PH domain is anchored to membranes through its interaction with phosphoinositides (PIPs) while PTB domain is recruited to the membrane by binding to a juxtamembraneous NPXY motif of cellular receptors, such as RTKs. The common knowledge of binding specificity of PTB domain to the peptide ligand is that ligand recognition is most commonly determined by the tertiary binding sites within the core [46,74,75]. Our experimental data evidently contradicts this theory. Both PTB and PH domains share a canonical structural core usually containing seven (PH) or eight (PTB) β -strands, flanked by one (PH) (C-terminal α 3) or two (PTB) (an additional N-terminal α 2) α -helices (Figs. 4, 5). Our structural data demonstrated that PTB domain might be evolved from PH domain, which have been validated from previous experimental data, either X-ray crystallography [34–39] or NMR structure [26,27,39–46]. Although the evolution of the structure of PTB domain has been noticed before [42,43], no attempt to use this criteria to re-classify the PTB domain has been performed. Domain-mediated oligomerization for enhancing membrane targeting and phosphoinositide binding of scaffold proteins has been reported in PTB-containing proteins [76]. The finding of NPXY-fPH domain-mediated oligomerization for CCM1 protein further enhanced our understanding of scaffold proteins in this aspect. However, the purpose and precise regulatory mechanism of CCM1 oligomerization needs to be further investigated.

Some PTB-containing proteins, such as DAB1/2, have been found to have independent binding to both peptide and phosphoinositide simultaneously [36,77], suggesting that PH and PTB domains can simultaneously interact with the protein and phosphoinositide targets using structurally separated binding sites [36,76,77]. Actually the separate binding sites for NPXY motifs and phosphoinositides have been shown to be non-cooperative and energetically independent [77]. PTB domain has long been recognized as one of inositide-recognition modules [61,78–81], in fact, PTB domains in SHC [81,82], DAB1 [37,77,83,84] and DAB2 [36,85], APPL1 and APPL2 [76,86], LDLRAP1 [87], TBC1D4 [50], NUMB1 [48,51,52], CAPON [49], and TENSIN1 [88] have been well documented for their phosphoinositide-binding, while only one attempt to measure the binding of PTB domain of APBB1 to phosphoinositide was reportedly failed so far [34]. Therefore, our data not only validated the previous findings but also defined many new phosphoinositide-binding PTB domains, enriching our understanding of the functions of PTB domains.

Our findings underscore the importance of functional diversity of repetitive motifs within a single molecule. The underlying molecular mechanisms of this binding specificity remain unknown. Further investigations should elucidate the diversified cellular functions of PTB domains, such as subcellular shuttling, cellular membrane inositol lipids binding, etc.

4.6. PH domain can be an independent functional module within FERM domain

FERM domains are typically known to link cytoskeleton to membrane proteins and phospholipids [89]. FERM domain generally forms a 'clover leaf structure of three globular modules or lobes (F1, F2 and F3) that fold independently. The F1 lobe has an ubiquitin-like fold, the F2 lobe adopts the structure of acyl-CoA binding protein (ACBP), and the F3 lobe

is the pleckstrin homology (PH) domain [58,90,91]. The majority of FERM domain is located at the N-terminus of FERM containing proteins. N-terminal FERM domain containing proteins commonly use their FERM domain to bind intra-molecularly to the other portion of molecule. This intramolecular allosteric interaction structurally reduces competence of other functional domains (such as kinase domain) serving as an “auto-inhibition” state for very precise spatial and temporal control of their activity and scaffolding functions [89,92]. Structural data have revealed that the F1 and F2 lobes (sometimes with part of F3) coordinately work together to play their roles in the N-terminal FERM domain auto-inhibition event [90,92–94], while F3 lobe in the N-terminal FERM domain of TALIN clearly play its role during its auto-inhibition state [95]. The auto-inhibition mechanism has even been reported in PTB domain [70] and PH domain [96], further supporting the significant role of F3 lobe in auto-inhibition. There are only a few reported C-terminal FERM domain containing proteins [58,90,97], CCM1 being one of them [98]. In contrast to the well-defined auto-inhibition mechanism for N-terminal FERM domain containing proteins, the function regulation of C-terminal FERM domain is less known [58,90,97]. There have been reports that C-terminal FERM domain can bind intra-molecularly to its N-terminal head in myosin VIIA to form an auto-inhibition state [99,100]. Likewise, reports regarding the inter-molecular interactions between FERM domain and other proteins also existed [101]. Interestingly, there is a report that the fPH (lobe 3) within its FERM domain of CCM1 can interact intra-molecularly with its 3rd NPXY motif for self-inhibition [63]. This is the only report showing that fPH in C-terminal FERM domain can function independently for auto-inhibition for CCM1 protein. However, our data in this experiment demonstrated the intermolecular interaction of CCM1 proteins, excluding the possibility of fPH in FERM domain directed auto-inhibition for CCM1.

