The Potassium Channel Interacting Protein 3 (DREAM/KChIP3) Heterodimerizes with and Regulates Calmodulin Function*

Received for publication, July 5, 2012, and in revised form, September 13, 2012. Published, JBC Papers in Press, September 27, 2012, DOI 10.1074/jbc.M112.398495

From the 1Division of Nephrology and Hypertension, Departments of 2Medicine and 3Biochemistry and Molecular Biology, 4Division of Surgical Research, **Proteomics Research Center, Mayo Clinic, Rochester, Minnesota 55905

**Background:** The calcium-binding protein DREAM/KChIP3 binds DNA and other proteins to regulate neuronal function.

**Results:** DREAM/KChIP3 binds the EF-hand protein, calmodulin, in the presence but not in the absence of calcium. Calcium-bound DREAM/KChIP3 enhances calmodulin-dependent calcineurin activity.

**Conclusion:** DREAM/KChIP3 binds and regulates calmodulin activity.

**Significance:** DREAM/KChIP3 heterodimerizes with the EF-hand protein, calmodulin, and regulates calmodulin activation of calcineurin.

Downstream regulatory element antagonistic modulator (DREAM/KChIP3), a neuronal EF-hand protein, modulates pain, potassium channel activity, and binds presenilin 1. Using affinity capture of neuronal proteins by immobilized DREAM/KChIP3 in the presence and absence of calcium (Ca\(^{2+}\)) followed by mass spectroscopic identification of interacting proteins, we demonstrate that in the presence of Ca\(^{2+}\), DREAM/KChIP3 interacts with the EF-hand protein, calmodulin (CaM). The interaction of DREAM/KChIP3 with CaM does not occur in the absence of Ca\(^{2+}\). In the absence of Ca\(^{2+}\), DREAM/KChIP3 binds the EF-hand protein, calcineurin subunit-B. Ca\(^{2+}\)-bound DREAM/KChIP3 binds CaM with a dissociation constant of \(\sim 3 \mu M\) as assessed by changes in DREAM/KChIP3 intrinsic protein fluorescence in the presence of CaM. Two-dimensional \(^1H,^{15}N\) heteronuclear single quantum coherence spectra reveal changes in chemical shifts and line broadening upon the addition of CaM to \(^{15}N\) DREAM/KChIP3. The amino-terminal portion of DREAM/KChIP3 is required for its binding to CaM because a construct of DREAM/KChIP3 lacking the first 94 amino-terminal residues fails to bind CaM as assessed by fluorescence spectroscopy. The addition of Ca\(^{2+}\)-bound DREAM/KChIP3 increases the activation of calcineurin (CN) by calcium CaM. A DREAM/KChIP3 mutant incapable of binding Ca\(^{2+}\) also stimulates calmodulin-dependent CN activity. The shortened form of DREAM/KChIP3 lacking the NH\(_2\)-terminal amino acids fails to activate CN in the presence of calcium CaM. Our data demonstrate the interaction of DREAM/KChIP3 with the important EF-hand protein, CaM, and show that the interaction alters CN activity.

---

\* This work was supported, in whole or in part, by National Institutes of Health Grants AR-058003 and AR-60869. This work was also supported by a grant from the Marion and Ralph Falk Medical Trust.

1 To whom correspondence should be addressed: MS 1-120, Mayo Clinic, 200 First St. S.W., Rochester, MN 55905. Fax: 507-538-3954. E-mail: rkumar@mayo.edu.

2 The abbreviations used are: DREAM, downstream regulatory element antagonist modulator; apoDREAM/KChIP3, metal-free DREAM/KChIP3; Ca\(^{2+}\)-DREAM/KChIP3, Ca\(^{2+}\)-bound DREAM/KChIP3; CaM, calmodulin; CN, calcineurin; NCS, neuronal calcium sensor; FL, full-length; sFL, short FL; sFL-DREAM, NH\(_2\)-terminal truncated variant of DREAM containing residues from 95–256; HSQC, heteronuclear single quantum coherence; KChIP3, potassium channel interacting protein-3; DRE, downstream regulatory element.
Not all functions of DREAM/KChIP3 are readily explained by known interactions with DNA and proteins. To gain further insights into how DREAM/KChIP3 functions in cells, we investigated whether DREAM/KChIP3 interacts with other previously unidentified neuronal proteins and regulates their activity in a Ca\(^{2+}\)-dependent manner. We employed a DREAM/KChIP3 affinity capture method followed by mass spectrometric identification of bound proteins to identify a number of significant Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent DREAM/KChIP3 heterologous protein interactions. In particular, we found that DREAM/KChIP3 interacted with the EF-hand, CaM, and other NCS proteins in a Ca\(^{2+}\)-dependent manner. We confirmed the interaction of DREAM/KChIP3 with CaM with in vitro experiments and demonstrated the functional relevance of the interaction by examining the effect of DREAM/KChIP3 on Ca\(^{2+}\)-CaM stimulation of CN.

**MATERIALS AND METHODS**

**Preparation of EF-hand Mut-1234-DREAM/KChIP3 Expression Vector**—The full-length DREAM–KChIP3 cDNA in pGEX 6P1 (6) and the QuickChange Lightning Multi-site Mutagenesis kit (Agilent Technologies, Santa Clara, CA) were used for mutagenesis of nucleotide residues encoding amino acids in EF-hand 1 (E103A,D110A), EF-hand 2 (D139A,D141A), EF-hand 3 (D175A,N177A), and EF-hand 4 (D223A,N225A). As noted, acidic residues (Glu/Asp) and a basic residue (Arg) were mutated to alanine residues. The chimeric mutant pGEX 6P1-EF-hand Mut-1234-DREAM/KChIP3 plasmid was used to transform *Escherichia coli* NEB Turbo cells. The plasmid was amplified and sequenced to verify the presence of mutations.

