Mg$^{2+}$ and Ca$^{2+}$ Differentially Regulate DNA Binding and Dimerization of DREAM*

DREAM (calsenilin/KChIP3) is an EF-hand calcium-binding protein that represses transcription of prodynorphin and c-fos genes. Here we present structural and binding studies on single-site mutants of DREAM designed to disable Ca$^{2+}$ binding to each of the functional EF-hands (EF-2: D150N; EF-3: E180Q; and EF-4: E234Q). Isothermal titration calorimetry (ITC) analysis of Ca$^{2+}$ binding to the various mutants revealed that, in the absence of Mg$^{2+}$, Ca$^{2+}$ binds independently and sequentially to EF-3 ($\Delta H = -2.4$ kcal/mol), EF-4 ($\Delta H = +5.2$ kcal/mol), and EF-2 ($\Delta H = +1.1$ kcal/mol). By contrast, only two Ca$^{2+}$ bind to DREAM in the presence of physiological levels of Mg$^{2+}$ for both wild-type and D150N, suggesting that EF-2 binds constitutively to Mg$^{2+}$. ITC measurements demonstrate that one Mg$^{2+}$ binds enthalpically with high affinity ($K_d = 13 \mu$m and $\Delta H = -0.79$ kcal/mol) and two or more Mg$^{2+}$ bind entropically in the millimolar range. Size-exclusion chromatography studies revealed that Mg$^{2+}$ stabilizes DREAM as a monomer, whereas Ca$^{2+}$ induces protein dimerization. Electrophoretic mobility shift assays indicated that Mg$^{2+}$ is essential for sequence-specific binding of DREAM to DNA response elements (DREs) in prodynorphin and c-fos genes. The EF-hand mutants bind specifically to DRE, suggesting they are functionally intact. None of the EF-hand mutants bind DRE at saturating Ca$^{2+}$ levels, suggesting that binding of a single Ca$^{2+}$ at either EF-3 or EF-4 is sufficient to drive conformational changes that abolish DNA binding. NMR structural analysis indicates that metal-free DREAM adopts a folded yet flexible molten globule-like structure. Both Ca$^{2+}$ and Mg$^{2+}$ induce distinct conformational changes, which stabilize tertiary structure of DREAM. We propose that Mg$^{2+}$ binding at EF-2 may structurally bridge DREAM to DNA targets and that Ca$^{2+}$-induced protein dimerization disrupts DNA binding.

DREAM$^1$ (downstream regulatory element antagonist modulator also known as calsenilin (1) and KChIP3 (2)) is a 29-kDa EF-hand Ca$^{2+}$-binding protein that serves as a transcriptional repressor for pain modulation (3–5). DREAM translocates into the nucleus during calcium signaling (6) and blocks transcription of prodynorphin and c-fos genes by binding to specific DNA sequences (DRE, downstream regulatory element (7, 8)) in a Ca$^{2+}$-dependent fashion (9–11). The importance of DREAM as a calcium-sensitive transcriptional repressor has been demonstrated in DREAM-deficient mice that exhibit the very striking phenotype of ongoing analgesia due to up-regulated expression of prodynorphin (12, 13). The DREAM knock-out mice also exhibit mild effects on $\beta$-amyloid production and long-term potentiation (13), but do not exhibit any motor or behavioral abnormalities. Hence, DREAM regulates pain transmission by controlling prodynorphin expression and represents an attractive therapeutic opportunity for managing pain.

