This is a repository copy of Distinctive phosphoinositide and Ca2+ binding properties of normal and cognitive performance-linked variant forms of KIBRA C2 domain.

White Rose Research Online URL for this paper:
http://eprints.whiterose.ac.uk/131014/

Version: Accepted Version

Article:

https://doi.org/10.1074/jbc.RA118.002279

This research was originally published in the Journal of Biological Chemistry. Posner, et al., Distinctive phosphoinositide and Ca2+ binding properties of normal and cognitive performance-linked variant forms of KIBRA C2 domain J. Biol. Chem. jbc.RA118.002279. doi:10.1074/jbc.RA118.002279. © the Author(s) (or their Institutions) 2018.

Reuse
Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.
Distinctive phosphoinositide and Ca$^{2+}$ binding properties of normal and cognitive performance-linked variant forms of KIBRA C2 domain

Mareike G. Posner¹, Abhishek Upadhyay¹, Rieko Ishima², Antreas C. Kalli³, Gemma Harris⁵, Joachim Kremerskothen⁶, Mark S. P. Sansom⁷, Susan J. Crennell¹, and Stefan Bagby¹*

From the ¹Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY; ²Department of Structural Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, 15260; ³Leeds Institute of Cancer and Pathology, University of Leeds, Leeds LS9 7TF; ⁴Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, LS2 9JT; ⁵Research Complex at Harwell, Rutherford Appleton Laboratory, Didcot OX11 0FA; ⁶Internal Medicine D, Department of Nephrology, Hypertension and Rheumatology, University Hospital Münster, Münster; ⁷Department of Biochemistry, University of Oxford, Oxford OX1 3QU

Running title: KIBRA C2: distinctive phosphoinositide and Ca$^{2+}$ binding

*To whom correspondence should be addressed: Stefan Bagby, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK, bsssb@bath.ac.uk, Tel. 0044(0)1225 386436; Fax. 0044(0)1225 386779.

Keywords
Analytical ultracentrifugation, C2 domain, calcium binding, human cognition, KIBRA, molecular dynamics, NMR, phosphoinositide, WWC protein family, X-ray crystallography
KIBRA C2: distinctive phosphoinositide and Ca\(^{2+}\) binding

**ABSTRACT**

KIBRA, a multi-functional scaffold protein with around twenty known binding partners, is involved in memory and cognition, organ size control via the Hippo pathway, cell polarity, and membrane trafficking. KIBRA includes tandem N-terminal WW domains, a C2 domain and motifs for binding aPKC and PDZ domains. A naturally occurring human KIBRA variant involving residue changes at positions 734 (M-to-I) and 735 (S-to-A) within the C2 domain affects cognitive performance. We have elucidated 3D structures, and calcium and phosphoinositide binding properties, of human KIBRA C2 domain. Both wild type and variant C2 adopt a canonical type I topology C2 domain fold. Neither Ca\(^{2+}\) nor any other metal ion was bound to wild type or variant KIBRA C2 in crystal structures, and Ca\(^{2+}\) titration produced no significant reproducible changes in NMR spectra. NMR and X-ray diffraction data indicate that KIBRA C2 binds phosphoinositides via an atypical site involving β-strands 5, 2, 1, and 8. Molecular dynamics simulations indicate that KIBRA C2 interacts with membranes via primary and secondary sites on the same domain face as the experimentally identified phosphoinositide binding site. Our results indicate that KIBRA C2 domain association with membranes is calcium-independent and involves distinctive C2 domain-membrane relative orientations.

Kidney- and BRAin-expressed protein (KIBRA, also called WW1 for WW- and C2-domain-containing protein 1) is a large (1119, 1118 and 1113 amino acid isoforms), multi-functional scaffold protein that has two N-terminal WW domains, a putative coiled coil regions, a C2 domain, a glutamic acid-rich motif, an atypical PKC (aPKC) binding region, and a C-terminal PDZ binding motif (Fig. 1). KIBRA has about twenty reported binding partners (2) and disease links including dementia (3-5), kidney disease (6), Tourette disorder (7), and some cancers (8, 9). Among its multiple functions, KIBRA is an upstream component of the Hippo pathway (8, 10), a central mechanism of organ size control and cellular homeostasis. KIBRA also has a role in cell polarity and migration, functioning as a link between polarity proteins and cytoskeleton components (2, 11-14). KIBRA also functions in membrane trafficking via regulation of vesicular transport (12, 15-17).

KIBRA is linked to memory, cognition, and neurological disorders in humans and in rodent models (reviewed in (2)). A single nucleotide polymorphism (SNP), rs17070145, in the ninth intron of KIBRA, for example, has been implicated in human cognition (15), a finding corroborated by numerous subsequent studies, including a meta-analysis (16). Rs17070145 is associated with Alzheimer’s disease (AD) (3, 17). KIBRA, moreover, has additive and epistatic interactions with APOE (18), the ε4 allele of which is the strongest genetic risk factor for sporadic AD. The effect of SNP rs17070145 on memory and AD may arise due to differential activation of the MAPK pathway (19), important for memory and learning processes. In human, rat and mouse brains, KIBRA is mainly expressed in memory-related regions, including hippocampus and cortex, as well as cerebellum and hypothalamus (15, 20, 21).