**Protein Preparation and Purification**—Full-length (FL) DREAM/KChIP3 (amino acids 1–256), a truncated DREAM/KChIP3 variant, short full-length (sFL)-DREAM/KChIP3 (EF-hands 1–4, amino acids 95–256), and the calcium-insensitive mutant EF-hand Mut-1234-DREAM/KChIP3 were expressed and purified as previously described (6). Briefly, the proteins were expressed as NH\(_2\)-terminal glutathione S-transferase (GST) fusion products containing an intervening PreScission protease site by transforming *E. coli* BL21 cells with chimeric DREAM/KChIP3-pGEX-6P-1 plasmids (GE Healthcare). Unless stated otherwise, GST was proteolytically cleaved from the expressed chimeric proteins using PreScission protease (GE Healthcare) leaving five residues (GPLGS) as an NH\(_2\)-terminal addition to the DREAM/KChIP3 sequence. Proteins were further purified on an HR 200 Superdex preparatory column with an AKTA fast performance liquid chromatography system (GE Healthcare). Proteolytically cleaved GST was saved and used in control experiments during pulldown assays.

Human CaM was prepared as previously described (18). Briefly, human CaM cDNA was subcloned into a pET-15b expression vector (GE Healthcare). CaM was overexpressed in BL21(DE3) *E. coli* cells. CaM was purified by incubating the clarified cell lysate with 5 mM CaCl\(_2\) at 52 °C for 5 min. The precipitated protein formed during the high temperature incubation was removed by centrifugation at 4000 \(\times\) g for 10 min. The soluble protein was filter-sterilized using a 0.45 \(\mu\)m filter and applied to a phenyl-Sepharose column equilibrated with 50 mM Tris-HCl, pH 7.5, 2 mM CaCl\(_2\), 150 mM NaCl, and 2 mM dithiothreitol (DTT). The column was washed with 200 column volumes of buffer. CaM was eluted with 50 mM Tris-HCl, pH 7.5, 10 mM EGTA, 10 mM EDTA, 150 mM NaCl, and 2 mM DTT. CaM was further purified by gel filtration chromatography using a HR 200 Superdex preparatory grade column at a flow rate of 0.25 ml/min in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 2 mM DTT.

**Size Exclusion Chromatography and Analytical Ultracentrifugation**—Size exclusion chromatography experiments of 10 \(\mu\)M FL-DREAM/KChIP3 were conducted at a flow rate of 0.25 ml/min in either 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 500 mM EDTA, 2 mM DTT, or 50 mM CaCl\(_2\). Sample loading volume was 200 \(\mu\)l. Standards for size exclusion chromatography runs were purchased from Sigma. Analytical ultracentrifugation experiments were conducted at 4 °C at 15,000 rpm and repeated at 12,000 rpm with an ANTi60 rotor and a Beckman Optima XL-I centrifuge (Beckman Coulter Instruments, Indianapolis, IN) equipped with an ultraviolet/interfacial detection system as described elsewhere (19–21). Centrifugation was continued until equilibrium was achieved as determined by the super-imposition of sequential scans obtained at 4-h intervals. Samples were analyzed in duplicate within the same run. Data were fit to single species and/or self-association models with SEDPHAT (22). Buffer conditions were 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM DTT, 1.5 mM EDTA, or 500 \(\mu\)M Ca\(^{2+}\) as indicated.

**DREAM/KChIP3 Affinity Capture Assay**—Sprague-Dawley Rats fed Lab Diet 5053 were euthanized at age 2 months. Two brains without spinal cord and brain stem weighing 1.67 and 1.63 g were rinsed in phosphate-buffered saline and stored for future use. The brains were subsequently thawed and placed in 10-m1 buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM DTT, 1 mini complete, EDTA-free, protease inhibitor mixture (Roche Diagnostics) and either 2 mM EDTA (buffer A) or 5 mM CaCl\(_2\) (buffer B), respectively. Rat brains were homogenized with three, 60-s pulses of a Polytron (Brinkman Instruments) with a power setting of 8. Brain homogenate was then clarified by centrifugation at 18,000 \(\times\) g for 30 min. The soluble supernatant fractions were collected, and total protein content was quantitated by Bradford analysis.

Four columns were prepared by incubating GST-DREAM/KChIP3 or GST for 2 h at 4 °C with glutathione-Sepharose resin (Miltenyi Biotec, Auburn, CA). Total protein bound to the resin was calculated by Bradford analysis (Bio-Rad) of the protein content in solution before binding to the glutathione resin and comparing this to the flow-through after resin incubation. Two columns containing 250 \(\mu\)l of glutathione-Sepharose resin saturated with purified GST-DREAM/KChIP3 and two columns containing 250 \(\mu\)l of glutathione-Sepharose resin saturated with purified GST were prepared. All four columns were treated as noted in the steps that follow. One column of GST-DREAM/KChIP3 and one control column of GST were paired and equilibrated with 20 column volumes of buffer A, whereas the other two remaining columns were equilibrated with 20 column volumes of buffer B. Clarified rat brain homogenate in either buffer A or buffer B was divided into two equivalent volumes (4.5 ml), and 1.5 ml of each was incubated for 15 min with GST-DREAM/KChIP3 or GST equilibrated with the...
respective buffers. The remaining 3 ml of rat brain homogenate was allowed to flow through each respective column. All resins were washed with 200 column volumes of buffer A or buffer B without protease inhibitors. The resin was then washed with 10 column volumes of the previous buffer containing 2 M NaCl. Residual proteins bound to the column were eluted with 500 μl of buffer A and buffer B containing 50 mM reduced glutathione. Fractions were kept separate and run on an SDS-PAGE gel. Gels were stained with 0.02% PhastGel Blue R in 30% methanol and 10% acetic acid.