DREAM contains four EF-hands similar to the Ca$^{2+}$-binding motifs found in calmodulin and troponin C (14, 15). The four EF-hands of DREAM are $>$45% identical in sequence to recoverin and related Ca$^{2+}$ sensors of the neuronal calcium sensor (NCS) subclass of the EF-hand superfamily (16, 17) (see Fig. 1). A characteristic feature of DREAM and the NCS family is the sequence CPXG (Cys-104 and Pro-105, see Fig. 1) that prevents the binding of Ca$^{2+}$ to the first EF-hand (EF-1) as seen in the crystal structures of recoverin (18), KChIP1 (19, 20), neurocalcin (21), and frequenin (22). The second EF-hand (EF-2) of DREAM contains aspartate (Asp-150) instead of the usual glutamate at the 12-position of the EF-hand binding loop (Fig. 1). The presence of aspartate at the 12-position in other EF-hand proteins is known to diminish its binding selectivity of Ca$^{2+}$ versus Mg$^{2+}$ (23). Mg$^{2+}$ binding to the second EF-hand of DREAM might be sufficient to affect DREAM structure and DNA binding. Indeed, a recent mass spectrometry study revealed that both Ca$^{2+}$ and Mg$^{2+}$ are bound by DREAM (24). Magnesium is not normally considered a regulator, but recent in vivo measurements have detected changes in free Mg$^{2+}$ concentrations in cortical neurons after treatment with neurotransmitter (25). Other NCS proteins such as GCAPs, VILIP, and NCS-1 also bind Mg$^{2+}$ and exhibit Mg$^{2+}$-induced effects (26, 27). Therefore, it is of interest to investigate the functional role of Mg$^{2+}$ binding to DREAM and determine whether Mg$^{2+}$ might control the binding of DREAM to DNA targets.

Here, we present a structural analysis of Mg$^{2+}$ and Ca$^{2+}$

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binding to the individual EF-hands in DREAM. The results reveal that Mg\textsuperscript{2+} is required for sequence-specific DNA binding; Mg\textsuperscript{2+} binds constitutively at EF-2; Mg\textsuperscript{2+} stabilizes a monomeric form of DREAM; Ca\textsuperscript{2+} induces protein dimerization; and Ca\textsuperscript{2+} binding at either EF-3 or EF-4 is sufficient to abolish DNA binding at high Ca\textsuperscript{2+}. On the basis of these results, a structural model for Ca\textsuperscript{2+}-sensitive DNA binding by DREAM will be presented.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Mouse DREAM-C and Its Mutants**—A deletion mutant of mouse DREAM (residues 65–256, named DREAM-C) was shown previously to exhibit functional binding to DNA and Ca\textsuperscript{2+} (10). The recombinant DREAM-C protein was expressed in E. coli supplemented with 1 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100, 5 mM MgCl\textsubscript{2} and CaCl\textsubscript{2} (0.5–2.0 mM), used as the titrant, were prepared using 1 M HCl twice followed by a rinse with decalcified buffer. EDTA was removed from protein solution using Econo-Pac 10 DG gel filtration column (Bio-Rad Laboratories, Inc.) pre-equilibrated in the decalcified buffer. Before the protein solution used in typical ITC experiment was performed at 25 °C on a protein solution containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM MgCl\textsubscript{2}, 1 mM dithiothreitol, and 5 mM CaCl\textsubscript{2} for Ca\textsuperscript{2+}-bound DREAM-C, or 1 mM EGTA for Mg\textsuperscript{2+}-bound DREAM-C. 0.1 ml of DREAM-C (protein concentration ranged from 10 to 460 μM) was loaded onto the column and eluted at a flow rate of 0.5 ml/min. Apparent molecular weights were calculated using a standard curve of V\textsubscript{o}/V\textsubscript{e} versus the log of the molecular masses of standard proteins: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), transferrin (81 kDa), carbonic anhydrase (29 kDa), and myoglobin (17 kDa). V\textsubscript{o} is a void volume obtained using blue dextran (2000 kDa), and V\textsubscript{e} is a volume of elution.

**Preparation of Decalcified Protein Solutions**—Decalcified ITC buffer (150 mM NaCl and 10 mM Tris-HCl, pH 7.6) was prepared by treatment with Chelex 100 resin (Bio-Rad). 1 mM dithiothreitol and 5 mM MgCl\textsubscript{2} were added after treatment with Chelex 100 and immediately before use. Containers and pipette tips were rinsed with 1 M HCl twice followed by a rinse with decalcified buffer. EDTA was removed from protein solution using Econo-Pac 10 DG gel filtration column (Bio-Rad Laboratories, Inc.) pre-equilibrated in the decalcified buffer. Before the experiment, the sample cell and injection syringe of ITC machine (Microcal Inc.) was cleaned by the decalcified buffer extensively.