---

1 The abbreviations used are: AD, Alzheimer’s disease; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; AUC, analytical ultracentrifugation; CBR, calcium binding region; ENM, elastic network model; KIBRA, Kidney- and BRAin-expressed protein; MD, molecular dynamics; C2(C771A), C771A mutant of KIBRA C2 domain; NLS, nuclear localization signal; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PI(3)P, phosphatidylinositol 3-phosphate; PI(4,5)P\(_2\), phosphatidylinositol 4,5 bisphosphate; PI(3,4,5)P\(_3\), phosphatidylinositol 3,4,5 trisphosphate; PME, particle mesh Ewald; RMSD, root mean square deviation; SNP, single nucleotide polymorphism; varC2, naturally occurring variant (M734I, S735A) KIBRA C2 domain; varC2(C771A), C771A mutant of variant KIBRA C2 domain; wtC2, wild type KIBRA C2 domain; WWC, WW- and C2-domain-containing protein; XRC, X-ray crystallography.
KIBRA C2: distinctive phosphoinositide and Ca\(^{2+}\) binding

KIBRA acts in the same pathway as protein kinase Mζ (PKMζ), a brain-specific kinase thought to be involved in long term memory storage through its control of neurotransmitter trafficking, particularly trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA-Rs), the major excitatory neurotransmitter receptors in the brain (22). KIBRA binds, co-localises with and is phosphorylated by PKMζ (11, 23), and KIBRA is involved in AMPAR trafficking (24). KIBRA counteracts proteasomal degradation of PKMζ (25).

In addition, KIBRA binds to dendrin (26) and synaptopodin (13, 27), postsynaptic cytoskeleton organisers important for synaptic transmission and cognition. Aberrant acetylation of tau, linked to cognitive deterioration in dementia, disrupts postsynaptic function by reducing postsynaptic KIBRA (4, 28). Consistent with these accumulated observations, KIBRA overexpression and knockdown exert positive and negative effects respectively on key molecular and cellular processes in neurons (29). It has been shown by immunoprecipitation that KIBRA can form homodimers (20) and heterodimers with the two other WWC protein family members, WWC2 and WWC3 (10), although the functional significance of this remains unknown.

Here we characterize the calcium and phosphoinositide binding properties of KIBRA C2 domain (Fig. 1), including a variant that arises from two SNPs (rs3822660G/T and rs3822659T/G) in human KIBRA that result in substitution of two adjacent residues (M734I, S735A) (30). Although the molecular consequences of these exonic SNPs have not been fully elucidated, they affect cognitive performance and there is almost complete linkage disequilibrium between the aforementioned intronic SNP rs17070145 (15) and rs3822660G/T and rs3822659T/G (30).

The C2 domain is found in more than 125 different human proteins (31, 32). Whilst most C2 domains are Ca\(^{2+}\)-dependent membrane association domains, some C2 domains do not bind Ca\(^{2+}\) and some mediate protein-protein rather than protein-lipid interactions (31, 32). Our experimental and computational investigation of wild type and variant KIBRA C2 domains indicate that KIBRA C2 is a non-canonical C2 domain that in vitro both binds phosphoinositides and associates with membranes in an atypical, calcium-independent manner.

Results

Wild type and variant KIBRA C2 adopt a typical C2 fold

Our 2.6 Å resolution crystal structure (PDB 6FD0) shows that varC2 adopts a typical eight-stranded β-sandwich C2 domain fold. The crystal form comprises a parallel dimer (i.e. the two monomers are parallel to each other) formed via an inter-monomer disulfide bond involving C771, the fifth and only unpaired cysteine in KIBRA C2 (Fig. 2A). The previously available crystal structure of wild type KIBRA C2 (PDB 220U) involves a similar parallel, C771 disulfide-linked dimer. According to AUC measurements (SI Fig. 1, SI Table 2), wtC2 and varC2 samples both comprise a mixture of monomer and dimer in solution.

On the other hand, AUC and NMR relaxation measurements (SI Figs. 1 and 2, SI Tables 2 and 3) indicate that the C2(C771A) and varC2(C771A) mutants are monomeric in solution. NMR samples of C2(C771A) and varC2(C771A) were homogeneous and stable, permitting backbone resonance assignments to be made using standard NMR methods (SI Fig. 3). Although AUC and NMR indicate that C2(C771A) is monomeric in solution, its crystal structure PDB 6FB4 (2.5 Å resolution) comprises an anti-parallel dimer (Fig. 2B), most likely formed due to crystal packing.

The backbone ¹H, ¹³C and ¹⁵N NMR chemical shifts of C2(C771A) and varC2(C771A) match closely the corresponding chemical shifts back-calculated using Sparta (33) from the C2(C771A) crystal structure (PDB 6FB4). Correspondingly, the TALOS+ (34) and Sparta (33) predictions are that the secondary structure compositions of C2(C771A) and varC2(C771A) in solution are very similar to the C2(C771A) crystal structure (SI Fig. 4). The β-sheet NOE patterns of C2(C771A) and varC2(C771A), moreover, are as expected based on the C2 topology I fold, allowing for peak overlap and absence. NMR data therefore indicate that C2(C771A) and varC2(C771A) in solution are conformationally very similar to each other (SI Fig. 5) and to C2 monomers.
in C2(C771A), varC2 (6FB4, 6FD0, respectively) and wtC2 (PDB 2Z0U) crystal structures.

