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Common exonic missense variants in the C2 domain of the human KIBRA protein modify lipid binding and cognitive performance

K Duning[1,10], DO Wennmann[1,10], A Bokemeyer[1,10], C Reissner[2], H Wersching[3], C Thomas[1], J Buschert[1], K Guske[1], V Franzke[1], A Flöel[4,11], H Lohmann[4], S Knecht[4], S-M Brand[5], M Pöter[6], U Rescher[6], M Missler[2], P Seelheim[7], C Pröpper[8], TM Boeckers[8], L Makuch[9], R Huganir[9], T Weide[1], E Brand[1], H Pavenstädt[1,10] and J Kremerskothen[1,10]

The human *KIBRA* gene has been linked to human cognition through a lead intronic single-nucleotide polymorphism (SNP; rs17070145) that is associated with episodic memory performance and the risk to develop Alzheimer's disease. However, it remains unknown how this relates to the function of the KIBRA protein. Here, we identified two common missense SNPs (rs3822660G/T [M734I], rs3822659T/G [S735A]) in exon 15 of the human *KIBRA* gene to affect cognitive performance, and to be in almost complete linkage disequilibrium with rs17070145. The identified SNPs encode variants of the KIBRA C2 domain with distinct Ca^{2+} dependent binding preferences for monophosphorylated phosphatidylinositols likely due to differences in the dynamics and folding of the lipid-binding pocket. Our results further implicate the KIBRA protein in higher brain function and provide direction to the cellular pathways involved.
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Introduction

KIBRA (also called WWC1 for WW-and-C2-domain–containing-protein-1) represents a cytoplasmic protein highly expressed in kidney and brain that was recently linked to higher brain functions,[1–5] presumably based on differences in hippocampal processing.[6,7] Intriguingly, a genome-wide screen for candidate genes affecting human cognitive functions and memory formation revealed an intronic single-nucleotide polymorphism (SNP; rs17070145, residing in the ninth intron of the human *KIBRA* gene) that was associated with superior performance in episodic memory tasks.[5] Although a number of subsequent studies reproduced and confirmed the association between rs17070145 SNP and cognitive performance in different cohorts,[4–6,8] including a meta analysis[9] and a putative protective association against Alzheimer's disease,[10] the impact of the intronic variant on KIBRA protein function remains elusive.

KIBRA is a member of the WWC family of proteins that was initially identified to interact with the actin-binding protein dendrin[1] as well as with the atypical protein kinase C ζ (PKCζ).[2] KIBRA contains two amino terminal WW domains that are known to bind to PPxY motifs in target molecules (for example, dendrin and synaptopodin), a C2 domain and a carboxyterminal-binding motif for PSD-95/Discs-large/ZO-1 modules.[1,4,11] C2 domains were initially identified as Ca^{2+}-sensitive lipid-binding motifs in PKC, but are also found in various other proteins including the family of synaptotagmins and phosphoinositide-specific phospholipase C.[12] In contrast to other lipid-binding motifs (for example, Pleckstrin homology domains, Bin–Amphiphysin–Rvs domains, Phox-homology domains, Fab-YOTB-Vac1-EEA1 domains), C2 domains display a unique structure that lacks a defined lipid-binding pocket and a conserved cationic patch.[13] Whereas the majority of known C2 domains have a role in Ca^{2+}-dependent vesicle membrane association, these protein modules are also involved in Ca^{2+}-insensitive membrane targeting as well as in intracellular protein–protein interactions.[13] Although the function of the KIBRA C2 domain in membrane association has not been analyzed yet, a recent study in null-mutant mice demonstrated that KIBRA is involved in the regulation of the vesicle-based turnover of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the hippocampus.[7] Additional studies suggested that KIBRA may also function in other cellular processes including regulation of endosomal vesicle sorting,[14] directed cell migration[11,15] and establishment of cell polarity.[16]

In this study, we describe the identification of two novel adjacent SNPs in the human *KIBRA* gene that results in

[1]Internal Medicine D, Department of Nephrology, Hypertension and Rheumatology, University Hospital Münster, Münster, Germany; [2]Department of Anatomy and Molecular Neurobiology, University Münster, Münster, Germany; [3]Institute of Epidemiology and Social Medicine, University of Münster, Münster, Germany; [4]Department of Neurology, University Hospital Münster, Münster, Germany; [5]Institute of Sports Medicine, University of Münster, Münster, Germany; [6]Institute of Medical Biochemistry, Center for Molecular Biology of Inflammation, Münster, Germany; [7]Institute of Biochemistry, University of Münster, Münster, Germany; [8]Institute of Anatomy and Cell Biology, University Ulm, Ulm, Germany and [9]Howard Hughes Medical Center, John Hopkins University, Baltimore, MD, USA
Correspondence: Dr J Kremerskothen, Internal Medicine D, Department of Nephrology, Hypertension and Rheumatology, University Hospital Münster, Albert-Schweitzer-Campus 1, 48149 Münster, Germany.
E-mail: kremers@uni-muenster.de
[10]These authors contributed equally to this study.
[11]Current address: Department of Neurology, Center for Stroke Research Berlin, Cluster of Excellence NeuroCure, Charite Universitätsmedizin, Berlin, Germany.
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structural changes within the C2 domain of the encoded protein. Genetic analysis demonstrated a striking linkage between the exonic SNPs affecting the C2 domain and the well-characterized intronic SNP known to be associated with human cognitive performance. A biochemical characterization revealed a Ca^{2+}-dependent-binding specificity of the KIBRA C2 domain for monophosphorylated phosphatidylinositols. Finally, the identified exonic SNPs were shown to affect the lipid-binding specificities of the C2 domain accompanied by changes in the structural dynamics of the protein motif. In conclusion, our data elucidate the function of the KIBRA C2 domain in membrane association and present for the first time a linkage between natural KIBRA protein variants and cognitive performance in humans.