As a complex domain, the FERM domain consists of three independent modules; FERM domain itself also can act as a module to form a new function domain, such as MyTH4-FERM domain in myosin [102], which reflects the evolving nature of new structures generated from the preserved functional modules. Interestingly, a recent report showed that ankyrin repeat domain (ARD) in CCM1 acts as an additional F0 lobe of FERM, as previously observed in Kindling and Talin [103,104], to form a stable and globular ARD-FERM domain [105]. However, whether or how the formation of ARD-FERM domain affects the function of each single lobe (F1, F2, F3) of FERM domain needs to be further elucidated.

4.7. Possible involvement of CCM1 in tumorigenesis

Upon finding multiple potential binding proteins of CCM1, we re-examined the expression patterns of the *CCM1* gene in different human tissues by *qPCR* and found this gene is nearly ubiquitously expressed in all major tissues enriching our previous findings using other methods [5], and indicating potentially diverse functions of CCM1. Notably, we found the expression pattern of *CCM1* in the brain does not closely match with the location of CCM lesions. This observation differs from the immunohistochemistry (IHC) results performed till now [106]. We were intrigued to find that the CCM1 expression is up-regulated in tumor cell lines which was further confirmed in several human tumor specimens (Fig. 8). Interestingly, endometrial and testis tumors have higher expression of *CCM1*. Future elucidation of the

role of the CSC in tumorigenesis will enhance our understanding of molecular pathophysiology and may suggest new options for diagnosis, prognosis, and therapy in tumors of the reproductive system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CCM	Cerebral cavernous malformation
CSC	CCM Signaling complex
PTB	Phosphotyrosine binding domain
PH	Pleckstrin homology domain
FERM domain	Band 4.1 and ERM homology domain
I-TASSER	Iterative Threading ASSEmbly Refinement

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Fig. 1.

Interactions between NPXY motifs of CCM1 and PTB domains. A). β -galactosidase activity of each transformant was measured, normalized and converted to relative β -galactosidase activity (RBGA). Negative control (light grey) between fusions GAL4BD-Lamin C (LAM) and GAL4AD-large T antigen (pGAD-T); and positive control (dark grey) between GAL4BD-p53 (p53) and pGAD-T. B). RBGA values were obtained by further normalizing data to the wild-type (W, light grey) of NPXY motifs of CCM1 respectively. Mutant form (M, dark grey) of NPXY motifs dramatically decrease or even abolish the interaction with

various PTB domains. Mutant K5 and K8 are constructs harboring compound mutation N231A/F234A and N250A/F253A, respectively. Normalized data represents at least three independent assays. RBGA⁺⁺⁺: very significantly higher than that observed in any negative controls ($P < 0.001$). K5 and K8 are fragments containing the second and third NPXY motif respectively in CCM1.

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Fig. 2.

Binding between NPXY motifs of CCM1 and PTB-containing proteins. In Co-IP of GST-tagged PTB domains with HIS-tagged NPXY motifs of CCM1, protein lysates from transformed BL21 clones were pulled down with: A) MagneGST beads for either GST-tagged PTB domains only (upper panels) or with their associated targets (lower panels), full-length (FLHK) and CCM1 fragments, K2 (1st NPXY motifs), K5 (2nd NPXY motif), K8 (3rd NPXY motif) and K12 (both 2nd and 3rd NPXY motifs). Expression of GST-tagged PTB domains were confirmed by IP with MagneGST beads and followed by Coomassie

staining (upper panel). B) Dynabeads for HIS-tagged full-length CCM1 (FLHK) and its associated PTB domains. C) Dynabeads for HIS-tagged full-length PTB-containing proteins and their associated full-length CCM1. Lack of any interaction was seen between any fragment of CCM1 and negative controls, DLC1 (Residues 359–403 of DLC1 Protein, non-NPXY PTB domain-binding motif), CCM2–1209 (CCM2 C-terminal fragment, Residues 303–444, no PTB domain), ICAP1 β (ICAP1 isoform β , no PTB domain), and mock control. Mock control is host cell lysate.

