The role of calcium-binding proteins in the control of transcription: structure to function

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Summary
Transcriptional regulation is coupled with numerous intracellular signaling processes often mediated by second messengers. Now, growing evidence points to the importance of Ca2+, one of the most versatile second messengers, in activating or inhibiting gene transcription through actions frequently mediated by members of the EF-hand superfamily of Ca2+-binding proteins. Calmodulin and calcineurin, representative members of this EF-hand superfamily, indirectly regulate transcription through phosphorylation/dephosphorylation of transcription factors in response to a Ca2+ increase in the cell. Recently, a novel EF-hand Ca2+-binding protein called DREAM has been found to interact with regulatory sequences of DNA, thereby acting as a direct regulator of transcription. Finally, S100B, a dimeric EF-hand Ca2+-binding protein, interacts with the tumor suppressor p53 and controls its transcriptional activity. In light of the structural studies reported to date, this review provides an overview of the structural basis of EF-hand Ca2+-binding proteins linked with transcriptional regulation.

Introduction
Calcium (Ca2+) signals in the cell are highly regulated and are generated by ion influx through voltage- and ligand-gated Ca2+-permeable ion channels, release from internal stores, and sequestration by Ca2+ pumps and exchangers.1 Changes in intracellular Ca2+ levels, in response to agonist stimulation or cell depolarization, can be displayed as highly localized pools of Ca2+ in subcellular structures, or as Ca2+ waves that spread throughout the cell including the nucleus.2 The effects of fluctuations in intracellular Ca2+ levels are mediated primarily by Ca2+-binding proteins that belong to the EF-hand superfamily.3

Calmodulin (CaM), a ubiquitous and abundant EF-hand Ca2+-binding protein, interacts with and regulates various proteins including calmodulin-dependent protein kinases (CaMKs), calcineurin, NF-AT and AP-1, which are all involved in transcriptional regulation. The S100B protein, a member of the S100 subfamily of the EF-hand superfamily, interacts with and regulates the tumor suppressor protein, p53, which functions as a transcriptional activator or repressor of numerous genes. More recently, another EF-hand protein variously named calsenilin/DREAM/KChIP3 (referred here as DREAM) has been identified in the human brain to serve as a Ca2+-regulated transcriptional repressor by binding directly to DNA elements downstream from the promoter sequence. DREAM, therefore, in contrast to calmodulin, is the first example of an EF-hand Ca2+-binding protein, which is also capable of direct binding to DNA.

It is now clear that numerous pathways link Ca2+ signaling to transcription. In this review, we discuss structural aspects and possible mechanisms of transcriptional regulation by various Ca2+-binding proteins including calmodulin, calcineurin B, S100B and DREAM. For more functional aspects of these biological processes, we suggest excellent reviews on CaM,4,5 CaMKs,6–8 calcineurin/NF-AT,9–13 and S100B.14

Abbreviations: CaM, calmodulin; CaMK, calmodulin-dependent protein kinase, CaMKK, CaMK kinase, PKA, cAMP-dependent protein kinase; PKB, protein kinase B; CREB, cAMP response element binding protein; CBP, CREB-binding protein; MLCK, myosin light-chain kinase; Cn, calcineurin; FKBP, FK506-binding protein; DRE, downstream regulatory element; DREAM, downstream regulatory element antagonist modulator; KchIP, potassium channel interacting protein; GCAP, guanylyl cyclase activating protein

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CaM-dependent protein kinase cascades in transcriptional regulation

**CaM kinase cascades**
Recent studies on Ca\(^{2+}\)/CaM-dependent protein kinases revealed the existence of a cascade that consists of three Ca\(^{2+}\)/CaM-dependent kinases (Fig. 1): CaM kinase I (CaMKI), CaM kinase IV (CaMKIV), and CaM kinase kinase (CaMKK). CaMKK acts as an upstream activator of CaMKI and CaMKIV by phosphorylating Thr 177 in CaMKI and Thr 196 in CaMKIV. As a result, CaMKI and CaMKIV become more efficient in phosphorylating various protein substrates including MAP kinases and CREB (see below). CaMKK can also phosphorylate and activate PKB in vitro and in the NG108 cell, thereby inhibiting apoptosis through phosphorylation of the pro-apoptotic Bcl-2-family member BAD.