Materials and methods

Population-based cohort study and neuropsychological testing. We used clinical, neuropsychological and genetic data of 545 participants aged above 50 years from the population-based SEARCH Health study (Systematic evaluation and alteration of risk factors for cognitive health). Details of the study protocol were described elsewhere.[8,17] Sixteen single-neuropsychological tests were condensed into four composite scores using principal component analysis (varimax rotation and including coefficients with absolute values above 0.4), reflecting the cognitive domains of verbal memory, word fluency, working memory and executive functions/psychomotor speed.[8,17]

Identification and genotyping of KIBRA polymorphisms. Genomic DNA was extracted from white blood cells using QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). We screened 8773 bp for genetic variants using single-strand conformation polymorphism analysis and subsequent direct sequencing (ABI 3730). As both exon 15 SNP variants (rs3822660 and rs3822659) were in complete linkage disequilibrium with one another (tested by direct sequencing in 100 individuals), only the frequency of exonic SNP variant rs3822660 in the study population was determined by restriction fragment length polymorphism. For rs3822660, genomic DNA of all individuals of the study population was amplified with primers 5′-GGCTGACCCAAGATTTCCTGC-3′ and 5′-GCTGACCGGCCTTCTGTGTT-3′, respectively, using a touch-down PCR program. The annealing temperature started at 70 °C and was decreased by 1 °C/cycle for the first 12 cycles, followed by 25 cycles at 56 °C. The resulting PCR products (308 bp) were digested with *Hin*1II restriction endonuclease and visualized on ethidium bromide-stained agarose gels. The T allele lacks the *Hin*1II site, so only in the presence of the G allele, the PCR product was cut into two fragments (153 bp and 155 bp). The frequency of the intronic variant rs17070145 was determined as previously described.[8]

Statistical analysis. As both exon 15 SNP variants (rs3822660 and rs3822659) were in complete linkage disequilibrium with one another, only KIBRA rs3822660 GT (heterozygote for the variant) and TT (homozygote for the variant) genotypes were combined and tested against KIBRA rs3822660 GG ('wild type', homozygote) genotype. Effects of the KIBRA rs3822660 on the cognitive domains were tested with multivariable analyses of variance using the general linear model procedure in SAS 9.2 (www.sas.com/software/sas9/). The model included the following covariates: age, gender, education, history of arterial hypertension, intake of antihypertensive medication, history of diabetes, lifestyle index, history of depression and intake of antidepressive medication. Allelic associations of KIBRA rs3822660 and rs17070145 were tested using a χ^2 test.

Reagents. Standard highest-grade reagents were from Sigma-Aldrich (Deisenhofen, Germany).

Antibodies. Monoclonal antibodies directed against glutathion-S-transferase (GST) were purchased from Sigma-Aldrich. Horseradish peroxidase-conjugated secondary antibodies were purchased from Dianova (Hamburg, Germany).

Preparation of GST fusion proteins. Human KIBRA cDNA[1] (Acc. No. AF506799) fragments were cloned into a modified pGEX-KG vector. The constructs pGEX-KG, pGEX-KG-human KIBRA C2 wild-type (WT; aa 661–776), pGEX-KG-KIBRAC2 var. (I734/A735) were used for the isopropyl β-D-1-thiogalactopyranoside-induced expression of GST fusion proteins in *Escherichia coli* BL21 as previously described.[11] GST and GST-C2 fusion proteins were purified from bacterial lysates using glutathione sepharose beads (Amersham Bioscience, Freiburg, Germany) according to the manufacturer's instructions.

Western blotting. Western blotting experiments were performed using standard techniques.[11] Antibodies were diluted in blocking buffer containing 5% skim milk powder in phosphate-buffered saline (PBS)/Tween. After washing, the membranes were incubated with respective secondary antibodies coupled to horseradish peroxidase. Finally, the membranes were washed and developed using the Lumi-Light chemiluminescence detection kit (Roche, Mannheim, Germany) and an X-ray film developer.

Liposome co-sedimentation assay. Lipid-binding assay was performed as described earlier.[18] In brief, brain lipid extracts (total bovine brain lipids, Folch fraction I, Sigma, Munich, Germany) were resuspended at 2 mg ml^{-1} in binding buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol) at 50 °C, and liposomes were formed using a LiposoFast mini extruder (Avestin, Mannheim, Germany). Subsequently, 0.6 mg ml^{-1} liposomes were incubated with purified proteins (0.5 μM) in 100 μl buffer for 15 min at 37 °C and were centrifuged at 140 000 *g* for 15 min at 4 °C. After removing the supernatants, pellets were washed once with binding buffer and subjected to SDS-polyacrylamide gel electrophoresis. Sedimented GST or GST-KIBRA C2 proteins were detected using blotting analysis with anti-GST antibodies.

Phospholipid overlay (phosphatidylinositolphosphate (PIP) strip) assay. For the PIP strip assays, phospholipid membranes (Echelon, Salt Lake City, UT, USA) were blocked with 3% bovine serum albumin (BSA) in TRIS-buffered saline

containing 0.2% Tween-20 (TBST) for 1 h at room temperature. Afterwards, membranes were incubated in buffer containing GST–C2 fusion proteins or GST alone (each 0.5 $\mu g\,ml^{-1}$) in the presence or absence of calcium (1 mM $CaCl_2$ or 5 mM ethylene glycol tetraacetic acid) in 3% BSA/TBST at 4 °C overnight.