**Mass Spectrometric Identification of Interacting Proteins**—Gel bands and the interband regions were groupied and excised according to Fig. 2A. Gel bands were destained in 50% acetonitrile, 50 mM Tris, pH 8.1. Proteins within the bands were reduced with 50 mM tris(2-carboxyethyl)phosphine, 50 mM Tris, pH 8.1, at 55 °C for 40 min and alkylated with 40 mM iodoacetamide, 50 mM Tris, pH 8.1, at 22 °C for 40 min. Proteins were digested in situ with 30 μl (0.005 μg/μl) of trypsin (Promega Corp., Madison, WI) in 20 mM Tris, pH 8.1, 0.0002% H9262. The LTQ Orbitrap mass spectrometer was set to perform a Fourier transform full scans using an Eksigent nanoLC-2D HPLC system (Eksigent, Dublin, CA) and a Thermo Finnigan LTQ Orbitrap Hybrid Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany). Digested peptide mixtures were loaded onto a 250-nl OPTI-Pak trap (Optimize Technologies, Oregon City, OR) packed with a C8 solid phase (Michrom Bioresources, Auburn, CA). Chromatography was performed using 0.2% formic acid using a solvent (98% water, 2% acetonitrile) and B solvent (80% acetonitrile, 50 mM Tris, pH 8.1, at 0.2% trifluoroacetic acid for protein identification by nano-flow liquid chromatography electrospray tandem mass spectrometry using an Eksigent nanoLC-2D HPLC system (Eksigent, Dublin, CA) and a Thermo Finnigan LTQ Orbitrap Hybrid Mass Spectrometer. Two-dimensional 1H,15N heteronuclear single quantum coherence (HSQC) spectra of 15N-labeled DREAM/KChIP3 were recorded in the presence and absence of CaM. Samples contained 100 μM 15N-labeled DREAM/KChIP3 with 2 mM CaCl2, 10 mM Tris-HCl, 50 mM NaCl, 10% D2O, 10 mM DTT at pH 7.0. 50 μM unlabeled CaM was added to a second identically prepared sample.

**Identification of Significant Proteins Interacting with DREAM/KChIP3—Scaffold** (Version Scaffold_2.00.06, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identification. All identified proteins were first sorted and accepted if the number of unique peptides found during MS/MS analysis was greater that 2 and if the protein identification probability was greater than 98.8% as specified by the Peptide Prophet algorithm. Of these, proteins were eliminated if they were found in the eluates of the GST-DREAM/KChIP3 column and the GST column for each paired condition.

**Fluorescence Measurements**—Tryptophan fluorescence measurements were acquired on FluoroLog-3 spectrophotometer (Horiba Scientific, Dallas, TX) with an excitation wavelength of 295 nm and detection wavelength between 305 and 400 nm. Excitation and emission slit widths were set to 2.5 nm, whereas step size and integration time were set to 1 nm and 1 nm/s, respectively. Equilibrium dissociation constant (Kd) measurements were acquired by monitoring the tryptophan fluorescence of 5 μM DREAM/KChIP3 in 25 mM HEPES, pH 7.5, 50 mM NaCl, 100 μM CaCl2 at 4 °C as a function of the CaM concentration. Calcium titration experiments were performed by monitoring tryptophan fluorescence of 10 μM DREAM/KChIP3 in 25 mM HEPES, pH 7.5, 50 mM NaCl, 2 mM EGTA with or without 10 μM CaM. Calcium was titrated in, and the total free Ca2+ in solution was estimated by the MaxChelator Ca-EGTA calculator v1.2 using NIST critical stability constants (Maxchelator).

**NMR Spectroscopy of DREAM/KChIP3 and DREAM/KChIP3-CaM Complex**—FL DREAM/KChIP3 were expressed in minimal media, supplemented with 15NH4Cl salts, and purified as previously described (6). NMR experiments were conducted on a Bruker Avance 700 MHz NMR spectrometer equipped with a triple resonance cryogenic probe. Two-dimensional 1H,15N heteronuclear single quantum coherence (HSQC) spectra of 15N-labeled DREAM/KChIP3 were recorded in the presence and absence of CaM. Samples contained 100 μM 15N-labeled DREAM/KChIP3 with 2 mM CaCl2, 10 mM Tris-HCl, 50 mM NaCl, 10% D2O, 10 mM DTT at pH 7.0. 50 μM unlabeled CaM was added to a second identically prepared sample.

**Calcium Binding to DREAM Protein Constructs**—Calcium binding to full-length DREAM/KChIP3 and mutant EF-hand Mut1234-DREAM/KChIP3 was measured using a blotting procedure. An Immun-Blot PVDF membrane (Bio-Rad) was used in a MINIFOLD II (Schleicher & Schuell) assembled according to the manufacturer’s instructions. 60 μl of 10, 20, and 40 μg of DREAM/KChIP3 and EFMut1234-DREAM/KChIP3 in 25 mM Tris-HCl, 150 mM NaCl, pH 7.5, were incubated with 2.2 × 107 cpm of 45CaCl2 (specific activity 14.03 mCi/ml, PerkinElmer Life Sciences) for 1 h at 4 °C. Half of each solution of DREAM/KChIP3 and EFMut1234-DREAM/KChIP3 was applied to separate wells. After the protein was adsorbed to the PVDF membrane, the membrane was cut in half. Each membrane half was incubated for 15 min in a 25 mM Tris-HCl, 150 mM NaCl, pH 7.5, buffered solution containing 1 μCi/ml 45CaCl2 with or without 10 mM CaCl2. Both membranes were washed in buffer for 5 s, dried, and exposed to a phosphor screen for 3 h. The image was captured using STORM 840 phosphorimaging (GE Healthcare) set to a pixel resolution of 50 microns. Images were processed using the ImageJ program (23).

**Calcineurin Activity Assay**—A colorimetric CN phosphatase assay kit was purchased from ENZO (ENZO Life Sciences, Farmingdale, NY). Reactions were carried out in the following buffer: 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 0.1
mg/ml BSA, 4 μM CaCl₂. The final reaction was 25 μl and contained 20 units of CN, 10 μM CaM, 150 mM RII phosphopeptide substrate (DLDPVPGRFDDRVPVAAE) and varying concentrations of DREAM/KChIP3 or sFL-DREAM/KChIP3. The reactions were performed in a half-volume 96-microwell plate and were conducted in parallel with six repeats. Reactions were initiated by the addition of substrate to each well. Plates were mixed by vortexing for 15 s at a speed setting of 4 and then covered and incubated at 30 °C for 45 min. Reactions were quenched by the addition of 100 μl of BIOMOL GREEN reagent (ENZO scientific), vortexed for 15 s, and incubated for 20 min at 21 °C. The absorbance at 620 nm was recorded using the SpectraMax M2 (Molecular Devices) microplate reader. For the assay conducted using EF-hand 1234Mut-DREAM/KChIP3, all reaction volumes were doubled along with the units of CN used, and the assay was performed in a full-area, clear bottom 96-well plate. All values were compared with the experimentally determined standard phosphate curve as detailed in the phosphatase assay kit. Data were analyzed for significance using a two-tailed, two-sample equal variance Student’s t test. DREAM/KChIP3 did not change the phosphatase activity of CN when CaM was not present in the reaction.