Fig. 3.

Binding between the first NPXY motif of CCM1 and PTB/PH domains. In co-IP of GST-tagged PTB/PH domains with HIS-tagged K2 (1st NPXY motifs) fragment of CCM1, Protein lysates from transformed BL21 clones were pulled down with: A) MagneGST beads for GST-tagged PTB/PH domains, expression of GST-tagged PTB/PH domains were confirmed by IP with MagneGST beads and followed anti-GST antibody (upper panels) or Dynabeads for HIS-tagged K2 fragment followed by anti-GST antibody for detection of K2 (1st NPXY motifs) associated PTB/PH domains (lower panels). B) Dynabeads for HIS-tagged K2 (1st NPXY motifs of CCM1) fragment, expression of HIS-tagged K2 fragment were confirmed by IP with Dynabeads followed by anti-HIS antibody (upper panels) or MagneGST beads followed by anti-HIS antibody for detection of PTB/PH domains associated K2 fragments. Lack of any interaction was seen between K2 fragment of CCM1 and negative controls, DLC1 (Residues 359–403 of DLC1 Protein, non-NPXY PTB domain-binding motif).

A

B

Fig. 4.

Comparison of structural similarities among PTB and PH domains. A). structural similarity among the PTB domains of normal form (NUMB-PTB), the insertion isoform (NUMB-L-PTB) and the truncated form of NUMB (NUMB-ss-PTB). The insertion isoform (NUMB-L-PTB) has an elongated variable loop from N-terminal $\alpha 2$ helix (orange arrow), $\alpha 2$ loop, which contributes to phospholipid-binding (Middle panel). The truncated form of NUMB (NUMB-ss-PTB) has a N-terminal $\alpha 2$ helix and one β strand each (blue colored ribbon) from each side of two orthogonal antiparallel β sheets missing (Lower panel). B). structural

similarity between PH domains and fPH domain embedded in FERM domains. Ribbon representations of the tertiary structures of PTB domains in NUMB isoforms and various PH/fPH domains were created from I-TASSER. Red color: c-terminus and blue color: N-terminus.

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Fig. 5.

The difference of melting curves between PTB and PH domains. Estimated temperature midpoints for the coupled unfolding/self-association of protein monomers were determined by dynamic light scattering. The midpoint of temperature-dependent conformational change (or partial unfolding) of PTB/PH domains was estimated as melting temperature (T_m). ** indicates a significant difference of T_m values between PTB and PH domain.

Fig. 6. Binding between the NPXY motif of CCM1 and PH domains reaffirmed by competition-binding assays. GST-tagged PH domains were titrated with increasing volumes of ICAP1α PTB domains. Band intensities of PH domains immuno-precipitated with HIS tagged 1st NPXY motifs of CCM1 were measured by quantitative densitometry at least three independent experiments. The binding of the 1st NPXY motifs of CCM1 to either N-terminal truncated NUMB PTB domain (black cycle, $n = 6$) or PLEK PH domain (red square, $n = 3$) were dramatically decreased with increasing volumes of GST tagged ICAP1α PTB domains. At each measure point, only half of the error bars were shown (either up or down).

Fig. 7.

CCM1 protein self-assembly through intermolecular interactions. A). Equal amount of purified GST-tagged full-length CCM1 proteins were separated by electrophoresis under either native or denatured conditions, two major aggregation peaks were observed. B). DLS profile and size distribution of full-length CCM1 at 25 °C. The x-axis represents hydrodynamic diameter, and the y-axis, the % mass estimated using the Raleigh spheres model. The inset displays the temperature dependence of the midpoint of Peak 1 vs. temperature. DSL analysis further showed that although majority of full-length CCM1

proteins exist as monomer (80%), another group of self-assembled oligomers are formed, which is estimated to be 40 mers (20%). The process appears to be highly cooperative, with emphasis on the end states, i.e., monomer vs. high-order multimers.