Unbound protein was washed away with TBST (three times for 10 min). For the detection of bound protein, anti-GST antibodies were used. Blots were incubated for 1 h at room temperature with primary antibodies diluted in 3% BSA/TBST followed by incubation with appropriate secondary antibodies coupled to horseradish peroxidase. After three washing steps with TBST the immunoreactivity was visualized with the enhanced chemiluminescence detection system.

Lipid plate-binding assays. Wells of 96-well microtiter plates (Immuno Plate Maxisorp, Nunc) were coated with 100 ng per well of the respective lipid and air-dried overnight. After blocking the wells with 5% BSA in PBS for 1 h, purified GST-C2 proteins (500 ng per well) in 1 mM Ca^{2+}-containing PBS was added for 1 h at room temperature. Subsequently, wells were washed three times with PBS containing 0.05% Tween-20 in an ELISA plate washer (Tecan, Crailsheim, Germany). The amount of bound complex was determined by a colorimetric reaction using GST-specific antibodies (Sigma), peroxidase-coupled secondary antibodies (Dianova) and tetramethylbenzidine substrate (Thermo Scientific, Dreieich, Germany) measured at 450 nm in an ELISA plate reader (MWG-Biotech, Ebersberg, Germany).

Structural analysis. We used the recently deposited coordinates of the calcium- and ligand-free C2 structure of KIBRA (protein data bank (PDB) Id: 2Z0U) at the PDB to generate structural models of the single (M734I, S735A) and double variant (M734I + S735A). Two calcium ions have been placed according to the predictions of 3DLigandSite and the calcium coordination found in rabphilin-3A (PDB Id: 2K3H). Stabilities of wild-type and variant structures were compared by calculating difference binding energies $\Delta\Delta G$ using FoldX. Dynamic structures were generated using the method of normal and geometric parameters that have been determined with DynDom.[19] Model structures were visualized using pymol (http://www.pymol.org).

Results

Identification of genomic KIBRA variants. To search for novel genetic variations that may have a functional impact on the KIBRA protein, we performed a screening of the human *KIBRA* gene by PCR/single stranded conformation polymorphism and subsequent direct sequencing, and identified 21 genetic variants (Figure 1a). Importantly, among the four missense SNPs identified in our screen, two variants (rs3822660 and rs3822659) were located in exon 15, leading to adjacent amino acid substitutions (M734I and S735A) within the KIBRA C2 domain (Figure 1b). In addition, we observed that both SNPs were in complete linkage disequilibrium with one another (see Materials and Methods for details). To evaluate a putative effect of these exonic SNPs on human cognition, we next performed cognitive testing in a large cohort of volunteers (SEARCH Health study, $n = 545$),[17] and genotyped the entire study population for rs3822660 (as described in Materials and Methods). Of the 545 participants studied, 485 carried rs3822660 GG ('C2 wild

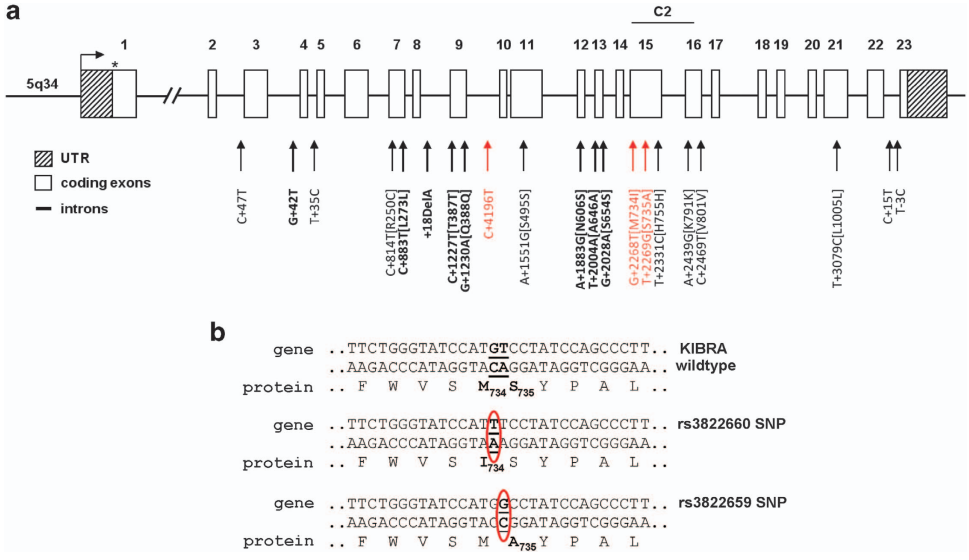

Figure 1 Identification of *KIBRA* gene variants. (**a**) Schematic representation of the *KIBRA* gene structure with exons 1–23. A right angled arrow indicates the transcription start site and an asterisk the translation start site. Using single-strand conformation polymorphism analysis and subsequent direct sequencing of 8773 bp of the *KIBRA* gene in total, 21 genetic variants were detected. Black arrows indicate the location of identified, annotated variants in exonic and/or intronic regions (C + 47T (intron 2) rs2287703, T + 35C (intron 3) rs10064053, C + 814T[R250C] rs17551608, C + 4196T (intron 9) rs17070145, A + 1551G[S495S] rs12054944; G + 2268T[M734I] rs3822660, T + 2269G[S735A] rs3822659, T + 2331C[H755H] rs3822658, A + 2439G[K791K] rs3733981, C + 2469T[V801V] rs3733980, T + 3079C[L1005L] rs3203960, C + 15T (intron 22) rs2305724, T-3C (intron 22) rs17731898). Red arrows indicate the location of detected newly identified variants within the *KIBRA* gene (G + 42T (intron 3), C + 883T[L273L], + 18DelA (intron 8), C + 1227T[T387T], G + 1230A[Q388Q], A + 1883G[N606S], T + 2004A[A646A] and G + 2028A[S654S]). (**b**) Exonic rs3822660 or rs3822659 single-nucleotide polymorphisms (SNPs) lead to a M734I or S735A amino-acid replacement, respectively. UTR, untranslated region.