RESULTS

Analytical Ultracentrifugation and Size Exclusion Chromatography Reveal Dissociation of DREAM/KChIP3 Dimers upon Ca²⁺ Binding—We hypothesized that conformational and oligomeric state changes occur upon the binding of Ca²⁺ to DREAM/KChIP3 that free up DREAM/KChIP3 monomers for interaction with proteins within neuronal cells. To clarify previously described changes in the oligomerization state of DREAM/KChIP3 upon Ca²⁺ binding (6, 24, 25), we performed analytical ultracentrifugation experiments on full-length DREAM/KChIP3 and sFL-DREAM/KChIP3. Size exclusion chromatography (Fig. 1) demonstrates that DREAM/KChIP3 exists solely as a dimer in the apo-state with a \( V_e/V_o \) ratio of 2.03, corresponding to a molecular mass of ~49 kDa. In the presence of 50 mM Ca²⁺, DREAM/KChIP3 dissociates to exist in equilibrium between monomeric and dimeric states and has a bi-modal distribution with a second \( V_e/V_o \) of 2.15, corresponding to a molecular mass of ~25 kDa. These data are confirmed by analytical ultracentrifugation (data not shown). The \( K_d \) values for the Ca²⁺-bound DREAM dimer, determined by fitting the data to a monomer to dimer equilibrium model, are ~1.17 and 1.52 μM for Fl-DREAM/KChIP3 and sFL-DREAM/KChIP3, respectively.

DREAM/KChIP3 Binds Several Neuronal Proteins in the Presence and Absence of Ca²⁺—To determine Ca²⁺-dependent and Ca²⁺-independent interactions of neuronal cell proteins with DREAM/KChIP3, we performed affinity pulldown assays using clarified rat brain homogenate with GST or GST-DREAM/KChIP3 columns. Four conditions were tested. Two columns contained glutathione resin saturated with GST-DREAM/KChIP3 and equilibrated with buffer containing either Ca²⁺ or EDTA (buffer A and B, respectively; see “Material and Methods”). The other two control columns contained glutathione resin saturated with GST and equilibrated with buffer A or buffer B. Total GST-DREAM/KChIP3 and GST bound to the glutathione resin were 18.4, and 9.0 mg protein/ml resin, respectively. Total protein from rat brain homogenate prepared in buffer A and applied to the glutathione columns equilibrated with buffer A was 16.63 mg. Total protein from rat brain homogenate in buffer B applied to the glutathione columns equilibrated with buffer B was 15.28 mg. Total protein bound to each column after applying 3.5 ml of homogenate to each column is summarized in Table 1. The relative molecular mass of proteins on SDS/PAGE was similar to the calculated theoretical molecular weight of identified proteins and is a good indication of minimal proteolysis. The final high salt wash before elution with reduced glutathione was included to remove any nonspecific interactions with the column resin and bound proteins. As a result, the SDS-PAGE analysis and the following mass spectrometry of gel lanes resulted in consistent and clear analysis of the sequencing data with minimal bacterial proteins identified.

A greater number of protein bands of varying molecular weight were visible by Coomassie staining of SDS-PAGE lanes in eluates from GST-DREAM/KChIP3 columns in the presence or absence of Ca²⁺ compared with the eluates from GST col-
umns run under identical conditions (Fig. 2A). Forty proteins were identified as having significant interactions with Ca\(^{2+}\)/H\(_{11001}\)-bound DREAM/KChIP3 (Table 2). Of these, 22 interacted exclusively with Ca\(^{2+}\)/H\(_{11001}\)-bound DREAM/KChIP3 and 3 with apo-DREAM/KChIP3. There were a total of 18 proteins identified as having a Ca\(^{2+}\)/H\(_{11001}\)-independent interactions with DREAM/KChIP3. Five proteins containing EF-hand motifs bound to Ca\(^{2+}\)/H\(_{11001}\)-bound DREAM/KChIP3 exclusively in the presence of Ca\(^{2+}\). Four of the bound EF-hand proteins belong to the NCS family of proteins (neuron-specific hippocalcin, neurocalcin-δ, hippocalcin-like protein-4, visinin-like protein-1) (Fig. 2B). The final EF-hand protein bound to Ca\(^{2+}\)/DREAM/KChIP3 was CaM, an important Ca\(^{2+}\)-dependent second messenger protein (Fig. 2B). One EF-hand protein, CN subunit β type 1, was found to bind to apoDREAM/KChIP3.

A large proportion of proteins that were identified as having Ca\(^{2+}\)-independent interactions with DREAM/KChIP3 belonged to the Ras-related Rab family of proteins that regulate intracellular protein and membrane traffic. Proteins that interacted exclusively with apoDREAM/KChIP3 were CN subunit β type 1, complement C3, and 40 S ribosomal protein S18. Other proteins identified include contactin-1, peroxiredoxin-2, mitochondrial fission 1 protein, and ATP synthase subunit B. The numbers of unique peptides found for proteins that interact with DREAM/KChIP3 in a Ca\(^{2+}\)-independent manner are of similar magnitude (Table 2).

**CaM Binds DREAM/KChIP3 with Micromolar Affinity**—We investigated the role of DREAM/KChIP3 in binding to and regulating the activity of CaM as the latter protein is an important EF-hand protein with multiple roles in cellular signaling (26). We investigated the Ca\(^{2+}\)-dependent interaction of DREAM/KChIP3 with EF-hand protein CaM by monitoring the intrinsic tryptophan fluorescence of DREAM/KChIP3 and sFL-DREAM/KChIP3 as a function of the Ca\(^{2+}\) concentration in the presence and absence of an equivalent molar concentration of CaM. CaM does not contain tryptophan residues, so the fluorescence contribution of CaM is minimized by selectively exciting at 295 nm to allow the observation of changes in tryptophan fluorescence of DREAM/KChIP3. The fluorescence spectrum of DREAM/KChIP3 was blue-shifted and decreased in intensity with increasing Ca\(^{2+}\) concentrations, consistent with previously reported measurements (6). In the presence of equimolar CaM, the fluorescence spectrum of DREAM/KChIP3 was strikingly different. The fluorescence emission spectrum was blue-shifted and decreased in intensity with increasing Ca\(^{2+}\) concentrations, consistent with previously reported measurements (6). In the presence of equimolar CaM, the fluorescence spectrum of DREAM/KChIP3 was strikingly different. The fluorescence emission spectrum was blue-shifted and decreased in intensity with increasing Ca\(^{2+}\) concentrations, consistent with previously reported measurements (6).