Since KIBRA dimerisation has been indicated by cell-based data (10, 20), MD simulations were conducted to compare conformational stabilities of the C2 dimers observed in crystal structures. Both parallel dimers (wtC2/2Z0U and varC2/6FD0) exhibited significant changes in relative monomer position (Fig. 3) with the changes varying between repeat simulations and force fields. In wtC2 dimer simulations the mean RMSD value after three repeat simulations was 0.52 ± 0.04 nm and 0.49 ± 0.05 nm for GROMOS and OPLS forcefields, respectively. The corresponding values in varC2 simulations were 0.51 ± 0.08 nm and 1.4 ± 0.23 nm. In equivalent simulations, in contrast, the C2(C771A) anti-parallel dimer (PDB 6FB4) did not undergo significant changes in relative monomer position. There were no significant conformational changes within C2 monomers in any simulation (Fig. 3).

**NMR and X-ray diffraction data indicate that KIBRA C2-Ca\(^{2+}\) interaction is very low affinity and non-specific**

Ca\(^{2+}\) binding was investigated by NMR, XRC and simulation. In numerous \(^1\)H-\(^15\)N HSQC-monitored titrations with C2(C771A), and one with wtC2, calcium chloride addition to 10-20 mM and sometimes higher concentration (up to 76 mM) produced no significant reproducible chemical shift change, with the largest composite \(^1\)H-\(^15\)N changes around 0.05 ppm. Such small changes may be due to a very weak Ca\(^{2+}\) ion interaction (K\(_d\) > 10 mM), possibly non-specific Ca\(^{2+}\) binding to oxygen-rich surface clusters. Such a non-specific interaction is supported by simulation: when eight Ca\(^{2+}\) ions were initially positioned randomly around KIBRA C2, the final Ca\(^{2+}\) positions in eight simulations included the CBRs plus six other locations around the domain (SI Fig. 6).

It is also possible that the domain scavenged Ca\(^{2+}\) during expression and purification and was therefore Ca\(^{2+}\)-bound prior to the experiments, although this seems unlikely since particular effort was made during some C2 NMR sample preparations to exclude Ca\(^{2+}\). Initial \(^1\)H-\(^15\)N HSQC spectra were very similar, furthermore, irrespective of whether or not measures were taken to exclude Ca\(^{2+}\) from NMR samples.

In several crystal structures including wtC2 (PDB 2Z0U), and our C2(C771A) and varC2 structures, no bound Ca\(^{2+}\) has been observed, although the protein for 2Z0U was produced by cell free synthesis and the 2Z0U PDB entry does not mention any attempt to introduce metal ions. The crystal structures determined here used E. coli-expressed protein with no attempt to exclude Ca\(^{2+}\), however, so the C2 domains presumably encountered Ca\(^{2+}\) and other divalent metal ions during expression and purification. Several times C2 crystals were soaked in solutions containing divalent metal ions including 20 mM CaCl\(_2\), 100 mM CaCl\(_2\) and 100 mM MnSO\(_4\). Soaking did not cause visible disintegration of the crystals. Diffraction after soaking was still good, but these experiments yielded only one metal-bound structure which had one Ca\(^{2+}\) located in the inter-monomer interface of a C2 dimer rather than in any of the CBRs.

Overall, our NMR and XRC data are consistent with KIBRA C2 domain having very low (K\(_d\) > 10 mM), non-specific affinity for Ca\(^{2+}\). Together with PDB entry 2Z0U, moreover, our NMR and XRC data show that lack of bound Ca\(^{2+}\) does not disrupt KIBRA C2 from adopting a typical C2 domain fold.

**KIBRA C2 exhibits an unusual phosphoinositide interaction mode**

NMR and XRC studies were conducted in order to define the phosphoinositide binding site location(s) on KIBRA C2. Three NMR titrations were conducted by recording \(^1\)H-\(^15\)N HSQC spectra as a function of increasing concentration of phosphoinositide and subsequently calcium chloride. These titrations, involving C2(C771A) with PI(3)P, C2(C771A) with PI(4,5)P\(_2\), and varC2 with PI(4,5)P\(_2\), produced very similar results in which phosphoinositide-induced chemical shift changes were observed for numerous peaks, almost all located in the β-sheet comprising β-strands 5, 2, 1 and 8 (Fig. 4). L666 (β1), I677 (β2) and L678 (β2) backbone \(^1\)H-\(^15\)N peaks exhibited the largest chemical shift perturbations with changes observed for around twenty further backbone \(^1\)H-\(^15\)N peaks plus two pairs of unassigned side chain NH\(_2\) peaks
(Fig. 4). The $K_d$ for C2(C771A)-PI(3)P binding was determined as a representative case using 13 perturbed residues (SI Fig. 7); the average $K_d$ was 1 mM. After phosphoinositide titration, CaCl$_2$ was titrated into the same NMR samples to establish whether Ca$^{2+}$ ions influence C2-phosphoinositide interaction or vice versa; there was no further spectral change upon CaCl$_2$ addition.