type (WT)'; 89%), 56 were carriers for GT (10.3%) and 4 individuals bore TT ('C2 var.'; 0.7%; Table 1). In this population, rs3822660 T carriers showed a significantly better performance in word fluency before and after adjustment for common risk factors compared with non-carriers (Table 1). Although these results indicate that exonic polymorphisms in KIBRA can have gain-of-function potential, T carriers did not generally fare better in cognitive tests because we also found a significantly reduced performance in executive functions as evidenced by reduced psychomotor speed in the same population (Table 1). Both effects showed a tendency to be stronger in women, albeit this was not significant based on the number of individuals included in our study (Table 1). Interestingly, we determined that exonic rs3822660 identified here and the previously reported intronic rs17070145[4,5] were in almost complete linkage disequilibrium as 96.7% rs3822660 T allele carriers also carried the rs17070145 T allele (Table 2; χ^2 test, $P<0.0001$). These findings suggest that not the intronic rs17070145 SNP on its own but the linked exonic rs3822660 and rs3822659 SNPs have an influence on the structural characteristics and biological function of KIBRA.

Lipid-binding specificity of KIBRA C2 domain variants. Our discovery of two common exonic SNPs allows us to relate the polymorphisms to testable defects in protein function. Sequence analysis of the KIBRA C2 domain that is affected by the exonic variants shows the prototypical arrangement of two four-stranded β sheets (β1-8) that are connected by three loops (loop 1–3) and a single short α helix (Figure 2a). Sequence alignment of the C2 domains from human KIBRA, rabphilin-3a and synaptotagminI (Figure 2a) and with KIBRA from other species (Supplementary Figure 1A) and related WWC2 and WWC3 proteins (Supplementary Figure 1B) demonstrated the presence of conserved amino acid residues in the β sheet and loop regions (Figure 2a), and revealed that the position of our exonic variants coincides with a moderately conserved region preceding the α helix (Figure 2a; Supplementary Figure 1A and B). To test whether these common amino acid substitutions affect the function of the KIBRA C2 domain, we first analyzed the membrane-binding capacity of the wild-type domain. We purified recombinant GST-KIBRA C2 fusion proteins (hereafter named GST-C2 WT, see Materials and Methods for details) and used them in a co-sedimentation assay with purified liposomes from bovine brain (Folch fraction I). Although GST as a negative control did not precipitate in the presence of Folch liposomes (Figure 2b, upper row, lane 4), a significant amount of GST-C2 WT was found in the liposome-containing pellet fraction after ultra-centrifugation (Figure 2b, lower row, lane 4). Precipitation of GST-C2 WT was not observed in samples without liposomes (Figure 2b, lower row, lane 2), demonstrating that the KIBRA C2 domain possesses binding activity toward brain liposomes. Next, we assessed the lipid-binding specificity of the recombinant GST-C2 WT protein using a commercially available array of common cellular phospholipids (PIP strips). In this assay, GST-C2 WT protein bound specifically and strongly to phosphatidylinositol-3-phosphate (PI(3)P) but not to other poly-phosphorylated PIPs (for example, PI(4,5)P_2) or phosphatidylserine and phosphatidylcholine (Figure 2c). GST alone as a negative control showed no binding to the phospholipids spotted on the membranes (Figure 2c). To test whether binding of the GST-C2 WT protein to PI(3)P is Ca^{2+} dependent as determined for other C2 domain proteins,[12] we performed the PIP strip assays in the presence of the calcium chelator ethylene glycol tetraacetic acid. Ethylene glycol tetraacetic acid completely abolished binding of GST-C2 WT to PI(3)P, indicating that the KIBRA C2 domain is a Ca^{2+}-sensitive lipid-binding domain (Figure 2c). To finally confirm the results from the PIP strip assays, we established an ELISA-based lipid-binding assay for KIBRA C2 domain. For this, defined concentrations of PI(3)P, PI(4)P, PI(5)P or PI(4,5)P_2 were immobilized and incubated with recombinant GST or GST-C2 WT. Quantification of bound GST fusion proteins mostly validated our data from the PIP strip experiments because it revealed a binding preference of GST-C2WT for PI(3)P. However, using this highly sensitive assay, we also detected a weaker interaction between GST-C2 WT and PI(4)P, PI(5)P and PI(4,5)P_2 (Figure 2d). These data give evidence for a function of the KIBRA C2 domain as Ca^{2+}-sensitive lipid-binding domain with preference for PI(3)P but also raised the question if this function was altered by the amino acid substitutions described above.

To evaluate the impact of rs3822660 and rs3822659 on protein function, we amplified the DNA encoding the C2 domain from an individual carrying both minor alleles, and

Table 1 Association of rs3822660 genotype with cognitive domains

	Verbal memory	*Word fluency*	*Working memory*	*Executive functions/ psychomotor speed*
All participants ($n=545$)	$\beta=-0.08$ $P=0.53$	$\beta=0.27$ $P=0.04$	$\beta=-0.01$ $P=0.95$	$\beta=-0.26$ $P=0.04$
Women ($n=304$)	$\beta=-0.09$ $P=0.56$	$\beta=0.33$ $P=0.07$	$\beta=-0.25$ $P=0.14$	$\beta=-0.28$ $P=0.11$
Men ($n=241$)	$\beta=-0.03$ $P=0.89$	$\beta=0.26$ $P=0.20$	$\beta=0.29$ $P=0.13$	$\beta=-0.22$ $P=0.25$

Regression coefficient β and corresponding *P*-value for the association of the GT/TT genotype (GG = reference) with the four cognitive domains in all participants and in subgroups according to gender. Based on multivariable linear regression models adjusted for age, gender, education, intake of antihypertensive medication, history of hypertension, history of diabetes, lifestyle index, history of depression and intake of antidepressive medication.