---

**TABLE 1**

| Condition  | Column             | Protein bound to resin (mg proteins/ml resin) | Protein from rat brain homogenate applied to columns (mg) | Total unbound homogenate (mg) | Total protein from homogenate bound to column (mg) | Total protein eluted with 50 mM glutathione after all wash steps (mg) |
|------------|-------------------|---------------------------------------------|---------------------------------------------------------|-------------------------------|-------------------------------------------------|---------------------------------------------------------------------|
| Ca\(^{2+}\) buffer | GST-DREAM/KChIP3  | 18.4                                        | 16.63                                                   | 12.04                         | 4.59                                            | 0.145                                                               |
|            | GST               | 9.0                                          |                                                        |                               |                                                 |                                                                     |
| EDTA buffer | GST-DREAM/KChIP3  | 18.4                                        | 15.28                                                   | 8.62                         | 6.66                                            | 0.146                                                               |
|            | GST               | 9.0                                          |                                                        |                               |                                                 |                                                                     |

**FIGURE 2.** A, shown is a SDS-PAGE analysis of eluate from each respective column. Oblong rectangles represent the sections that were dissected and then analyzed by LC/MS after tryptic digestion. The ladder is displayed in the left-most lane followed by the elutions from each respective experiment, as labeled above each lane. M.W., molecular mass. B, of the 22 proteins that were found to interact exclusively with Ca\(^{2+}\)-bound DREAM/KChIP3, 5 belonged to EF-hand proteins. For the bar graphs, light gray bars represent EF-hand proteins that interact with Ca\(^{2+}\)/DREAM/KChIP3, and the dark gray bar represents the EF-hand protein that interacts with apoDREAM/KChIP3. Only interactions determined to be significant are displayed. * denotes the gel band corresponding to ~17 kDa and displays the contrasting band intensities for that region.
shifted and increased in intensity as the Ca$^{2+}$ concentrations were increased (Fig. 3A). The increase in intensity was maximal between Ca$^{2+}$ concentrations of 1 μM and 1 mM and decreased linearly at higher concentrations of Ca$^{2+}$. In contrast, sFL-DREAM/KChIP3 and sFL-DREAM/KChIP3 in the presence of CaM had identical tryptophan fluorescence changes with increasing Ca$^{2+}$ concentrations (Fig. 3A). Similar titrations conducted with CaM alone displayed no change in fluorescence intensity, and in addition the fluorescence response curve of sFL-DREAM/KChIP3 in the presence and absence of CaM suggests that CaM does not complicate the fluorescence spectra, confirming method specificity for observing changes in DREAM/KChIP3 fluorescence. The results clearly indicate that the NH$_2$-terminal portion of DREAM/KChIP3 is necessary for binding CaM and that Ca$^{2+}$ binding to DREAM/KChIP3 results in a conformational change in DREAM/KChIP3 to confer this specificity.

Because we observed a Ca$^{2+}$-dependent fluorescence change of DREAM/KChIP3 in the presence of CaM, we sought to determine the apparent $K_d$ of the DREAM/KChIP3-CaM complex by monitoring the intrinsic tryptophan fluorescence of DREAM/KChIP3 in the presence of 100 μM Ca$^{2+}$ and as a function of the CaM concentration. A concentration of 100 μM Ca$^{2+}$ was used because of the maximal change in fluorescence and because this concentration provided a buffering window from small changes in Ca$^{2+}$ concentrations as observed at the two limits where large changes begin (1 μM and 1 mM, Fig. 3B). The fluorescence intensity increases to a maximum of 20% upon saturation. Fitting the normalized data to a nonlinear hyperbolic binding curve results in an average $K_d$ of 2.92 ± 1.11 μM (Fig. 3C). The equilibrium binding curve does not indicate nonspecific binding. A change in tryptophan fluorescence was not observed when similar experiments were conducted with sFL protein.

**NMR Spectroscopy of 15N-Labeled DREAM/KChIP3 Demonstrates Binding to CaM**—We recorded $^1$H, $^{15}$N HSQC spectra of 15N-labeled DREAM/KChIP3 to probe resonance changes upon binding to unlabeled CaM. Several resonances in the

---

**TABLE 2**

Proteins identified as having significant interactions with Ca$^{2+}$-bound and apo-DREAM/KChIP3

Unique peptide count is reported as * $^\dagger$ where * is the number of unique peptides found for the Ca$^{2+}$-DREAM condition, and $^\dagger$ is the number of unique peptides for the apo-DREAM condition. EF-hand proteins that interact with DREAM/KChIP3 are italicized.