**Mg\textsuperscript{2+} and Ca\textsuperscript{2+} Titration into DREAM-C by ITC**—Binding of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} to DREAM-C was measured by ITC (28) using a MicroCal VP-ITC MicroCalorimeter (MicroCal Inc.). The protein solution used in the titration was exchanged with decalcified buffer using Econo-Pac 10 desalting gel-filtration column as described above. Stock solutions of MgCl\textsubscript{2} and CaCl\textsubscript{2} (0.5–2.0 mM), used as the titrant, were prepared using decalcified buffer as a diluent. A stock solution of CaCl\textsubscript{2} with 5 mM MgCl\textsubscript{2} was the titrant for Ca\textsuperscript{2+} titration of Mg\textsuperscript{2+}-bound DREAM-C. A typical ITC experiment was performed at 25 °C on a protein solution containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 1 mM dithiothreitol for 12 h. The dialyzed sample was applied onto a HiTrap DEAR-FF column (Amersham Biosciences) previously equilibrated in the buffer, and eluted with NaCl gradient (0 to 0.3 M NaCl over 3 h) at a flow rate of 5 ml/min.

**Size Exclusion Chromatography**—Determination of the molecular weight of the purified DREAM-C in solution was carried out on a Hitachi HR 10/30 column (Amersham Biosciences) in the buffers containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM MgCl\textsubscript{2}, 1 mM dithiothreitol, and 5 mM CaCl\textsubscript{2} for Ca\textsuperscript{2+}-DREAM-C, or 1 mM EGTA for Mg\textsuperscript{2+}-bound DREAM-C. 0.1 ml of DREAM-C (protein concentration ranged from 10 to 460 μM) was loaded onto the column and eluted at a flow rate of 0.5 ml/min. Apparent molecular weights were calculated using a standard curve of V\textsubscript{o}/V\textsubscript{e} vs. the log of the molecular masses of standard proteins: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), transferrin (81 kDa), carbonic anhydrase (29 kDa), and myoglobin (17 kDa). V\textsubscript{o} is a void volume obtained using blue dextran (2000 kDa), and V\textsubscript{e} is a volume of elution.
ApoDREAM-C binds three Ca\(^{2+}\) ions—Isothermal titration calorimetric analysis of Ca\(^{2+}\) binding to apoDREAM-C. The trace of the calorimetric titration of 29 \(\times\) 10-\(\mu\)l aliquots of 1.3 mM CaCl\(_2\) into the cell containing 46 \(\mu\)m apo-DREAM-C (A), and integrated binding isotherm (B). No correction was needed for the heat of dilution, because negligible heat was observed when adding aliquots of CaCl\(_2\) (or MgCl\(_2\)) into a decalcified buffer blank.

An overlay of the Ca\(^{2+}\)-binding isotherms for each of the EF-hand mutants is shown in Fig. 3. The ITC isotherm of D150N exhibits stoichiometric binding of two Ca\(^{2+}\) ions but lacks the low affinity endothermic phase seen in wild-type, suggesting that EF-2 is the low affinity site. The isotherm of E186Q exhibits a strongly endothermic phase (assignment described above to EF-2). The isotherm of E234Q exhibits an exothermic phase (4.2 kcal/mol) assigned to Ca\(^{2+}\) binding at EF-3 and U-shaped isotherm suggest that a protein conformational change might accompany binding at EF-3.

In summary, EF-2 is an endothermic site with low affinity that gives rise to the non-stoichiometric binding in the wild-type isotherm. EF-3 and EF-4 are relatively high affinity sites (stoichiometric binding) whose enthalpies nearly cancel one another in the wild-type isotherm. A recent mass spectrometry analysis of DREAM suggested that a fourth Ca\(^{2+}\) might bind to DREAM at EF-1 (24), although the affinity of this site was not reported, and the binding of a fourth Ca\(^{2+}\) might be artificially
enhanced in gas phase species analyzed in mass spectrometry experiments. The primary sequence of EF-1 contains unfavorable substitutions (e.g. Cys-104, Pro-105, and Thr-113) that would be expected to abolish physiological Ca\(^{2+}\) binding at this site as has been demonstrated in other NCS proteins (16, 18, 19).

