Phosphorylation sites in the cerebral cavernous malformations complex

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Studies in the past four years in humans, mice, zebrafish and cultured cells have identified the cerebral cavernous malformation (CCM) multiprotein complex, which is localized in part to endothelial and epithelial cell–cell junctions, and is important in the stability of these junctions and in vascular development (Glading et al., 2007; Mably et al., 2006; Mably et al., 2003; Whitehead et al., 2004; Wustehube et al., 2010). In humans, mutations that affect at least two members of this complex are associated with a common (~0.5% prevalence) vascular malformation that leads to substantial morbidity and mortality. In animals, mutations in components of this complex lead to defects in cardiovascular development, increased vascular permeability (Boulday et al., 2009; Guclu et al., 2005; Laberge-le Couteulx et al., 1999) and are associated with exacerbation of Wnt/β-catenin-driven pathologies, such as intestinal adenomas (Glading and Ginsberg, 2010). Recent studies have identified a physical association of this complex with the transmembrane receptor heart of glass (HEG1) and have established the role of this complex in inhibiting Rho and Rho-associated protein kinase 1/2 (ROCK1/2), to stabilize endothelial and epithelial cell–cell junctions (Crose et al., 2009; Kleaveland et al., 2009; Stockton et al., 2010; Whitehead et al., 2009), in limiting permeability of the endothelial monolayer and in regulating Wnt/β-catenin-driven transcription (Glading and Ginsberg, 2010).

KRIT1 is a component of a multiprotein CCM complex

KRIT1 (also known as CCM1) contains a C-terminal FERM (for 4.1, ezrin, radixin, moesin) domain and several ankyrin repeats. The FERM domain is subdivided into three subdomains; F1 resembles a Ras association domain and F3 resembles a phosphorylated-tyrosine-binding (PTB) domain (Hamada et al., 2003; Serebriiskii et al., 1997; Wohlgemuth et al., 2005). The N-terminal region contains multiple NPxY/F (x=any residue) motifs, one of which (N^{192}PAY) mediates binding to the integrin-binding protein ICAP1α (also known as ITGB1BP1) (Zawistowski et al., 2002). N^{192}PAY has been reported to bind to the KRIT1 FERM domain (Beraud-Dufour et al., 2007); however, another study failed to confirm the role of N^{192}PAY, although that study did confirm the KRIT1 N-terminal–FERM-domain intramolecular interaction (Francalanci et al., 2009). In addition to *KRIT1*, heterozygous loss-of-function mutations in two other genes, *CCM2* and *CCM3*, are linked to the development of CCM (Bergametti et al., 2005; Denier et al., 2004). Two NPxF sequences (N^{231}PLF and N^{250}PYF) in KRIT1 mediate binding to the PTB domain of CCM2 (Zawistowski et al., 2005b). CCM2 acts as a scaffold for Rac1, actin, MEKK3 (also known as MAP3K3) and MKK3 (also known as MAP2K3) (Uhlik et al., 2003) in macrophages. CCM3 has been reported to bind to CCM2 (Voss et al., 2007); however, others have found that CCM3 associates with protein phosphatases and germinal center kinases to a much greater extent than it does with KRIT1 or CCM2 (Goudreault et al., 2009). Importantly, null mutations of the gene encoding CCM2 in both zebrafish (Mably et al., 2006) and mice (Whitehead et al., 2009) phenocopy the loss of KRIT1. Furthermore, depletion of KRIT1 or CCM2 from endothelial cells in vitro leads to similar effects on vascular permeability (Glading et al., 2007b; Stockton et al., 2010; Whitehead et al., 2009), and genetic and biochemical studies indicate that the major phenotypic effect of deficiency of KRIT1 or CCM2 is endothelial cell autonomous (Akers et al., 2008; Glading et al., 2007b; Pagenstecher et al., 2009; Stockton et al., 2010; Whitehead et al., 2009). Although studies have suggested that CCM3 might also act in an endothelial cell autonomous manner to cause CCMs (Akers et al., 2008; Pagenstecher et al., 2009), available evidence is less compelling than for the other two genes encoding CCM proteins (e.g. Louvi et al., 2011). Taken together, the combination of genetic, biochemical and cell biological studies show that a protein complex assembles around KRIT1 and CCM2, and that it acts in a cell autonomous fashion to regulate endothelial and epithelial cell–cell junctions and vascular development.