| Identified protein | Number of unique peptides (Ca$^{2+}$/EDTA) | Molecular weight (Da) |
|-------------------|------------------------------------------|-----------------------|
| **Ca$^{2+}$ interactions only** | | |
| AP-2 complex subunit α-1 | 7/— | 107,548.70 |
| Na$^+$/K$^+$-transporting ATPase subunit α1 | 3/— | 111,751.50 |
| Active breakpoint cluster region-related protein | 4/— | 97,615.20 |
| Glycogen phosphorylase, brain form | 4/— | 96,342.60 |
| Matrin-3 | 5/— | 94,626.70 |
| Trifunctional enzyme subunit α, mitochondrial | 4/— | 82,666.90 |
| Ubiquilin-4 | 3/— | 63,506.30 |
| T-complex protein 1 subunit γ | 12/— | 60,587.20 |
| 1,25-Dihydroxyvitamin D3 24-hydroxylase, mitochondrial | 3/— | 59,450.60 |
| Isocitrate dehydrogenase | 6/— | 39,668.70 |
| NAD subunit a NAD-dependent deacteylase sirtuin 2 | 12/— | 39,320.70 |
| ATPase ASNA 1 | 5/— | 38,793.50 |
| Anionic trypsin-1 | 2/1 | 25,959.10 |
| Cotillin-1 | 3/2 | 18,533.20 |
| GTPase NRas | 3/— | 21,256.30 |
| Transcription elongation factor B polypeptide 2 | 3/— | 13,170.20 |
| Reticulin-1 | 2/3 | 83,573.60 |
| Neuron-specific calcium-binding protein hippocalcin | 3/— | 22,428.50 |
| Neurocalcin-δ | 4/— | 22,246.30 |
| Hippocalcin-like protein 4 | 2/3 | 22,303.50 |
| Calmodulin | 12/1 | 16,838.00 |
| Visinin-like protein 1 | 11/— | 22,143.40 |
| **Interactions with apoDREAM only** | | |
| Complement C3 | —/3 | 187,255.10 |
| 40 S ribosomal protein S18 | —/5 | 17,719.30 |
| Calcineurin subunit β type 1 | 1/3 | 19,300.40 |
| **Ca$^{2+}$-independent interactions** | | |
| Contactin-1 | 3/3 | 113,386.80 |
| Ras-related protein Rab-3C | 9/9 | 25,872.70 |
| Ras-related protein Rab-11B | 4/3 | 24,889.00 |
| Ras-related protein Rab-14 | 11/8 | 23,897.60 |
| Ras-related protein Rab-6A | 3/4 | 23,491.40 |
| Ras-related protein Rab-4B | 1/3 | 23,587.20 |
| Ras-related protein Rab-2A | 3/7 | 23,546.20 |
| Ras-related protein Rab-7A | 10/5 | 23,544.10 |
| Ras-related protein Rab-1A | 7/9 | 22,678.50 |
| Ras-related protein Rab-10 | 4/4 | 22,570.10 |
| Transforming protein RhoA | 10/11 | 21,768.40 |
| Ras-related C3 botulinum toxin substrate 1 | 5/6 | 21,450.60 |
| Rho-related GTP-binding protein RhoG | 6/5 | 21,308.10 |
| Cell division control protein 42 homolog | 5/4 | 21,258.80 |
| Ras-related protein Rap-1A | 8/8 | 20,987.30 |
| ATP synthase subunit B | 6/7 | 17,264.50 |
| Mitochondrial fission 1 protein | 2/8 | 17,009.20 |
| Peroxiredoxin-2 | 3/2 | 21,779.10 |
HSQC spectrum of DREAM/KChIP3 disappeared due to exchange broadening upon the addition of CaM in the presence of saturating levels of Ca²⁺/H₁₁₀₀₁ (Fig. 4). Changes in downfield peaks are clearly visible between the 9.2- and 10.2-ppm region. Slight differences in peak intensity between free Ca²⁺/H₁₁₀₀₁-DREAM/KChIP3 and Ca²⁺/H₁₁₀₀₁-DREAM/KChIP3 in complex with CaM are observed for the downfield glycine residues typical for Ca²⁺-bound EF-hand protein (10.5–11.0 ppm). The observed disappearance of NMR signals is likely due to intermediate exchange on the time scale of NMR chemical shifts between free and CaM-bound DREAM/KChIP3. Exchange broadening is consistent with the measured $K_d$ of $\sim$3 μM for the DREAM/KChIP3-CaM interaction.

**DISCUSSION**

Our findings suggest novel functional roles of DREAM/KChIP3 within neuronal cells. Our salient observation is that DREAM/KChIP3 binds CaM, another EF-hand protein, in a
Ca\(^{2+}\)-dependent manner. In the absence of Ca\(^{2+}\), DREAM/KChIP3 fails to bind CaM, whereas upon the addition of Ca\(^{2+}\), binding occurs. In the presence of Ca\(^{2+}\), DREAM/KChIP3 has a $K_d$ for CaM of $\sim 3 \mu M$. Our data show that amino-terminal 94 residues of DREAM/KChIP3 that lack EF-hand motifs are required for binding of DREAM/KChIP3 to CaM. A mutant of DREAM incapable of binding Ca\(^{2+}\) enhances CaM-stimulated CN activity, demonstrating that Ca\(^{2+}\)-dependent changes in DREAM/KChIP3 are not essential for the activation process. This is also consistent with the stimulatory role of the NH\(_2\)-terminal portion of DREAM/KChIP3, which does bind calcium. Because the binding of Ca\(^{2+}\) brings about the dissociation of dimeric DREAM/KChIP3, it suggests but does not conclusively prove that monomeric DREAM/KChIP3 binds to CaM. We cannot, however, rule out the binding of the dimer of DREAM/KChIP3 to CaM. Interestingly and of relevance to the regulation of CN (see below), apoDREAM/KChIP3 binds the regulatory subunit of CN. This interaction is abolished in the presence of Ca\(^{2+}\).

The binding of Ca\(^{2+}\)-bound DREAM/KChIP3 to CaM alters the capacity of CaM to activate the serine/threonine phosphatase, CN, which functions in diverse biological roles including the modulation of immune function, cardiac hypertrophy, and neural processes (30–32). CN plays an important role in neuronal function (32). CN-deficient mice display behavioral characteristics similar to humans with schizophrenia, memory impairment, and attention deficit disorder (33). Inhibition of CN results in CN inhibitor-induced pain syndromes (34, 35). The activation of CN by DREAM/KChIP3 requires full-length protein, Ca\(^{2+}\), and CaM. The binding of Ca\(^{2+}\) to CN results in activation of its serine/threonine phosphatase activity, and the addition of

---

**FIGURE 5. Calcineurin activity assay.** A. Shown is phosphatase activity of CN in the presence of CaM only, CaM and equimolar DREAM/KChIP3, or sFL-DREAM/KChIP3 or a 10-to-1 molar ratio of DREAM/KChIP3-to-CaM. B. Shown is phosphatase activity of CN in the presence of CaM only and CaM with equimolar DREAM/KChIP3 or EFMut1234-DREAM/KChIP3. Student’s $t$ test was performed to evaluate differences among groups. ***, $p < 0.005$; **, $p < 0.05$. 