 Accordingly, our ITC Ca\(^{2+}\) binding measurements performed on samples of DREAM-C in solution do not detect any Ca\(^{2+}\) binding at EF-1, suggesting that EF-1 does not bind Ca\(^{2+}\) under physiological conditions or the enthalpy of binding is zero.

The wild-type Ca\(^{2+}\)-binding isotherm (in the absence of Mg\(^{2+}\)) is approximately represented as the normalized sum of isotherms for the three single-site mutants, implying three independent Ca\(^{2+}\)-binding sites. The Ca\(^{2+}\) binding also appears ordered. We suggest that Ca\(^{2+}\) binds to EF-3 first, because the beginning of the wild-type isotherm (0 to 1 equivalents) resembles that of E186Q. A second Ca\(^{2+}\) binds subsequently at EF-4, because the middle of the wild-type isotherm (from 1 to 2 equivalents) resembles that of E234Q. Lastly, a third Ca\(^{2+}\) binds to apoDREAM-C at EF-2, because the low affinity phase (beyond 2 equivalents) is abolished in the D150N isotherm. The independent and sequential Ca\(^{2+}\) binding to the EF-hands (in contrast to cooperative binding) suggests a molten globule or unstructured form of apoDREAM-C in the region, consistent with an unstructured apo-protein.

**Constitutive Mg\(^{2+}\) Binding to EF-2**—The presence of physiological levels of Mg\(^{2+}\) (5 mM Mg\(^{2+}\)) had a profound affect on the ITC Ca\(^{2+}\) binding isotherms of wild-type and EF-hand mutants (Fig. 3B). The Ca\(^{2+}\) binding isotherm of wild-type in the presence of physiological Mg\(^{2+}\) was saturated with a Ca\(^{2+}\) binding stoichiometry of two, in contrast to a stoichiometry of three in the absence of Mg\(^{2+}\) (Fig. 3A). The Ca\(^{2+}\) binding isotherm of D150N in the presence of physiological Mg\(^{2+}\) looked remarkably similar to that of wild-type and was saturated with a Ca\(^{2+}\) binding stoichiometry of two. The Ca\(^{2+}\) binding isotherms of E186Q and E234Q in the presence of physiological Mg\(^{2+}\) both saturated with a Ca\(^{2+}\) binding stoichiometry of one. These results imply that Ca\(^{2+}\) does not bind to EF-2 in the presence of Mg\(^{2+}\) (Fig. 3B), suggesting that EF-2 binds constitutively to Mg\(^{2+}\) under physiological conditions (i.e., 5 mM Mg\(^{2+}\) and 0.1–100 μM Ca\(^{2+}\)). The presence of Asp-150

**FIG. 3. Comparison of Ca\(^{2+}\) binding isotherms for DREAM-C and EF-hand mutants (D150N, E186Q, and E234Q).** Ca\(^{2+}\) binding isotherms of apoDREAM-C (without MgCl\(_2\)) and Mg\(^{2+}\)-saturated protein (5 mM MgCl\(_2\)) are depicted in A and B, respectively. Wild-type DREAM-C, D150N, E186Q, and E234Q are shown in black, red, green, and blue, respectively. A dotted line in panel B shows the normalized sum of isotherms for E186Q and E234Q mutants.

**FIG. 4. Isothermal titration microcalorimetric analysis of the Mg\(^{2+}\) binding to DREAM-C.** Trace of the calorimetric titration of 29 × 10\(^{-11}\) M aliquots of 2.0 mM MgCl\(_2\) into the cell containing 69 μM apoDREAM-C (A) and integrated binding isotherm (B). The binding isotherm in B was fit using a two-site model where \(K_1 = 0.91 ± 0.01\), \(K_2 = (7.5 ± 0.7) \times 10^4\) [M\(^{-1}\)], \(ΔH_1 = −791 ± 90\) [cal mol\(^{-1}\)]; \(ΔS_1 = 2\) (fixed), \(K_2 = (4.3 ± 0.2) × 10^3\) [M\(^{-1}\)], \(ΔH_2 = (3.97 ± 0.06) \times 10^4\) [cal mol\(^{-1}\)].
**Mg$^{2+}$ and Ca$^{2+}$ Binding to DREAM**

The enthalpic binding of Mg$^{2+}$ to DREAM-C suggests a possible Mg$^{2+}$-induced conformational change. NMR spectra of DREAM-C change quite dramatically upon the addition of saturating Mg$^{2+}$ (see below), suggesting that Mg$^{2+}$ binding stabilizes tertiary structure of DREAM-C.