Co-crystallization experiments involving C2(C771A) and varC2 with PI(4,5)P$_2$ or PI(3,4,5)P$_3$ yielded crystals of C2(C771A) with bound PI(4,5)P$_2$ and PI(3,4,5)P$_3$ in ProPlex HT-96 condition G1 (PDB 6FJD and 6JFC, 2.6 Å and 2.9 Å resolution, respectively). Both PI(4,5)P$_2$ and PI(3,4,5)P$_3$ bound to monomer A of an anti-parallel C2(C771A) dimer with polar interactions between R776 ($\beta$7-$\beta$8 loop) and negatively charged oxygen atoms of phosphoinositide phosphates (Fig. 5). L678 ($\beta$2) and V729 ($\beta$5) sidechains and main chain of L666 ($\beta$1) and I677 ($\beta$2) interact with the phosphoinositide inositol/aliphatic chains. The C2(C771A)-PI(4,5)P$_2$ and C2(C771A)-PI(3,4,5)P$_3$ crystal structure binding site is consistent with the phosphoinositide-induced spectral changes in the $^1$H-$^1$N HSQC titrations; the backbone NH groups of L666, I677 and L678, for example, showed the largest chemical shift changes. Phosphoinositide binding occurs with small changes in C2 structure e.g. a typical overall RMSD of 0.28 Å between unbound and phosphoinositide-bound structures.

Coarse-grained MD simulations were performed to further examine KIBRA C2 monomer and dimer association with phosphoinositides and membranes. In these simulations, which can predict the binding modes of peripheral proteins to model membrane (35, 36), the three experimentally observed forms of KIBRA C2 (monomer, anti-parallel dimer, and parallel dimer, all without Ca$^{2+}$) were displaced away from a pre-formed phosphoinositide-containing bilayer. In the primary (more frequent) binding mode observed with a C2 monomer, C2 interaction with phosphoinositides involves mainly R661, K671, R772, R776 and R779 (Fig. 6), slightly shifted from the crystal structure/NMR titration binding site. In a small number of simulations, the C2-phosphoinositide interaction involves mainly K667 and R776, coincident with the crystal structure/NMR titration binding site (Figs. 4 and 5). The parallel dimer (wtC2/2Z0U) shows two binding modes with one phosphoinositide often located close to the crystal structure/NMR titration binding site (Fig. 7). The anti-parallel dimer (C2(C771A)) exhibits a primary binding mode in which the main interactions with the membrane occur via one of the two C2 domains (Fig. 8). In this orientation, a phosphoinositide is observed near the crystal structure/NMR titration binding site. In all cases, KIBRA C2 domain binding to the bilayer causes a degree of phosphoinositide clustering around the domain. Phosphoinositides interact mainly with lysines and arginines that face the bilayer.

Discussion

KIBRA C2 structure

We have elucidated structural and functional characteristics of four forms of KIBRA C2 domain: wild type, naturally occurring variant, and C771A mutants of wild type and variant. Variant KIBRA, involving two amino acid changes in the C2 domain (M734I, S735A), affects human cognitive performance and is in almost complete linkage disequilibrium (30) with a previously identified intronic SNP that affects cognition (15, 16). wtC2 and varC2 adopt a type I topology C2 domain fold with slightly different dimerisation properties in solution (SI Fig. 1) indicating a possible effect of M734I/S735A residue differences on domain behaviour. The M734I substitution has previously been calculated to reduce C2 stability (30), although wtC2 and varC2 adopt very similar conformations in both crystal structures and in solution (SI Fig. 3 and SI Fig. 5).

It has been shown previously that full length wild type KIBRA forms dimers in mammalian cells (20). Over-expression of Flag-tagged KIBRA in HEK293 cells, moreover, resulted in large KIBRA-containing clusters (20). KIBRA also forms heterodimers with the two other WWC protein family members, WWC2 and WWC3 (10). Yeast two-hybrid mapping indicated that KIBRA dimerises in an anti-parallel orientation, with the C2 domain and one or more regions N-terminal of C2 required for this interaction (20). Whether or not
KIBRA C2: distinctive phosphoinositide and Ca\(^{2+}\) binding

KIBRA can dimerise in mammalian cells via intermolecular disulfide bonding remains open to question. Since redox potentials, and therefore likelihood of intermolecular disulfide bonding, vary according to cell type, cell status and cell compartment, a mixture of non-covalent and covalent WWC protein dimerisation modes is possible in vivo. The degree of KIBRA dimerisation and/or oligomerisation in the cell could, for example, fine tune KIBRA function as a hub for multi-protein complex assembly. Different dimerisation tendencies of wtC2 and varC2 could then provide an indication of the molecular mechanism(s) underpinning the link between variant KIBRA C2 and improved cognition (30). Other components of the Hippo pathway are modulated by dimerisation; indeed, YAP2L and TAZ form disulphide-mediated dimers that are more stable and more oncogenic than the corresponding monomers (37). Finally, since oxidative stress can promote disulfide bond formation in cytoplasmic proteins (38), the extent of disulfide-mediated KIBRA dimerisation, and hence KIBRA function, could be regulated by oxidative stress. It is potentially relevant in this context that KIBRA-associated pathways can be redox-modulated (39) and that oxidative stress is a hallmark of AD (40).