---

**DREAM/KChIP3 Binds EF-hand Proteins**
CaM results in approximately a 10-fold increase in CN activity (36). CN is a heterodimer composed of catalytic (CNA) and regulatory (CNB) subunits. The CNA is responsible for recognizing and dephosphorylating protein substrates, and CNB is an EF-hand protein that binds Ca\textsuperscript{2+} and increases the activity of CNA. Interaction of CNA with CaM and CNB enhances the phosphatase activity of CNA (36). Calcium-bound CaM and CNB activate CNA by different mechanisms. CaM increases the rate of dephosphorylation of CNA by displacing the autoinhibitory domain, whereas binding of CNB results in an increase in affinity of CNA for its substrate (36). Calcium binding to both low affinity sites of CNB and CaM is required for CNA activation (36).

We suggest that under basal intracellular Ca\textsuperscript{2+} conditions, apoDREAM/KChIP3 binds to CNB, thus inhibiting CNA activation of CN. When intracellular Ca\textsuperscript{2+} concentrations increase, DREAM/KChIP3 dissociates from CNB, allowing CNB to activate CNA. Ca\textsuperscript{2+}-bound DREAM/KChIP3 associates with CaM to further enhance CaM activation of CNA. The differences in affinities for Ca\textsuperscript{2+} between CNB, CaM, and DREAM/KChIP3 are likely to be an important factor regulating CN activity.

The Ca\textsuperscript{2+}-dependent interactions of DREAM/KChIP3 with other neuronal EF-hand proteins suggests a potential role of DREAM/KChIP3 as a regulator of other neuronal functions. To the best of our knowledge, the interaction of an EF-hand protein to multiple other EF-hand NCS family proteins has not been previously observed. NCS-1, another EF-hand protein, has been implicated in regulating neuronal processes in a Ca\textsuperscript{2+} dependent manner, but the precise mechanism by which this occurs has not been elucidated, and it is unknown whether this involves other EF-hand partners (27, 28). Among the interacting EF-hand proteins are neuron-specific hippocampal, neurocalcin-\(\delta\), hippocalcin-like protein-4, and visinin-like protein-1, which belong to the NCS family of proteins. All four proteins are 65% homologous, which suggests the presence of a common binding mechanism or motif to DREAM/KChIP3.

Other investigators have demonstrated interactions of DREAM/KChIP3 with other proteins. For example, Rivás et al. (29) have reported that DREAM/KChIP3 interacts with a mitochondrial thiol-specific antioxidant protein, peroxiredoxin-3. Interestingly, we found that DREAM/KChIP3 binds peroxiredoxin-2 in a calcium-independent manner. These findings provide further evidence for a role of DREAM/KChIP3 in the regulation of oxidative stress within neuronal cells. In conclusion, DREAM/KChIP3 binds CaM and regulates CN function through Ca\textsuperscript{2+}-dependent mechanisms. This novel mechanism could be exploited for therapeutic purposes.