Also noteworthy was our observation that each EF-hand mutant exhibited Mg$^{2+}$ binding similar to that of wild-type. Apparently, the EF-hand mutation at the 12-position in our study does not impair Mg$^{2+}$ binding like it does for Ca$^{2+}$. Different coordination numbers for Mg$^{2+}$ and Ca$^{2+}$ might explain their different binding behavior in this regard (33).

Mg$^{2+}$/Ca$^{2+}$ Effects on Oligomerization of DREAM-C—Previous dynamic light scattering and size-exclusion chromatography (SEC) analysis of metal-free DREAM-C (apoDREAM-C) originally indicated a mixture of aggregated protein species in solution (10). The presence of 10 mM lauryldimethylamine oxide detergent increased the solubility of apoDREAM-C and stabilized the metal-free protein in a tetrameric state. The DREAM tetramer is believed to bind functionally to DNA response elements in the prodynorphin and c-fos genes (9). In our current SEC study (Fig. 5), we now determine that DREAM-C in the presence of physiological Mg$^{2+}$ levels (5 mM MgCl$_2$ and absence of detergent or DNA) eluted with an apparent molecular mass of 30 kDa in solution, which is slightly higher than the mass calculated for a protein monomer (25 kDa). The slightly higher apparent molecular mass in solution (30 versus 25 kDa) might be attributed to a non-spherical shape factor or perhaps a small percentage of dimer formation and was independent of protein concentration below 0.4 mM. Dynamic light scattering analysis similarly revealed a monomeric hydrodynamic radius for Mg$^{2+}$-bound DREAM-C. These results suggest that Mg$^{2+}$ binding to one or more EF-hands (Fig. 4) stabilizes the protein mainly in a monomeric state at protein concentrations below 0.4 mM. By contrast, Ca$^{2+}$-saturated DREAM-C in the presence of Mg$^{2+}$ eluted with an apparent molecular mass of 50 kDa in solution (Fig. 5), indicating that the Ca$^{2+}$-bound protein forms a stable dimer even at very dilute protein concentrations (10). Similar calcium-sensitive hydrodynamic properties were observed for each of the EF-hand mutants (data not shown), suggesting that each mutant is structurally intact. In summary, DREAM-C appears to be a monomer under basal physiological conditions (5 mM Mg$^{2+}$ and 100 mM Ca$^{2+}$) and forms a stable dimer at saturating Ca$^{2+}$ levels. A similar Ca$^{2+}$-induced dimerization has been reported previously for other NCS proteins such as neurocalcin (21).

Mg$^{2+}$ and Ca$^{2+}$ Stabilizes Tertiary Structure of DREAM-C—NMR spectroscopy was used to probe protein conformational changes in DREAM-C induced by Mg$^{2+}$ and/or Ca$^{2+}$ binding (Fig. 6). The peaks in the $^1$H-$^1$5N HSQC NMR spectra represent main-chain and side-chain amide groups and provide a fingerprint of the overall protein conformation. The two-dimensional $^1$H-$^1$5N HSQC spectrum of apoDREAM-C exhibited poorly resolved and overlapping peaks with narrow chemical shift dispersion in the amide proton dimension (Fig. 6A). The number of observed peaks was far less than the expected number of amide groups, and the intensities of many peaks were quite weak perhaps due to exchange broadening caused by conformational