**Calcium binding**

Any KIBRA C2-Ca\(^{2+}\) interaction is low affinity (K\(_d\) > 10 mM); we did not observe bound metal ions in any of our six C2 crystal structures, with or without crystal soaking with Ca\(^{2+}\) or other divalent metal ions, or bound phosphoinositide; and there is no bound Ca\(^{2+}\) in the previous wtC2 structure (PDB 2Z0U). In NMR-monitored Ca\(^{2+}\) titrations, moreover, the C2(C771A) \(^1\)H-\(^{15}\)N HSQC spectrum did not change significantly. Unlike KIBRA C2, most C2 domains bind Ca\(^{2+}\) with sub-mM affinity. For example, synaptotagmin I C2B domain has a Ca\(^{2+}\) affinity of about 500 \(\mu\)M (41), perforin C2 ~200 \(\mu\)M (42), and rabphilin-3A C2B domain has a particularly high Ca\(^{2+}\) affinity with K\(_d\) values of 7 \(\mu\)M and 11 \(\mu\)M (43) explained by contributions from C2-flanking residues (44). Like KIBRA C2, however, some C2 domains show very weak or no residual calcium binding e.g. rat synaptotagmin 4 C2A domain binds Ca\(^{2+}\) with a K\(_d\) around 10 mM, and rat synaptotagmin 4 C2B domain essentially cannot bind Ca\(^{2+}\), despite the lack of an obvious sequence reason (45). The very low Ca\(^{2+}\) affinity of KIBRA C2 could at least partly arise from sub-optimal sequences for Ca\(^{2+}\) binding in CBR1 and CBR3 (SI Fig. 8).

**Phosphoinositide binding and membrane association**

C2 domains have been shown to bind phospholipids via the Ca\(^{2+}\) binding regions (31, 46), where the phospholipid contributes to the Ca\(^{2+}\) coordination sphere. C2 domains also bind phospholipids and phosphoinositides via a \(\beta3-\beta4\) lysine-rich cluster (31, 47, 48). Our XRC data involving C2(C771A), however, indicate that KIBRA C2 domain binds phosphoinositide in a novel way involving residues from strands \(\beta1, \beta2, \) and \(\beta8\) (Fig. 5, PDB 6FJC, 6FJD). Given this unusual binding mode, we checked whether C2 dimerisation and/or crystal packing hinders phosphoinositide binding at the previously identified C2-phosphoinositide binding sites: anti-parallel C2 domain dimerisation with strands \(\beta7\) and \(\beta8\) forming the inter-monomer interface, as observed in our C2(C771A) crystal structures, obscures neither CBRs nor \(\beta3-\beta4\) lysine-rich cluster. Similarly, phosphoinositide interaction at the lysine-rich cluster is not prevented by crystal packing: the cluster is not occluded by symmetry-related molecules. Hence steric factors do not explain why the previously observed phospholipid binding modes were not observed for KIBRA C2.

Essentially the same phosphoinositide binding site was also observed in NMR titrations of two phosphoinositides, PI(3)P and PI(4,5)P\(_2\), with C2(C771A) (monomeric), and of PI(4,5)P\(_2\) with varC2 (parallel dimer in crystal structure, Fig. 2), although the binding is relatively low affinity. The NMR-observed binding involved residues in \(\beta5\) as well as \(\beta1, \beta2, \) and \(\beta8\) (Fig. 4). Hence the observed \(\beta1, \beta2, \beta5\) and \(\beta8\) phosphoinositide binding site persisted, irrespective of whether the particular version of KIBRA C2 is predominantly monomer, anti-parallel dimer, or parallel dimer.
Possible reasons why phosphoinositide interaction with KIBRA C2 does not occur at previously identified C2-phosphoinositide binding sites include the fact that KIBRA C2 does not conform to the consensus sequence of the β3-β4 lysine-rich cluster (SI Fig. 9) and may not have sufficient positively charged residues in the vicinity to support phosphoinositide binding. The lack of a lysine-rich cluster may reflect the fact that KIBRA is not required to promote membrane fusion and hence does not need to induce membrane curvature. Also, clashes occur when C2(C771A) and PI(4,5)P₂-bound rabphilin 3A structures are overlaid; the side chain of KIBRA residue E757, for example, lies between phosphates 4 and 5 of PI(4,5)P₂, and apparently would repel these phosphate groups of the phosphoinositide; a glutamate occurs at this position in the C2 domains of all three WWC proteins, but an asparagine occurs here in seven of the eight other aligned C2 domains (SI Fig. 9). In addition, our observation that KIBRA C2 binds calcium very weakly, or perhaps not at all, with consequent lack of electrostatic bridging between KIBRA C2 negatively charged side chains and negatively charged phosphoinositides, helps to explain why phosphoinositide binding is not observed at the CBRs in KIBRA C2. Although the phosphoinositide head group is normally expected to be the key moiety, it is also worth considering whether the type of non-polar tail influences binding; we used diC₄ forms of phosphoinositides for reasonable solubility whilst retaining an aliphatic tail. Previous C2 studies have used a range of phosphoinositides, including just the PI(4,5)P₂ head group (50, 51), diC₈ tails (49), and seemingly full length tails (47), all resulting in typical C2-phosphoinositide binding sites. It seems unlikely therefore that phosphoinositide tail type is a significant factor in our observation of an unusual phosphoinositide binding site in KIBRA C2.

MD simulations indicate that when associated with membranes, KIBRA C2 can bind to multiple phosphoinositides. As observed for other membrane-bound proteins (52), this results in clustering of phosphoinositide lipids around the protein, with phosphoinositides binding to lysines and arginines that face the membrane. Such phosphoinositide lipid clustering around KIBRA C2 is expected to change the local lipid environment. This, together with the fact that in some simulations we observe secondary orientations of KIBRA C2 relative to the membrane, could influence partner protein recruitment and consequently processes such as trafficking of receptors involved in learning and memory.