Table 2 Association of rs3822660 SNP ('exonic variant') with rs17070145 ('intronic variant')

rs17070145	*rs3822660*		
	GG (n = *485*)	*GT* (n = *56*)	*TT* (n = *4*)
CC ($n=234$)	232 (47.84%)	2 (3.57%)	0 (0.0%)
CT ($n=234$)	196 (40.41%)	37 (66.07%)	1 (25.0%)
TT ($n=77$)	57 (11.75%)	17 (30.36%)	3 (75.0%)

Abbreviation: SNP, single-nucleotide polymorphism.
Frequencies shown in absolute numbers and percentage of the rs3822660 genotype. See Materials and Methods for details.

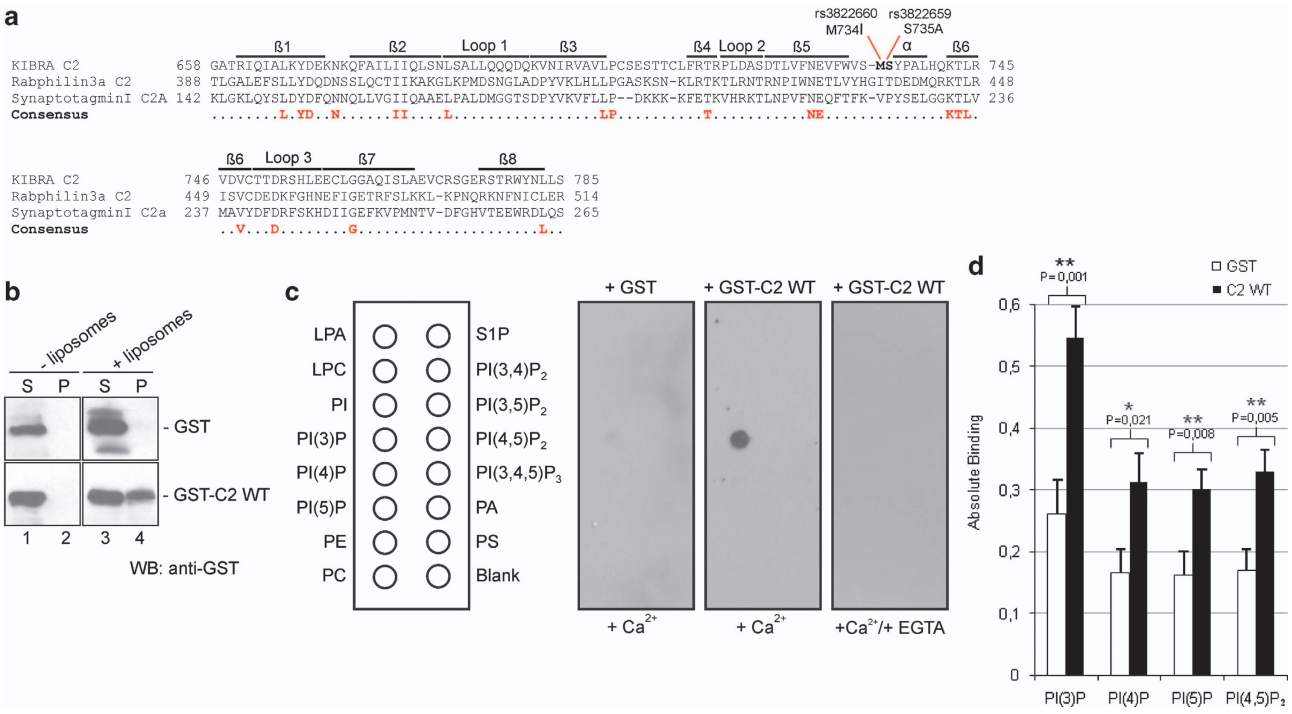

Figure 2 Structure and lipid-binding properties of the KIBRA C2 domain. (**a**) Sequence alignment of C2 domains from human KIBRA (GenBank Acc. No. NP_056053), human rabphilin-3A (GenBank Acc. No. AAH17259) and human synaptotagminI (GenBank Acc. No. EAW97344). Single-nucleotide polymorphisms rs3822660 and rs3822659 lead to amino acid replacements (M734I/S735A) in the KIBRA C2 domain close to the unique α-helix localized between β strands no. 5 and 6. (**b**) KIBRA C2 domain binds to brain liposomes. Whereas glutathion-*S*-transferase (GST) was exclusively found in the supernatant (S) fraction after sedimentation of liposome complexes (lane 3, upper panel), GST-C2 wild-type (WT) was detected in the supernatant and the liposome-containing pellet (P) fraction (lanes 3/4, lower panel). No sedimentation of GST and GST-C2 WT protein was found in the absence of brain liposomes (lane 2). (**c**) Binding of recombinant GST or GST-C2 WT to phosphoinositides and other lipids spotted onto a nitrocellulose membrane (phosphatidylinositolphosphate strips). GST-C2 WT bound strongly and exclusively to phosphatidylinositol-3-phosphate (PI(3)P) in the presence of Ca^{2+}. The Ca^{2+} chelator ethylene glycol tetraacetic acid (EGTA) completely abolished PI(3)P binding of GST-C2 WT. Abbreviations: LPA, lysophosphatidic acid; LPC, lysophosphocholine; PI, phosphatidylinositol; PI4P, PtdIns(4)P; PI5P, PtdIns(5)P; PI(3,4)P_2, PtdIns(3,4)P_2; PI(3,5)P_2, PtdIns(3,5)P_2; PI(4,5)P_2, PtdIns(4,5)P_2; PI(3,4,5)P_3, PtdIns(3,4,5)P_3; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S1P, sphingosine 1-phosphate; WB, Western blotting. (**d**) Lipid binding of the KIBRA C2 domain determined in an ELISA-based assay. GST C2 WT displays a binding preference for PI(3)P and a moderate affinity for PI(4)P, PI(5)P and PI(4,5)P_2. Binding assays were performed eight times. Bar graphs indicate the mean value ± s.e.m. *Significant; **highly significant.