To obtain insights into the basis and regulation of interactions amongst components of this complex we have used a tandem affinity purification (TAP) tag on the core scaffold (KRIT1/CCM1) to purify it as a complex with associated proteins from U2OS cells, cells that form typical adherens junctions (Xue et al., 2005) (Fig. 1A,B). Our TAP protocol led to recovery of isolated bait protein (average yield=22%) and confirmed that ICAP1, CCM2 and HEG1 are KRIT1/CCM1-interacting proteins (Fig. 1B); however, no CCM3 was detected, confirming previous results (Goudreault et al., 2009). Here, we report on the phosphorylation sites in this CCM complex.

S22 was the only high-stoichiometry phosphorylation site in KRIT1/CCM1 (Table 1 and Fig. 2A). The Human Protein Reference Database PhosphoMotif finder (http://www.hprd.org/PhosphoMotif_finder) (Amanchy et al., 2007) indicates that this residue is a possible target site for protein kinase A or casein kinase I. NPxY/F motifs of KRIT1/CCM1 are known to be crucial for the interaction of KRIT1/CCM1 with ICAP1 (Zawistowski et al., 2002) and CCM2 (Zawistowski et al., 2005a), and they regulate nuclear localization (Francalanci et al., 2009). We found a low-

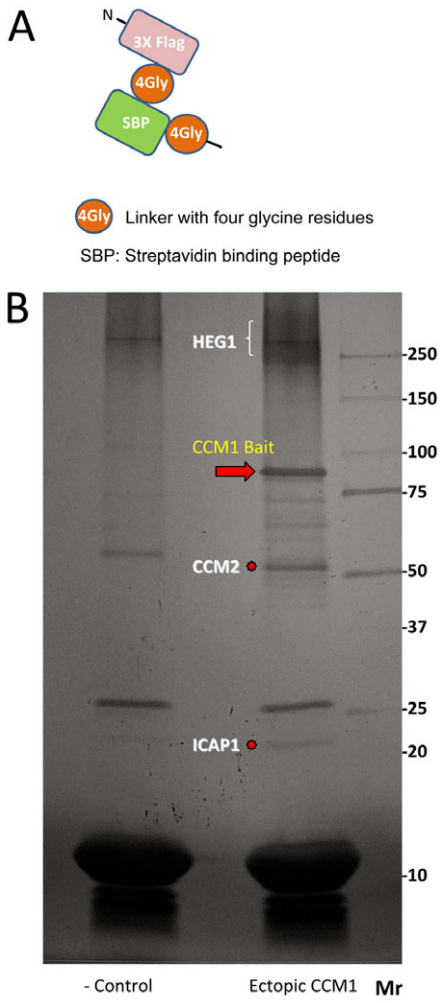

Fig. 1. Tandem affinity purification of KRIT1. (**A**) Tandem affinity purification tag. (**B**) Silver stain gel of polypeptides isolated by tagged KRIT1 (right lane) and a control tag with no KRIT1 bait (left lane).

Table 1. Phosphorylation sites in the proteins comprising the CCM complex

Peptide	Residue	Stoichiometry	Predicted kinase
KRIT1/CCM1			
IRPKNTApSLNSREY	22	+++	PKA, CKI
VCSESSTHFApTLTAR	151	++	?
VVINPpYFGLGAPDYSK	252	++	JAK2
CCM2			
GKKPGIVpSPFKR	15	+++	GSK3, ERK1/2, CDK5
TAQDPGIpSPSQSLCAESSR	164	+++	GSK3, ERK1/2, CDK5
TAQDPGISPpSQSLCAESSR	166	++	DNA-dependent kinase
TAQDPGISPSQpSLCAESSR	168	++	DNA-dependent kinase, GSK3 motif
AIFDGApSTPTHHL	238	++	CKII
IFDGASpTPT	239	++	GSK3, ERK1/2, CDK5
AIFDGASTPpTHHLSLHSDDSSTK	241	+	CKII
DGASTPTHHLSLHpSDDSSTK	248	+++	GPCRI
TFCFPESVDVGGApSPHSKT	280	++	GSK3, ERK1/2, CDK5
SKTIpSESELSASATELLQDY	287	++	CKII
MLpTLRTKLSSQEIQQF	305	++	PKA, PKC
GVKDGRGIITDpSFGRHRRALST	384	+++	β-adrenergic receptor kinase
ATGSSDDRpSAPSEGDEWDR	413	+++	CKI
ATGSSDDRpSAPpSEGDEWDR	416	++	CKII motif
GCSMDQDpSA	443	++	CKI
ICAP1α			
HSpSSSSQSSEISTK	11	+++	CaMKII, MAPKAPK1
KRHpSSSSSQSSEISTK or	10 or 14	+++	PKA
KRHSSSSpSQSSEISTK			CKI, DNA dependent kinase
KRHSSSSpSQSSEISTK or	14	++	MAPKAPK2,
KRHpSSSSpSQSSEISTK or			GSK3, MAPKAPK2
KRHSpSSSpSQSSEISTK			?
HSSSSSQSpSEISpTK	17 and 21	+	GPCR1, PKA, PKC, CKI
SKpSVDSSLGGLSR	25	+	PKA, PKC
STVApSLDTDSTK	41	+++	GSK3 motif
SSTVApSLDTDpS/pTK	46 or 47	+	?
SRSSTVASLDpTDSTKSSGQSNNNSDTCAEF	44	+	CKII

The peptide coverage for KRIT1/CCM1 was 735/736=100% by amino acid count, for CCM2 444/460=100% by amino acid count, and for ICAP1α 194/200=97% by amino acid count.