In summary, experimental and simulation data indicate that KIBRA C2 is a non-classical C2 domain that can associate with membranes in a distinctive side-on, Ca²⁺-independent manner. Further investigation is required to tease out the detailed functional significance of this unusual mode of membrane association, and consequently its implications for molecular mechanisms of learning and memory, organ size control, and major diseases. Finally, we caution that we have observed this interaction mode using an isolated C2 domain in vitro. Although we have identified several factors that explain the observed binding mode, in the cell it is possible that other factors such as protein binding partners and membrane composition/structure alter KIBRA C2-membrane association mode.

**Experimental procedures**

**Cloning, protein expression and protein purification**

Sequences encoding residues 658-785 of wild type and variant (M734I, S735A) human KIBRA, and C771A mutants of these, were PCR-amplified and inserted into pQE30 (Qiagen). Unless otherwise stated, the forms of KIBRA C2 are abbreviated as follows: wild type C2 = wtC2, variant C2 = varC2, C771A mutant C2 = C2(C771A), variant C771A mutant C2 = varC2(C771A) (details in SI Table 1). The resulting proteins, with the sequence MRGSHHHHHHGS N-terminal to G658 of human KIBRA, were expressed using BL21(DE3) with IPTG induction and purified as described previously (53). ¹⁵N- and ¹⁵N/¹³C-labelled C2 domain samples for nuclear magnetic resonance (NMR) were produced by expression in M9 minimal medium supplemented with 1 g/L ¹⁵NH₄Cl as the sole nitrogen source or 1 g/L ¹⁵NH₄Cl and 2 g/L ¹³C glucose as the sole nitrogen and carbon sources, respectively. For NMR and analytical ultracentrifugation (AUC), C2 was exchanged into
10-50 mM MES, pH 6.5, with 50-150 mM NaCl. For crystallisation screens, C2 was exchanged into 10 mM MES, pH 6.5, 75 mM NaCl.

**NMR**

NMR data for resonance assignment (SI Fig. 3) were acquired at 35 °C on a 600 MHz Varian Unity INOVA spectrometer with an ambient temperature probe at University of Bath, or on cryoprobe-equipped 700 MHz Bruker or 800 MHz Varian/Agilent spectrometers at the MRC Biomedical NMR Centre, Mill Hill. Protein concentration used for 3D NMR experiments was in the range 0.5 mM to 0.8 mM. NMR data were processed using NMRPipe/NMRDraw (54) and analyzed using CCPN Analysis (55). Backbone chemical shifts for C2(C771A) and varC2(C771A) were deposited in BMRB, accession numbers 27429 and 27430, respectively. Talos (34) was used to predict C2 domain secondary structure from the assigned NMR chemical shifts. For comparison, secondary structure was predicted from crystal structures by using Sparta (33) to convert the coordinates to chemical shifts, and then using those to predict secondary structure.

Numerous $^1$H-$^{15}$N HSQC-monitored titrations of C2(C771A), and one of wtC2, with CaCl$_2$ were conducted, including attempts to produce NMR samples that were initially calcium-free which involved buffer solutions made with commercial calcium-free water and treatment with EGTA. Typical initial sample conditions for these experiments were 50 mM MES, pH 6.5, 50 mM NaCl, and 10 mM MES pH 6.5, 150 mM NaCl, sometimes with EGTA (1 mM or 2 mM) as an additional measure to try to ensure that C2 was not Ca$^{2+}$-bound to begin with. Final CaCl$_2$ concentrations in some of these titrations exceeded 15 mM, in one case reaching 76 mM.

Titrations of C2(C771A) with phosphatidylinositol 3-phosphate (PI(3)P) and phosphatidylinositol 4,5 bisphosphate (PI(4,5)P$_2$) were monitored via $^1$H-$^{15}$N HSQC spectra recorded at Mill Hill (700 MHz cryoprobe system), and titration of varC2 with PI(4,5)P$_2$ was monitored via $^1$H-$^{15}$N HSQC spectra recorded in-house (600 MHz ambient temperature probe). Protein concentration used for these titrations was in the range 0.3 mM to 0.5 mM. DiC4 versions of phosphoinositides (Echelon Biosciences) were used in these NMR experiments and in X-ray crystallography (XRC) (described below). The molar ratio of C2 to diC4-phosphoinositide at each titration point was 1:0, 1:0.25, 1:0.50, 1:0.75, 1:1 and 1:1.5 (phosphoinositide cost prohibited higher phosphoinositide levels); a $^1$H-$^{15}$N-HSQC spectrum was recorded at each titration point. CaCl$_2$ was then titrated into the same NMR sample with ratios of C2 to CaCl$_2$ of 1:0.25, 1:0.50, 1:0.75, and 1:1; a $^1$H-$^{15}$N-HSQC spectrum was recorded after each CaCl$_2$ addition. $^1$H-$^{15}$N chemical shift changes are reported as $\Delta \delta_{av}$ (ppm), where $\Delta \delta_{av}$ (ppm) = $[(\Delta \delta_{av}^2 + \Delta \delta_{av}^2(\gamma_3/\gamma_1))/2]^{0.5}$ (56).