expressed this fragment as a GST-C2 I734/A735 fusion protein (hereafter named GST-C2 var). We first compared the lipid-binding specificity of the variant C2 domain with the wild-type C2 domain (M734/S735). PIP strip assays revealed that the GST-C2 var binds to PI(3)P at similar levels as the GST-C2 WT (Figure 3a), suggesting that I734 and A735 did not simply disrupt the integrity of the domain. However, we also observed that in contrast to the wild type the variant C2 domain interacts stronger with PI(4)P and even more so with PI(5)P (Figure 3a). These findings were then confirmed by the quantitative ELISA-based lipid-binding assay indicating that the two amino-acid substitutions within the KIBRA C2 domain in fact altered its binding preference for monophosphorylated phosphatidylinositols (Figure 3b).

Structural analysis of the KIBRA C2 domain variants. To uncover the molecular determinants of the altered phospholipid-binding profile caused by the novel SNP variants (Figures 2 and 3), we first analyzed the wild-type KIBRA C2 domain (PDB ID: 2Z0U), which has a fold similarity to other C2 domains in, for example, rabphilin, synaptotagmin or PKC. Unlike most C2 domains, the KIBRA C2 structure exists as a homophilic dimer that is covalently linked by a cysteine bridge between the C771 residues of different molecules (Figure 4a). Internally, the KIBRA C2 structure reveals two additional cysteine bridges between C705-C711 and C749-C759 that also constitute a unique feature of this particular C2 domain (Figure 4a). As the available structure is of high quality (Supplementary Figure 2) but was solved from a ligand-free crystal, we applied modeling to define the residues of the Ca^{2+}- and PIP-binding sites in KIBRA in analogy to known ligand-bound structures of rabphilin (PDB ID: 2K3H) and PKC (PDB ID: 2B3R). The data revealed demonstrated that KIBRA is most likely able to bind two Ca^{2+} ions with loops 1 and 3 (Figure 4b). In addition, our analysis predicted that PIP either participates in Ca^{2+} coordination at the same site or binds to basic residues at the concave surface that is build by helix α2 and loop 3 (Figure 4c). This is an important result because it has been shown for other C2 domains that the participating loops undergo conformational changes and are stabilized by the binding of Ca^{2+} ions, PIP or both.[20,21] Because of the involvement of loop 3 at both sites in KIBRA, the conformational changes induced by PIP most likely affect Ca^{2+} binding and vice versa as demonstrated in rabphilin-3A.[22] In fact, our observation that PIP binding is Ca^{2+} dependent in

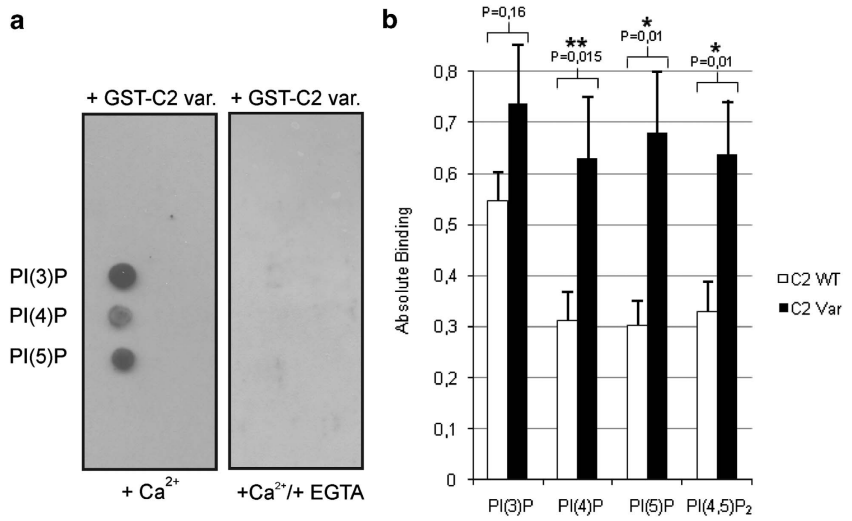

Figure 3 Different lipid-binding specificity of the KIBRA C2 domain isoforms. (**a**) Lipid-binding specificities of glutathion-*S*-transferase (GST)-KIBRA variant (var) determined in a phosphatidylinositolphosphate strip assay. The GST-C2 var protein interacts with phosphatidylinositol-3-phosphate (PI(3)P), but also with PI(4)P and PI(5)P, respectively. Similar to GST-C2 wild-type (WT), lipid binding of GST-C2 var was completely abolished in the presence of ethylene glycol tetraacetic acid (EGTA). **(b)** Comparison of the lipid-binding specificities of KIBRA C2 WT and KIBRA C2 var in the ELISA-based assay revealed a binding preference of GST-C2 WT for PI(3)P and a moderate affinity for PI(4)P, PI(5)P and PI(4,5)P_2. Compared with GST-C2 WT, the GST-C2 var displays a strong binding to PI(3)P and also to PI(4)P, PI(5)P or PI(4,5)P_2. Bar graphs indicate the mean value of ± s.e.m. calculated from eight performed binding assays. *Significant; **highly significant.