REFERENCES

1. Carrióñ, A. M., Link, W. A., Ledo, F., Mellström, B., and Naranjo, J. R. (1999) DREAM is a Ca\textsuperscript{2+}-regulated transcriptional repressor. Nature 398, 80–84
2. Ledo, F., Carrióñ, A. M., Link, W. A., Mellström, B., and Naranjo, J. R. (2000) DREAM-αCREM interaction via leucine-charged domains depresses downstream regulatory element-dependent transcription. Mol. Cell. Biol. 20, 9120–9126
3. Mellström, B., and Naranjo, J. R. (2001) Ca\textsuperscript{2+}-dependent transcriptional repression and derepression. DREAM, a direct effector. Semin. Cell Dev. Biol. 12, 59–63
4. Buxbaum, J. D., Choi, E. K., Luo, Y., Lilliehook, C., Crowley, A. C., Merriam, D. E., and Wasco, W. (1998) Calsenilin. A calcium-binding protein that interacts with the presenilins and regulates the levels of a presenilin fragment. Nat Med 4, 1177–1181
5. Choi, E. K., Zaidi, N. F., Miller, J. S., Crowley, A. C., Merriam, D. E., Lilliehook, C., Buxbaum, J. D., and Wasco, W. (2001) Calsenilin is a substrate for caspase-3 that preferentially interacts with the familial Alzheimer disease-associated C-terminal fragment of presenilin 2. J. Biol. Chem. 276, 19197–19204
6. Craig, T. A., Benson, L. M., Venyaminov, S. Y., Klimchuk, E. S., Bajzer, Z., Prendergast, F. G., Naylor, S., and Kumar, R. (2002) The metal-binding properties of DREAM. Evidence for calcium-mediated changes in DREAM structure. J. Biol. Chem. 277, 10955–10966
7. Hammond, P. I., Craig, T. A., Kumar, R., and Brimijoin, S. (2003) Regional and cellular distribution of DREAM in adult rat brain consistent with multiple sensory processing roles. Brain Res. Mol. Brain Res. 111, 104–110
8. Rivás, M., Mellström, B., Naranjo, J. R., and Santisteban, P. (2004) Transcriptional repressor DREAM interacts with thyroid transcription factor-1 and regulates thyroglobulin gene expression. J. Biol. Chem. 279, 33114–33122
9. Rivás, M., Villar, D., González, P., Dopazo, X. M., Melletstrom, B., and Naranjo, J. R. (2011) Building the DREAM interactome. Sci. China Life Sci 54, 786–792
10. Leisring, M. A., Yamaski, T. R., Wasco, W., Buxbaum, J. D., Parker, I., and LaFerla, F. M. (2000) Calsenilin reverses presenilin-mediated enhancement of calcium signaling. Proc. Natl. Acad. Sci. U.S.A. 97, 8590–8593
11. Lilliehook, C., Chan, S., Choi, E. K., Zaidi, N. F., Wasco, W., Mattson, M. P., and Buxbaum, J. D. (2002) Calsenilin enhances apoptosis by altering endoplasmic reticulum calcium signaling. Mol. Cell Neurosci. 19, 552–559
12. Alexander, J. C., McDermott, C. M., Tunur, T., Rands, V., Stelly, C., Karhson, D., Bowby, M. R., An, W. F., Sweatt, J. D., and Schrader, L. A. (2009) The role of calasin1/DREAM/KChIP3 in contextual fear conditioning. Learn. Mem. 16, 167–177
13. Cheng, H. Y., Pitcher, G. M., Laviolette, S. R., Whishaw, I. Q., Tong, K. L., Kockeritz, L. K., Wada, T., Joza, N. A., Crackower, M., Goncalves, J., Sarosi, I., Woodgett, J. R., Oliveira-dos-Santos, A. J., Ikura, M., van der Kooy, D., Salter, M. W., and Penninger, J. M. (2002) DREAM is a critical transcriptional repressor for pain modulation. Cell 108, 31–43
14. Lilliehook, C., Bozdagi, O., Yao, J., Gomez-Ramirez, M., Zaidi, N. F., Wasco, W., Gandy, S., Santucci, A. C., Haroutunian, V., Huntley, G. W., and Buxbaum, J. D. (2003) Altered DREAM/KChIP2 modulates the cardiac L-type calcium sensors. Eur. J. Cell Biol. 82, 553–556
15. Thomsen, M. B., Wang, C., Ozgen, N., Wang, H. G., Rosen, M. R., and Pitt, G. S. (2009) Accessory subunit KChIP2 modulates the cardiac L-type calcium current. Circ. Res. 104, 1382–1389
16. Zhang, Y., Su, P., Li, J., Zhu, Y. B., Yin, D. M., Li, J., Zhou, Z., Wang, K. W., and Wang, Y. (2010) The DREAM protein negatively regulates the NMDA receptor through interaction with the NR1 subunit. J. Neurosci. 30, 7575–7586
17. Juranić, N., Atanasova, E., Strefiñ, J. H., Macura, S., and Prendergast, F. G. (2007) Solvent-induced differentiation of protein backbone hydrogen bonds in calmodulin. Protein Sci. 16, 1329–1337
18. Baden, M. E., Owen, B. A., Peterson, F. C., Volkman, B. F., Ramirez-Alvarado, M., and Thompson, J. R. (2008) Altered dimer interface decreases stability in an amyloidogenic protein. J. Biol. Chem. 283, 15853–15860
19. Owen, B. A., Sullivan, W. P., Felts, S. J., and Toft, D. O. (2002) Regulation of heat shock protein 90 ATPase activity by sequences in the carboxyl terminus of Heat Shock Protein 90. Proc. Natl. Acad. Sci. U.S.A. 99, 16858–16863
20. Owen, B. A., Yang, Z., Lai, M., Gajec, M., Gajek, M., Badger, J. D., 2nd, Hayes, J. J., Edelmann, W., Kucherlapati, R., Wilson, T. M., and McMur-
ray, C. T. (2005) (CAG)(n)-hairpin DNA binds to Msh2-Msh3 and changes properties of mismatch recognition. Nat. Struct. Mol. Biol. 12, 663–670
22. Vistica, J., Dam, J., Balbo, A., Yikilmaz, E., Mariuzza, R. A., Rouault, T. A., and Schuck, P. (2004) Sedimentation equilibrium analysis of protein interactions with global implicit mass conservation constraints and systematic noise decomposition. Anal. Biochem. 326, 234–256
23. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ. 25 years of image analysis. Nat. Methods 9, 671–675
24. Osawa, M., Dace, A., Tong, K. I., Valiveti, A., Ikura, M., and Ames, J. B. (2005) Mg\(^{2+}\) and Ca\(^{2+}\) differentially regulate DNA binding and dimerization of DREAM. J. Biol. Chem. 280, 18008–18014
25. Osawa, M., Tong, K. I., Lilliehook, C., Wasco, W., Buxbaum, J. D., Cheng, H. Y., Penninger, J. M., Ikura, M., and Ames, J. B. (2001) Calcium-regulated DNA binding and oligomerization of the neuronal calcium-sensing protein, calсинelin/DREAM/KChIP3. J. Biol. Chem. 276, 41005–41013
26. Hanson, P. I., and Schulman, H. (1992) Neuronal Ca\(^{2+}\)/calmodulin-dependent protein kinases. Annu. Rev. Biochem. 61, 559–601
27. Ames, J. B., Lim, S., and Ikura, M. (2012) Molecular structure and target recognition of neuronal calcium sensor proteins. Front. Mol. Neurosci 5, 10
28. Kretsinger, R. H. (2012) Neuronal calcium sensor proteins. Recognizing a face in a crowd. Front. Mol. Neurosci. 5, 41

DREAM/KChIP3 Binds EF-hand Proteins

29. Rivas, M., Aurrekoetxea, K., Mellström, B., and Naranjo, J. R. (2011) Redox signaling regulates transcriptional activity of the Ca\(^{2+}\)-dependent repressor DREAM. Antioxid. Redox Signal. 14, 1237–1243
30. Li, H., Rao, A., and Hogan, P. G. (2011) Interaction of calcineurin with substrates and targeting proteins. Trends Cell Biol. 21, 91–103
31. Frost, R. J., and Olson, E. N. (2010) Separating the good and evil of cardiac growth by CIB1 and calcineurin. Cell Metab. 12, 205–206
32. Hara, M. R., and Snyder, S. H. (2007) Cell signaling and neuronal death. Annu. Rev. Pharmacol. Toxicol. 47, 117–141
33. Miyakawa, T., Leiter, L. M., Gerber, D. J., Gainetdinov, R. R., Sotnikova, T. D., Zeng, H., Caron, M. G., and Tonegawa, S. (2003) Conditional calcineurin knockout mice exhibit multiple abnormal behaviors related to schizophrenia. Proc. Natl. Acad. Sci. U.S.A. 100, 8987–8992
34. Sato, Y., Onaka, T., Kobayashi, E., and Seo, N. (2007) The differential effect of cyclosporine on hypnotic response and pain reaction in mice. Anesth. Analg. 105, 1489–1493
35. Kakihana, K., Ohashi, K., Murata, Y., Tsubokura, M., Kobayashi, T., Yamashita, T., Sakamaki, H., and Akiyama, H. (2012) Clinical features of calcineurin inhibitor-induced pain syndrome after allo-SCT. Bone Marrow Transplant. 47, 593–595
36. Stemmer, P. M., and Klee, C. B. (1994) Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B. Biochemistry 33, 6859–6866