**Analytical ultracentrifugation (AUC)**

Sedimentation velocity scans were recorded for four (varC2) or five (wtC2, C2(C771A), varC2(C771A)) concentrations of each construct; concentrations are listed in each panel of SI Fig. 1. All experiments were performed at 50000 rpm, using a Beckman XL-I analytical ultracentrifuge with an An-50Ti rotor. Data were recorded using the absorbance (at 280 nm) and interference optical detection systems. The density and viscosity of the buffers were measured using a DMA 5000M densitometer equipped with a Lovis 200ME viscometer module. The partial specific volume for each protein was calculated using Sednterp from the amino acid sequence. Data were processed using SEDFIT (57), fitting to the c(s) model (SI Table 2).

**X-ray crystallography**

Crystallization was performed using ProPlex and JCSG+ screens (Molecular Dimensions), utilizing the hanging drop vapour diffusion method at 18°C. VarC2 crystallised in 0.15 M (NH$_4$)$_2$SO$_4$, 0.1 M MES pH 6.0, 15% w/v PEG 4000, C2(C771A) (no ligand) crystallised in 0.1 M Na HEPES pH 7.5, 0.8 M NaH$_2$PO$_4$, 0.8 M KH$_2$PO$_4$, and for the lipid-bound studies, the NMR samples containing ligand were crystallised in 0.1 M Tris pH8.0, 1.5 M (NH$_4$)$_2$SO$_4$. Glycerol was used as a cryoprotectant at 25% v/v for all but the C2(C771A) (no ligand) crystals where 5% was sufficient. Diffraction data were collected using a Rigaku MicroMax 007HF...
KIBRA C2: distinctive phosphoinositide and Ca\(^{2+}\) binding

with a Saturn 944+ CCD detector. Data were processed with D*Trek or HKL-2000 (58). Molecular replacement was carried out with BALBES (59), and the model refined using Coot (60) and Phenix (61). The resultant structures were evaluated using MolProbity (62, 63). Data collection and processing statistics are given in S1 Table 4.

**Coarse-grained molecular dynamics (CG-MD) simulations**

CG-MD simulations were performed using the Martini 2.1 force field (64, 65) and GROMACS (66). In the CG-MD simulations the protein (in monomeric and dimeric forms) was displaced away from a preformed bilayer. Protein orientation relative to the bilayer was different at the beginning of each repeat simulation. 25 repeat simulations of 2 \(\mu\)s each were conducted. The bilayer consisted of POPC (~73%), POPS (~20%), PI(4,5)P\(_2\) (~5%) and phosphatidylinositol 3,4,5 trisphosphate (PI(3,4,5)P\(_3\)) (~2%). All systems were energy-minimized and subsequently equilibrated (for 500 ns) with the protein backbone particle restrained. For the production simulation the time step was 20 fs, the pressure was 1 bar and the temperature was 323 K. Berendsen's algorithm (67) was used to control the pressure and the temperature. An elastic network model (ENM) was applied to all backbone particles with a cut-off distance of 0.7 nm (68). The LINCS algorithm was used to constrain bond lengths (69) and the Lennard-Jones interactions were shifted to zero between 0.9 nm and 1.2 nm. Coulombic interactions were shifted to zero between 0 and 1.2 nm.

**Atomistic molecular dynamics (AT-MD) simulations**

AT-MD simulations were run at 310 K using GROMACS and two different force fields: the GROMOS96 43a1 force field (70) was used with SPC water molecules and the OPLS-AA force field with TIP4P water molecules. The velocity rescaling method (71) was used to control the temperature and the Parrinello-Rahman barostat (72) was used for pressure control. Isotropic pressure coupling was used. Bond lengths were constrained to equilibrium lengths using the LINCS method and the particle
KIBRA C2: distinctive phosphoinositide and $\text{Ca}^{2+}$ binding

Acknowledgments—We are very grateful to Geoff Kelly and Alain Oregioni, MRC Biomedical NMR Centre, Mill Hill (now Crick Institute), and Christina Redfield, Department of Biochemistry, University of Oxford, for their kind assistance with NMR data acquisition. This work was funded by the BBSRC (Biotechnology and Biological Sciences Research Council) grant number BB/J008176/1.

Conflict of interest—None of the authors has any conflict of interest to declare.


containing proteins regulate hepatic cell differentiation and tumorigenesis through the hippo signaling pathway. *Hepatology* **67**, 1546–1559


KIBRA C2: distinctive phosphoinositide and Ca$^{2+}$ binding


KIBRA C2: distinctive phosphoinositide and Ca\(^{2+}\) binding

insights into the association of PKC-C2 domain to PtdIns(4,5)P\(_2\). *Proc Natl Acad Sci USA* **106**, 6603–6607


KIBRA C2: distinctive phosphoinositide and Ca\(^{2+}\) binding

Figure 1. Schematic structure of human KIBRA protein showing locations of motifs and domains. The NLS and coiled coil regions are predicted rather than experimentally demonstrated.

Figure 2. Crystal structures of variant and mutant (C771A) KIBRA C2. (A) VarC2 parallel dimer (PDB 6FD0). (B) C2(C771A) anti-parallel dimer (PDB 6FB4). Termini, \(a\)-helices and residue 771 are labelled. In (A), the C771 label corresponds to the position of the inter-monomer disulfide bond which mediates parallel dimer formation in the wtC2 and varC2 crystal structures.