KIBRA (Figure 2c) suggests a mechanistic scenario similar to synaptotagmin, in which the negatively charged surface of the Ca^{2+}-free structure of the C2A domain repels association to phospholipid membranes but gets attracted by binding to one or more Ca^{2+} ions because of an electrostatic switch.[23] However, in contrast to synaptotagmin that contains two positionally flexible C2 domains (PDB ID: 1DQV),[24,25] the conformation of the C2 dimer of KIBRA is constrained through a cysteine bridge of C771. Consequently, the KIBRA C2 is only able to bind to phospholipid membranes in *cis* configuration (Supplementary Figure 3A) or with a single C2 domain (Supplementary Figure 3B).

We next addressed the question how our newly discovered variants from SNPs rs3822660G/T (M734I) and rs3822659T/G (S735) may affect the C2 domain structure and their properties. As both variant residues are located distant to the PIP- and Ca^{2+}-binding sites and to the membrane-binding loops 1 and 3 (Supplementary Figure 3), the effect of the double variant on the binding profile for phospholipids (Figure 3) must result from indirect modifications of the PIP-binding site. To investigate this possibility, we modeled a KIBRA C2 structure carrying the double variant M734I and S735A, and calculated the change of free binding energy ($\Delta\Delta G$) using FOLDX.[26] Interestingly, we observed that the amino-acid variant I734/A735 reduces protein stability because of a sterically non-favored hydrophobic interaction of I734 (Supplementary Figure 4). Although M734 of the wild-type C2 structure is in hydrophobic contact to the cysteine bridge of C705 and C711 (Figure 4d) that connects β-strands β6 and β7 and exposes the two conserved PIP-binding residues R699 and R714, the I734 variant structure lacks this hydrophobic contact (Figure 4d). As PIP2 binding to PKC changes the curvature of the C2 domain,[27] we propose that the M734I variant alters the connectivity of the interior hydrophobic cluster, which would change the dynamics of the curvature and the geometry of residues of the basic belt toward a preference for PI(4,5)P2. To test this idea, we performed an additional analysis of the protein dynamics of wild-type and variant C2 domains using the elastic normal mode method,[28] and indeed observed a change in the dynamics within the concave PIP-binding region and the Ca^{2+}-binding site (Cα-Cα-difference distance L712-L756 doubles for variant C2; and Supplementary Movie 1). Although nuclear magnetic resonance or electron spin resonance determination of C2 structures are desirable to further elucidate the exact residues that participate in PIP coordination and substrate specificity, an aim that has defied experimental efforts thus far for any C2 domain, our modeling-based analysis revealed a logical structural explanation for the effect of SNPs rs3822660G/T and rs3822659T/G in KIBRA.

Discussion

Earlier studies have clearly demonstrated a linkage between KIBRA expression and higher brain functions.[5–10] However, the impact of KIBRA on molecular processes within neurons remains elusive. Our biochemical and structural analyses demonstrated that the KIBRA C2 domain has a *bona fide* lipid-binding capacity with a preference toward PI(3)P that is altered in the variant carrying I734 and A735. These results clearly raise the question how the association of the C2 domain with PI(3)P-containing membranes contributes to KIBRA function in neurons and higher brain functions. As PI(3)P is enriched on membranes of subpopulations of endosomes,[29] KIBRA is likely involved in the regulation of vesicular trafficking within the endo-lysosomal system. Interestingly, in excitatory neurons, postsynaptic PI(3)P levels control endosome formation and destructive endosomal traffic