Fig. 8.

Significant expression changes of CCM1 found in certain tumor tissues with qPCR. The relative expression levels of CCM1 (2-ACT) were presented with bar plots, in which light grey bars represent the dark grey plots represented normal tissues (N) and light grey plots represented tumor tissues, among each tissue pair among major tissues: Endometrium (EN), Liver (LI), and Testis (TE) (***, **, and * above bar indicate $P = 0.001$, 0.01 , and 0.05 respectively for paired t -test).

Table 1

Kinetic analysis of the interactions between NPXY motif of CCM1 and PTB/PH domains. Affinity data obtained using three different platforms were presented. Binding Affinity was presented with the equilibrium dissociation constant (K_D). SB, strong binding but out of range of measurement; NB, no binding. K2a is a shorter form of K2.

PTB/PH domain	BLI		SPR		Full-kinetics			
	K2a	K8	K2	K8	SPR		ITC	
					K2	K8	K2	K8
APBB1-PTB1			1.31×10^{-5}	3.30×10^{-7}			1.32×10^{-7}	2.16×10^{-7}
APBB1-PTB2	SB		6.26×10^{-6}				3.23×10^{-6}	8.7×10^{-6}
DAB1-PTB	SB	4.18×10^{-8}	3.21×10^{-6}	4.13×10^{-8}			1.01×10^{-5}	9.8×10^{-7}
DAB2-PTB	SB	SB	1.85×10^{-7}	1.49×10^{-9}			3.61×10^{-7}	1.44×10^{-6}
CCM2-PTB	SB	2.17×10^{-7}	6.85×10^{-6}	1.29×10^{-7}			2.7×10^{-6}	1.27×10^{-6}
TBC1D1-PTB2			1.59×10^{-7}	2.20×10^{-8}	7.15×10^{-8}		1.07×10^{-6}	2.39×10^{-6}
TBC1D4-PTB1		8.19×10^{-7}	2.32×10^{-6}				3.7×10^{-6}	2.82×10^{-6}
TBC1D4-PTB2	SB	1.63×10^{-8}	3.52×10^{-7}	6.32×10^{-8}			8.55×10^{-7}	2.6×10^{-6}
LDLRAP1-PTB	SB	SB	1.20×10^{-7}	9.66×10^{-8}			2.18×10^{-7}	8.4×10^{-7}
APPL1-PTB		1.83×10^{-8}	4.15×10^{-7}	1.64×10^{-6}	9.05×10^{-8}		1.2×10^{-5}	3.34×10^{-7}
FRS2-PTB	SB		6.45×10^{-8}	2.24×10^{-9}	1.21×10^{-7}		2.23×10^{-6}	1.76×10^{-6}
ICAP1-PTB	SB		1.25×10^{-7}	1.35×10^{-7}	1.18×10^{-7}		2.96×10^{-7}	5.26×10^{-3}
mPID-s-PTB	SB	SB	1.42×10^{-7}				5.32×10^{-6}	1.31×10^{-5}
JIP-PTB	SB	SB	2.75×10^{-8}				6.49×10^{-6}	2.26×10^{-6}
RGS12-PTB	SB	SB	5.13×10^{-8}				2.6×10^{-5}	2.88×10^{-6}
ESP8-PTB							8.93×10^{-7}	2.34×10^{-6}
ESP8L3-PTB							8.62×10^{-7}	2.19×10^{-6}
CAPON-PTB		3.78×10^{-7}	4.69×10^{-8}	4.30×10^{-8}			5.65×10^{-7}	2.37×10^{-6}
RASGAP-PTB	SB						1.24×10^{-6}	1.87×10^{-6}
CCM2-1209	NB	NB	NB	NB				
SHC1-PTB	SB	SB	1.84×10^{-7}	1.34×10^{-7}				
DOK4-PTB		SB						