Italicized font indicates peptides that have possible phosphorylation but whose supporting spectra are weak.

Stoichiometry is defined as 100×(number of phosphopeptides/total number of identified peptides that harbor the phosphorylation site); +++: >10%, ++: 2–7%, +: <2%.

GSK3 or CKII motif indicates a motif that binds GSK3 or CKII when phosphorylated, otherwise it is the indicated kinases that are predicted to phosphorylate the phosphopeptide site.

Different phosphopeptides with a common phosphorylation site are categorized according to the common phosphorylation site (e.g. S14 in ICAP1).

stoichiometry tyrosine residue phosphorylation of N^{250}PYF, a site that has been implicated in both the CCM2 binding and nuclear retention of CCM1 (Francalanci et al., 2009), and this is a possible JAK2 kinase target site.

In CCM2, we found multiple serine/threonine phosphorylation sites scattered throughout the whole protein sequence (Table 1 and Fig. 2B). CCM2 might be the core functional unit of CCM complexes, the interaction of which with various proteins is regulated by multiple phosphorylation events. CCM2 is also a common binding partner of both KRIT1/CCM1 and CCM3. PhosphoMotif Analysis suggests that the two highest-stoichiometry sites, S15 and S164 in CCM2, are phosphorylated by GSK3 or ERK1/2. S164, S166 and S168 are phosphorylation sites within the PTB domain, suggesting that these residues might affect the interaction of CCM2 and KRIT1/CCM1. Tyrosine residue phosphorylation of CCM2 was not detected in our studies.

S11 and S41 were high-stoichiometry phosphorylation sites found in ICAP1 (Table 1 and Fig. 2C). S11 is a possible CaMKII or MAPK-activated protein kinase 1 (MAPKAPK1) target site. All of the phosphorylation sites identified on ICAP1 were located in the N-terminal serine/threonine-rich domain (amino acids 1–60). CaMKII is known to phosphorylate T38 in ICAP1, thereby increasing the binding of ICAP1 to the integrin β1 tail and thus blocking talin binding and suppressing integrin β1 integrin activation (Bouvard and Block, 1998; Harburger and Calderwood, 2009). Here, we found multiple serine/threonine phosphorylation sites in ICAP1. However, phosphorylation of T38 was not detected. Given that we specifically analyzed the fraction of ICAP1 that bound to KRIT1/CCM1 (Zawistowski et al., 2002; Zhang et al., 2001), it is possible that phosphorylation at this site leads to ICAP1 preferentially binding to integrin β1 rather than to CCM1.

Although we recovered HEG1 protein, we failed to identify any phosphorylation sites in this protein. The significance of this absence of phosphorylation is uncertain because this glycosylated membrane protein was present in multiple bands resulting in poor sequence coverage of the cytoplasmic domain.

Materials and Methods

A cDNA encoding a TAP tag of 3× FLAG peptide and a Strep-tag was subcloned 5′ of human KRIT1/CCM1 in pcDNA3.1c plasmid (TAP–CCM1). The human osteoblast U2OS cell line was transfected with TAP–CCM1 plasmid and a stable cell line was generated by geneticin selection. Cells were expanded on 10-cm culture plates and, to avoid possible keratin contamination, most procedures were performed in tissue culture hoods and all buffers were made with distilled and deionized water. In total, cells on 460 plates were scraped and lysed in the presence of Complete