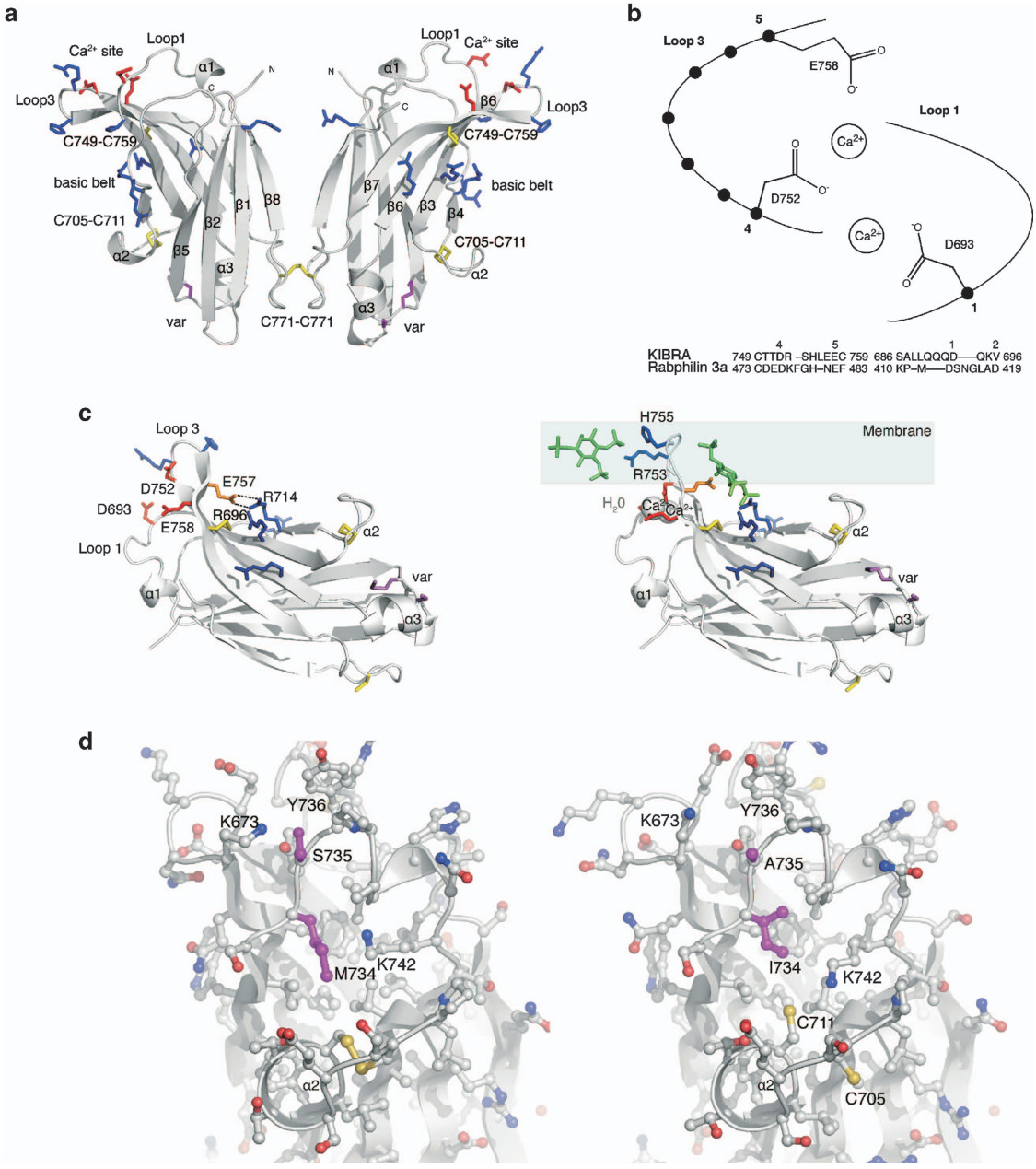

Figure 4 Structural analysis of wild-type and variant KIBRA C2 domains. (**a**) Structure of the homophilic dimeric C2 domain of human KIBRA (residues 658–785, PDB ID: 2Z0U) that represents the first C2 domain that is covalently linked (C771-C771). Two additional cysteine bridges stabilize helical loop α2 (C705-C711) and the Ca^{2+}-binding loop 3 (C749-C759). The proposed Ca^{2+}- (red) and phosphatidylinositolphosphate (PIP; blue)-coordinating residues are indicated. The single-nucleotide polymorphism variations M734I/S735A (Var) are located in a region of unknown function. (**b**) Ca^{2+} coordination in KIBRA C2 modeled in analogy to rabphilin-3A (PDB ID: 2K3H). Loop 3 is conserved with D752 binding to two and E758 to one Ca^{2+} ion, whereas loop 1 is in unique conformation because of the extended hydrophobic helical loop α1 (**a**) and a triple Q insertion. The highly conserved D693 binds to the second Ca^{2+} ion. (**c**) Proposed PIP (green) binding at the concave basic surface and to Ca^{2+}. In ligand-free KIBRA C2, the two-faced loop 3 is oriented toward the concave surface allowing E757 to make a side chain interaction to R714 (left panel). Ca^{2+} coordination requires a conformational change of loop 3 that opens the concave surface and exposes R714 to bind PIP2 (right panel). Phosphatidylserine or PIP2 may also bind to the conserved residues R753 or H755 and coordinate Ca^{2+} via a water molecule as determined for cPLA2.[35] Note that PIP to C2 interactions are Ca^{2+} dependent (Figure 2c). (**d**) Side chain geometry of wild-type (PDB ID: 2Z0U, left) and variant (model, right) KIBRA C2 domains. S735 makes hydrogen bonds to K673 that are released in the A735 variant. M734 fills a hydrophobic pocket, whereas I734 does not perfectly fit and makes non-favored sterical contacts (see also Supplementary Figure 4).

at postsynaptic sites.[30] Mutations in the human gene encoding the PI(3)P-phosphatase myotubularin 2 cause Charcot-Marie-Tooth disease type 4B1, an autosomal-recessive peripheral neuropathy associated with an impaired synaptic transmission,[31,32] implying that normal PI(3)P levels are crucial for human brain functions. KIBRA binds to protein-interacting-with-C-kinase-1, which is known to regulate the sorting of AMPA receptors from early endosomes to a specialized recycling compartment.[18] Consistently, the deletion of KIBRA expression in mice accelerated the recycling of

postsynaptic AMPA receptors in neurons indicating a role in retaining cytosolic AMPA receptor vesicles.[7] Based on these findings and our current data, we hypothesize that KIBRA may act as a local, postsynaptic docking station for AMPA receptors on PI(3)P-containing endosomes. A regulated anchoring of these vesicles probably depends on an KIBRA-mediated linkage between PI(3)P-bearing endosomal membranes and components of the postsynaptic cytoskeleton represented by the known KIBRA-interacting proteins dendrin and synaptopodin.[1,9,33,34] In this model, the lipid-binding capacity of the Ca^{2+}-sensitive KIBRA C2 domain may allow a fine-tuning of the constitutive docking-and-release process of endosomes at postsynaptic sites. Differences in the C2 domain lipid-binding specificity as demonstrated for our exonic SNPs may cause an altered AMPA receptor turnover in activated neurons and lead to changes in synaptic transmission, which in turn determines cognitive performance.

In summary, we have identified the first exonic KIBRA variants associated with cognitive functions. A detailed biochemical and structural characterization of the variant KIBRA C2 domains elucidated their distinct lipid-binding profiles. Our data provide novel insights into the mechanism how membrane-associated KIBRA might regulate neurotransmitter receptor trafficking and, thereby, determines cognitive performance.

Conflict of interest

The authors declare no conflict of interest.

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