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PTB/PH domain	BLI		SPR		Full-kinetics	
	K2a	K8	K2	K8	SPR	ITC
					K2	K8
GUILP1-PTB						2.23×10^{-5}
TENSIN2-s-PTB						4.57×10^{-6}
DOK1-PTB						1.38×10^{-5}
NUMB-L-PTB		5.54×10^{-7}	1.35×10^{-6}			2.27×10^{-5}
NUMB-SS-PTB			5.43×10^{-6}			1.04×10^{-7}
AGAP2-PH1		2.99×10^{-7}	1.24×10^{-13}		4.37×10^{-8}	2.79×10^{-6}
PLEK-PH2	SB		1.10×10^{-10}	6.98×10^{-6}	2.18×10^{-8}	3.1×10^{-5}
TALIN-4PH						3.31×10^{-7}
SNX17-4PH			1.32×10^{-6}			7.87×10^{-6}
CCM1-4PH						3.04×10^{-6}
Full-length CCM1						2.73×10^{-6}
						1.74×10^{-6}
						2.24×10^{-6}
						6.37×10^{-6}
						1.27×10^{-5}
						3.23×10^{-6}
						8.7×10^{-6}
						4.48×10^{-5}
						4.81×10^{-6}

Table 2

Purified recombinant PTB domains bind to phosphatidylinositol. No interaction of recombinant PTB domains was detected in Multiplex lipid bead mixes A (PI, PI(3)P, PI(4)P, PI(5)P, Ptdethanolamine (PE), No Lipid/blank control), while multiple interactions were found in Multiplex lipid bead mixes B which contain a mixture of 6 lipid types (PtdIns(3, 4)P2, PtdIns(3, 5)P2, PtdIns(4, 5)P2, PtdIns(3, 4, 5)P3, Phosphatidic Acid (PA) and Phosphatidylserine (PS)). Both GRIP1 and PLC Delta are positive controls for Multiplex lipid bead mixes provided by Echelon Biosciences. INT, interaction detected; NI, no interaction detected.

PTB/PH domains	PtdIns(3, 4)P2	PtdIns(3, 5)P2	PtdIns(4, 5)P2	PtdIns(3, 4, 5)P3	PA	PS
GRP1	INT	INT	INT	INT	NI	NI
PLC Delta	INT	INT	INT	INT	NI	NI
APPL1-PTB	INT	INT	INT	INT	NI	NI
FRS2-PTB	NI	NI	NI	NI	NI	NI
LDLRAP1-PTB	INT	INT	INT	INT	NI	NI
NUMB-PTB	INT	INT	INT	INT	NI	NI
CCM2-PTB	NI	NI	NI	NI	NI	NI
TBC1D4-PTB2	INT	INT	INT	INT	NI	NI
TBC1D4-PTB2	NI	NI	NI	NI	NI	NI
TALIN-fPH	NI	NI	NI	NI	NI	NI
ICAP1-PTB	NI	NI	NI	NI	NI	NI
CCM2-1209	NI	NI	NI	NI	NI	NI
APBB1-PTB1	NI	NI	NI	NI	NI	NI
APBB1-PTB2	NI	NI	NI	NI	NI	NI
mPID-PTB	NI	NI	NI	NI	NI	NI
JIP-PTB	INT	INT	INT	INT	NI	NI
RGS12-PTB	INT	INT	INT	INT	NI	NI
ESP8-PTB	NI	NI	NI	NI	NI	NI
ESP8L3-PTB	INT	INT	INT	INT	NI	NI
CAPON-PTB	NI	NI	NI	NI	NI	NI
RASGAP-PTB	NI	NI	NI	NI	NI	NI
TBC1D4-PTB1	NI	NI	NI	NI	NI	NI
TBC1D1-PTB2	INT	INT	INT	INT	NI	NI
FAM36A-PTB	INT	INT	INT	INT	NI	NI
SHC1-PTB	INT	INT	INT	INT	NI	NI
DAB1-PTB	INT	INT	INT	INT	NI	NI
DAB2-PTB	INT	INT	INT	INT	NI	NI
DOK4-PTB	INT	INT	INT	INT	NI	NI
AGAP2-PH1	INT	INT	INT	INT	NI	NI
PLEK-PH2	INT	INT	INT	INT	NI	NI