### FIG. 5. Molecular weight estimation of DREAM-C by size exclusion chromatography (SEC). **A.** Size exclusion chromatograms of Mg$^{2+}$- (solid line) and Mg$^{2+}$/Ca$^{2+}$-DREAM-C (dotted line). 100 μl of 200 μM protein solution were applied onto Superdex 200 HR (10/30) column (Amersham Biosciences) pre-equilibrated with the buffers containing 10 mM Hepes–NaOH (pH 7.6), 150 mM NaCl, 5 mM MgCl$_2$, 1 mM dithiothreitol, and 1 mM EGTA or 5 mM CaCl$_2$ for Mg$^{2+}$/Ca$^{2+}$-DREAM-C and Ca$^{2+}$-DREAM-C, respectively. B, the standard curve for the molecular mass determination was created using β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), transferrin (81 kDa), carbonic anhydrase (29 kDa), and myoglobin (17 kDa). Void volume of the column (Vo) was obtained as 7.81 ml using blue dextran (2000 kDa), and Ve is a volume of elution of each sample. Arrows indicate Ve/Vo values for Mg$^{2+}$- or Ca$^{2+}$-bound DREAM-C (50 μM). The molecular mass based on amino acid sequence of DREAM-C is 25 kDa.

heterogeneity. The poor chemical shift dispersion demonstrates that apoDREAM-C adopts an unstructured molten-globule state similar to that described for apo states of many other EF-hand proteins (30, 34, 35). Consistent with this interpretation is a lack of long range NOEs (particularly between aromatic and aliphatic resonances) in the homonuclear NOESY spectrum of apoDREAM-C (data not shown). However, circular dichroism analysis suggested that apoDREAM-C adopts a high degree of helical content, consistent with the formation of the four EF-hands, and the helical content did not change much upon binding Mg$^{2+}$ and/or Ca$^{2+}$. Taken together, our structural studies indicate that apoDREAM-C adopts native secondary structure but is in a flexible molten-globule state.

The HSQC spectrum of DREAM-C changed dramatically upon the addition of saturating Mg$^{2+}$ (Fig. 6B). Mg$^{2+}$ caused a greater number of peaks to appear that were in general sharper than those of apoDREAM-C. Mg$^{2+}$ binding to DREAM-C increased the NMR chemical shift dispersion and promoted long range NOEs in the NOESY spectrum (data not shown), demonstrating that Mg$^{2+}$-bound DREAM-C adopts at least some
stable tertiary structure in contrast to the metal-free protein. However, a number of peaks in the middle of the HSQC spectrum remained rather broad, suggesting that other parts of the Mg$^{2+}$/H11001-bound protein may not be structured or perhaps undergo conformational exchange. Pulsed-field gradient diffusion NMR studies (36) determined a hydrodynamic radius of 2.7 nm (corresponding to a spherical molecular mass of 34 kDa) for Mg$^{2+}$/H11001-bound DREAM-C under NMR conditions, which is slightly higher than that determined by SEC (Fig. 5). The apparent molecular mass for Mg$^{2+}$/H11001-bound DREAM-C observed by NMR increased monotonically as a function of protein concentration above 0.5 mM, consistent with low affinity dimer formation in the millimolar range.

The HSQC spectrum of DREAM-C changed even further upon the addition of two molar equivalents of Ca$^{2+}$/H11001 (Fig. 6C). Significant spectral changes induced by adding Ca$^{2+}$ to the Mg$^{2+}$/H11001-bound protein sample indicated that Ca$^{2+}$-induced conformational changes are distinct and separate from the Mg$^{2+}$-induced changes (Fig. 6, B and C). Two downfield-shifted peaks near 10.5 ppm are characteristic of conserved glycine residues at the 6-position of Ca$^{2+}$/H11001-occupied EF-hands, consistent with Ca$^{2+}$ bound at EF-3 and EF-4. Additional unique peaks of Ca$^{2+}$/H11001-bound DREAM-C (e.g. near 9.5 ppm) represent amino acid residues in EF-3 or EF-4 stabilized structurally by Ca$^{2+}$ binding. The HSQC spectrum of Ca$^{2+}$/Mg$^{2+}$/H11001-saturated DREAM-C exhibited variable peak intensities, suggesting exchange broadening perhaps associated with Ca$^{2+}$-induced dimerization (see Fig. 5). Pulsed-field gradient diffusion NMR studies determined a hydrodynamic radius of 3.2 nm, corresponding to a stable Ca$^{2+}$-bound protein dimer that persisted under a wide range of protein concentrations (0.05–1 mM).