Figure 3. MD simulation of KIBRA C2 dimer conformation in solution. RMSD for wtC2 (A), varC2 (B), and varC2(C771A) (C) with RMSD for the monomers in blue and red, and for the dimer in black is shown for one of the simulations of each system. Final structures from the three repeat simulations for each system are also shown, with one monomer superimposed in blue, red, and orange, with the corresponding crystal structure in grey.

Figure 4. C2(C771A)-P(3)P interaction monitored by NMR. (A) Overlay of C2(C771A) H\(^1\)-H\(^5\) HSQC spectra as a function of increasing P(3)P concentration. Perturbed but unassigned side chain NH peaks are connected by dashed lines and denoted as "scNH". The insets highlight the chemical shift changes of backbone NH peaks of I677 (strand \(b_2\)) and W731 (strand \(b_8\)) and of L678 (strand \(b_2\)), I679 (strand \(b_2\)) and R776 (loop). Peak colours and corresponding C2(C771A): P(3)P ratios are as follows: red 1:0, pink 1:0.25, green 1:0.5, cyan 1:0.75, purple 1:1, black 1:1.5. The secondary structure elements (\(\beta\)-strands and \(\alpha\)-helices) are labelled. Also, for ease of comparison with other C2 domains, the location of canonical calcium binding regions (CBR1, CBR2, and CBR3) is indicated.

Figure 5. X-ray diffraction structure of the C2(C771A)-P(3,4,5)P\(_3\) complex. Three views of the complex (PDB 6FJC) in which rotation of view (A) about the vertical axis through approximately 90° gives view (B).
Pl(3,4,5)P$_3$ binds to chain A of an anti-parallel C2(C771A) dimer, as does P(4,5)P$_2$. (C) A closer view of the C2(C771A)-Pl(3,4,5)P$_3$ interaction in the same orientation as (A), with electron density obtained in a simulated annealing omit map around the Pl(3,4,5)P$_3$ binding site shown in blue mesh, contoured at 1.1σ. Pl(3,4,5)P$_3$ is shown mainly in magenta. Some of the C2 residues that interact with Pl(3,4,5)P$_3$ are shown (hydrophobic residues in yellow, polar residues in cyan, and positively charged residues in blue), including the side chains of L678, Q681, N727 and R776, and backbone of L666 and I677. Oxygen and nitrogen atoms are shown in red and dark blue, respectively.

**Figure 6. Coarse-grained MD simulation of KIBRA C2 monomer association with membrane.** (A) Coarse-grained simulation set up. Snapshots are shown from one of the simulations at 0 μs, ~0.2 μs and 2 μs. KIBRA C2 domain is shown in grey, lipid phosphate atoms in orange and phosphoinositide lipids in green. (B) A snapshot of one of the simulations showing the preferred orientation of the C2 domain relative to the bilayer. (C) Alignment of C2 domain from the simulations (grey) with Pl(3,4,5)P$_3$-bound C2 crystal structure (blue). The phosphoinositides bound to C2 in the simulations are also shown. Oxygen atoms on lipids are shown in red, hydrogen atoms in white, and carbon atoms in cyan.

**Figure 7. Coarse-grained MD simulation of KIBRA C2 parallel dimer association with membrane.** (A) Primary and (B) secondary binding mode of the KIBRA C2 parallel dimer from simulation. KIBRA C2 domain is shown in grey, lipid phosphate atoms in orange and phosphoinositide lipids in green. Residues K667 and R776 are shown in blue. (C) and (D) Alignment of C2 domain from the simulations (grey) with the Pl(3,4,5)P$_3$-bound C2 crystal structure (blue). The phosphoinositides bound to C2 in the simulations are also shown. The phosphoinositide molecule that is close to the phosphoinositide binding site observed by XRC and NMR is shown in green. Oxygen atoms on lipids are shown in red, hydrogen atoms in white, and carbon atoms in cyan.

**Figure 8. Coarse-grained MD simulation of KIBRA C2 anti-parallel dimer association with membrane.** (A) Primary orientation of the varC2(C771A) anti-parallel dimer relative to the bilayer. The C2 dimer is shown in grey and lipid phosphate atoms in orange. (B) Phosphoinositide binding sites on KIBRA C2 resulting from simulation. The residues in each site are shown in a different color. (C) The varC2(C771A) crystal structure is shown in the same orientation as in (A) and (B). Residues K667 and R776 that interact with the phosphoinositide analogue are shown as yellow spheres. Oxygen atoms on lipids are shown in red, hydrogen atoms in white, and carbon atoms in cyan.
KIBRA C2: distinctive phosphoinositide and Ca$^{2+}$ binding

Figures

Figure 1

![Diagram of KIBRA C2 domain structure with WW1, WW2, NLS, C2, Glu rich binding, and PDZ binding regions marked.](image)
KIBRA C2: distinctive phosphoinositide and Ca^{2+} binding

Figure 2
Figure 3

A

B

C

Crystal structure

wtC2

varC2

C2(C771A)
KIBRA C2: distinctive phosphoinositide and Ca$^{2+}$ binding

Figure 4
**KIBRA C2: distinctive phosphoinositide and Ca$^{2+}$ binding**

Figure 5

![Image 1](image1)

Figure 6

![Image 2](image2)
Figure 7

A Primary binding mode

B Secondary binding mode

Figure 8

A MD simulations

B Crystal structure

772, 671, 673  667, 776  695, 716  699, 714  742
KIBRA C2: distinctive phosphoinositide and Ca$^{2+}$ binding