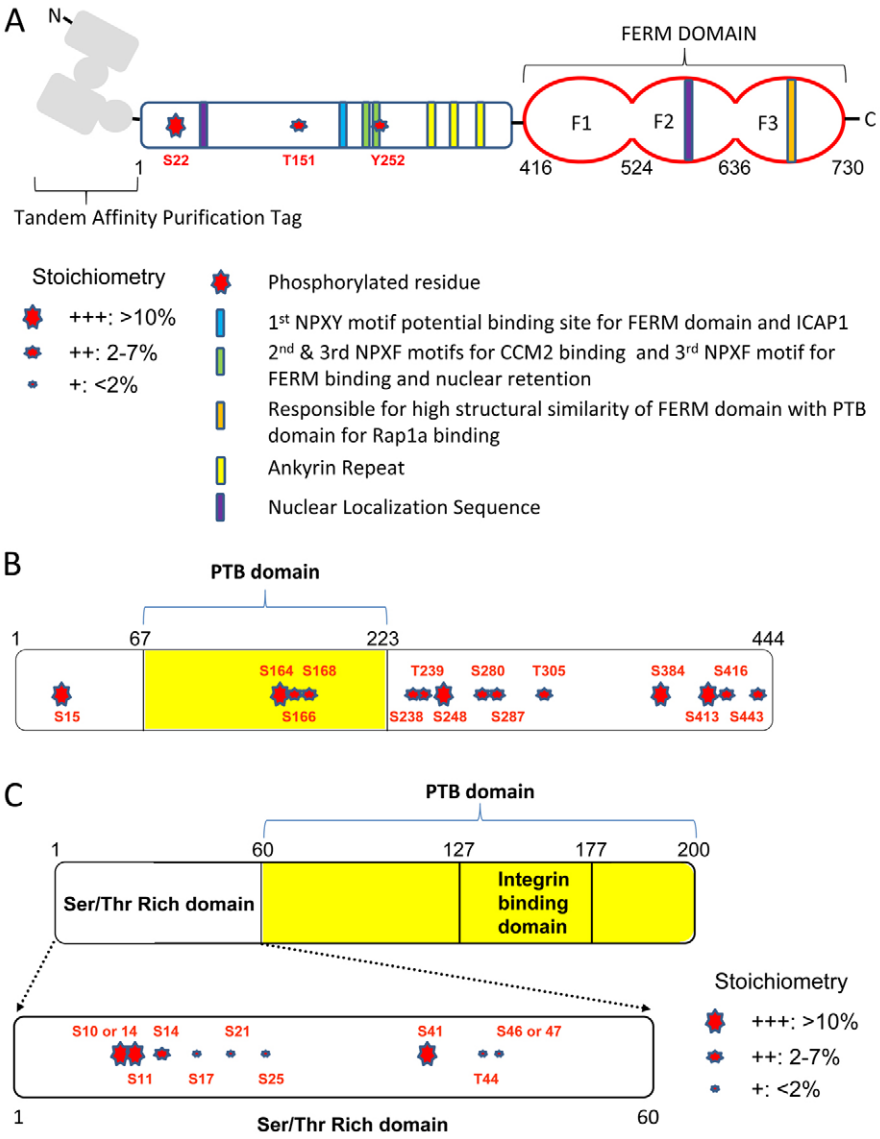

Fig. 2. Schematic of identified phosphorylation sites in the CCM complex. (**A**) Phosphorylation sites in KRIT1/CCM1. (**B**) Phosphorylation sites in CCM2. (**C**) Phosphorylation sites in ICAP1α. Note that the estimated stoichiometry is indicated by the size of the stars.

protease inhibitor cocktail (Roche), PhosphoStop (Roche), sodium orthovanadate (Sigma), sodium fluoride (Sigma) and calycurin (Cell Signaling). Lysates were precleared by centrifugation at 20,000 ***g*** for 15 minutes, and the supernatant was incubated with anti-FLAG M2 affinity gel (Sigma) overnight. Elution of anti-FLAG-antibody-bound bait proteins was performed with 3× FLAG peptide (Sigma) for 90 minutes, and the eluate was incubated with streptavidin ultralink resin (Pierce) for 6 hours before the resin was boiled in 2× sample buffer. Eluted proteins were separated in a Tris-glycine mini gel (4–12%; Invitrogen), and the gel was stained with the silver stain SNAPII kit (Pierce). The proteins were cut out as bands from the gel. Each band was divided into ~1-mm cubes and placed into a clean Eppendorf tube. The protein was reduced with DTT, alkylated with iodoacetamide and digested with enzymes (trypsin, chymotrypsin and elastase). Peptides extracted from each enzyme digest were eluted into a LTQ Orbitrap Velos mass spectrometer by microcapillary reverse-phase high-performance liquid chromatography (rpHPLC) coupled to nano-electrospray ionization. The mass spectrometer was set to acquire a MS spectrum in high resolution (orbitrap) followed by up to 20 MS/MS spectra in low resolution (ion trap) using dynamic exclusion. The MS/MS spectra were searched against the specific protein sequence using the Sequest algorithm. Any peptides passing minimal scoring criteria and having putative phosphorylation sites were verified manually. All analyses were performed in the Mass Spectrometry & Proteomics Resource Core at Harvard University.

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