**DNA Binding by EF-hand Mutants versus Ca$^{2+}$—Electrophoresis mobility shift assays (EMSA) were performed on the EF-hand mutants to monitor their Ca$^{2+}$-sensitive binding to duplex oligonucleotide derived from the downstream response element (DRE) of the prodynorphin gene (Fig. 7). The EMSA binding data for wild-type and single-site EF-hand mutants look very similar, suggesting that each of the mutants bind to the DRE and are functionally intact. A typical EMSA for DREAM is shown in Fig. 7 and exhibits two shifted bands that represent different oligomeric species of DREAM bound to
**Mg^{2+} and Ca^{2+} Binding to DREAM**

DRE. The lower shifted band has variable intensity, is not sequence-specific, is less responsive to Ca^{2+}, and may represent DNA binding by the DREAM dimer or monomer. The uppermost band (believed to represent a DREAM tetramer) has been characterized previously to represent sequence-specific binding to DRE (9, 10), and we found in this study that this band requires saturating levels of Mg^{2+}. The Mg^{2+}-dependent DRE binding to D150N mutant suggests that this mutation does not impair high affinity Mg^{2+} binding at EF-2 (Fig. 4) perhaps needed for DRE binding. The EMSA shifted bands for wild-type and each of the EF-hand mutants (D150N, E186Q, and E234Q) disappear upon the addition of saturating Ca^{2+}, suggesting that the Ca^{2+}-bound forms of the EF-hand mutants (like wild-type) do not bind to DRE. In summary, Mg^{2+} is required for sequence-specific binding to DRE and Ca^{2+} binding at either EF-3 or EF-4 is sufficient to abolish DNA binding.

**Mechanism of Ca^{2+}-sensitive DNA Binding**—In this study, we showed that Mg^{2+} is essential for DNA binding by DREAM-C; EF-2 binds Mg^{2+} (and not Ca^{2+}) under physiological conditions; EF-3 and EF-4 bind functionally to Ca^{2+} and induce protein dimerization; and binding of a single Ca^{2+} at either EF-3 or EF-4 is sufficient to drive protein conformational changes responsible for abolishing DNA binding at high Ca^{2+}. What do these structural facts imply about the mechanism of Ca^{2+}-sensitive DNA binding by DREAM? One clear implication is that Mg^{2+} binding at EF-2 may be important for promoting DREAM binding to DNA targets. The bound Mg^{2+} at EF-2 could structurally bridge DREAM to DNA, perhaps by forming an axial coordinate covalent bond with a phosphoryl oxygen atom from DNA. Previous x-ray structures have illustrated many examples where divalent cations bridge phosphate groups of DNA with various DNA-binding proteins (37–39). A similar type of metal ion bridge serves to link membrane proteins (e.g. C2 domains and annexin V) with the phosphoryl oxygen from lipid head groups (40, 41).

A second implication of this study is that Ca^{2+} binding at EF-3 and EF-4 promotes protein dimerization that somehow disrupts DNA binding by DREAM. In the Ca^{2+}-bound protein, residues at the dimer interface (perhaps in EF-3 or EF-4) might sterically block residues of the Ca^{2+}-free protein that contact DNA. The recent x-ray structure of a closely related DREAM-like protein, KChIP1, shows a dimeric structure for the Ca^{2+}-bound protein with intermolecular contacts between a hydrophobic groove (formed by EF-1 and EF-2) and a C-terminal helix adjacent to EF-4 (19). The C-terminal helix of KChIP1 (residues 205–215) forms extensive intramolecular contacts with both EF-3 and EF-4, whereas the opposite face of the C-terminal helix forms intermolecular contacts with EF-1 and EF-2. It is plausible that Ca^{2+}-induced conformational changes in either EF-3 or EF-4 would alter the disposition of the C-terminal helix and promote intermolecular contact with EF-1 and EF-2. An intriguing hypothesis follows that Ca^{2+}-induced dimerization might sterically alter the structure of EF-2 (due to an interaction with the C-terminal helix) and thereby disrupt an Mg^{2+} ion bridge with DNA. In the future, we plan to determine the atomic-level structure of DREAM-DNA complex and Ca^{2+}-induced conformational changes to more rigorously understand the structural basis of Ca^{2+}-sensitive DNA binding by DREAM.

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