All these Ca\(^{2+}\)/CaM-dependent kinases are abundant in the brain and thymus where they predominately exist in the cytoplasm of the cell. CaMKIV and CaMKK can also localize to the nucleus, while CaMKI does not translocate to any organelle from the cytoplasmic pool. One of the well-characterized functions of nuclear CaMKIV involves the regulation of transcription through phosphorylation of CREB, the cAMP-regulated transcriptional activator that activates transcription of target genes in part through direct interactions with the co-activator CBP (CREB-binding protein)/p300. Both cAMP-dependent protein kinase (PKA) and CaMKIV phosphorylate Ser 133 in CREB, stimulating the interaction of CREB with CBP/p300. Interestingly, transfection experiments indicated that both CaMKIV and CaMKK are required to achieve the same level of CREB-mediated transcription activation that PKA stimulates. CaMKI can also phosphorylate CREB in vitro, but the physiological significance of this phosphorylation remains unclear, as CaMKI is found only in the cytoplasm.

CREB, however, may not be the only nuclear target for CaMKIV. Recent studies indicated that CaMKIV regulates gene transcription through CBP. CBP/p300 is a versatile transcriptional co-activator that often works together with CREB and interacts with numerous transcription factors such as p53, c-Jun, c-fos, Sap-1a, c-Myb, MyoD, E12/E47, Tax and nuclear hormone receptors. CBP contains two transactivation domains that are controlled by nuclear Ca\(^{2+}\) and cAMP. Indeed, CBP contains a number of putative phosphorylation sites for CaMKIV and PKA, which could be phosphorylated upon stimulation with either glutamate or Ca\(^{2+}\). In vitro experiments using GAL4-CBP-dependent transcription assays, phosphorylation of CBP indeed enhances transcriptional activities. Under physiological conditions, however, phosphorylation of CBP alone is unlikely to occur since CREB and CBP are probably both phosphorylated by CaMKIV when nuclear Ca\(^{2+}\) increases.

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**Figure 1.** Schematic diagram of the CaM kinase cascade. When the Ca\(^{2+}\) concentration increases within the cell, CaM binds and activates its target kinases including CaM kinase IV (CaMKIV) and CaM kinase kinase (CaMKK). CaMKK phosphorylates and fully activates CaMKIV, resulting in transcriptional activation through phosphorylation of transcriptional factors. CaMKK also phosphorylates protein kinase B (PKB), which leads to the inhibition of apoptosis.
**Ca^{2+}/CaM-dependent activation mechanisms of CaM kinases**

A number of biochemical and structural studies have revealed a general mechanism by which Ca^{2+} activates protein kinases. This was first documented for myosin light-chain kinase (MLCK), a well-characterized Ca^{2+}/CaM-dependent protein kinase, whose three-dimensional structure was modeled on the basis of a structural homology with PKA. When the intracellular Ca^{2+} level is low, CaM assumes a so-called closed conformation that does not allow CaM to bind protein kinases. In this resting state of the cell, kinases are inactive as the autoinhibitory region masks the catalytic cleft, and, therefore, substrates have no access to the active site (Fig. 2A). When the intracellular Ca^{2+} level is elevated, CaM binds four Ca^{2+} ions and undergoes a large conformational change. This conformational change enables CaM to bind to a kinase at the binding site that overlaps with the autoinhibitory region, leading to the removal of this polypeptide segment from the catalytic cleft, thus activating the kinase (Fig. 2B). This model has been widely accepted and serves as a working hypothesis for other Ca^{2+}/CaM-dependent protein kinases.

This general model applies to most, if not all, Ca^{2+}/CaM-dependent kinases, based on the global sequence similarity in the domain architecture where the ATP-binding and catalytic domains span the N-terminal region, followed by the autoinhibitory and CaM-binding regions (Fig. 2). The regulatory domain containing the CaM-binding region, however, significantly differs in sequence from one kinase to another. Interestingly, these differences result in a variety of CaM recognition modes. To date, three-dimensional structures of CaM in complex with the binding domain of protein kinases such as MLCK, CaMKII, and CaMKK have been elucidated and detailed comparisons have been reported. Three basic modes of CaM binding to target peptides have been characterized namely by the distance between two key hydrophobic residues in the target peptide, which forms an α-helix in the core of the binding region (Fig. 3). The 1-10-type, first identified in CaMKII, is the shortest CaM-binding sequence that uses Leu 299 and Leu 308 as anchoring amino acids, each of which interacts with the C- and N-terminal domain of CaM, respectively (Fig. 3A). In an earlier NMR-derived structure of CaM bound to the skeletal muscle MLCK peptide, M13, and the crystal structure of CaM complexed with smooth muscle MLCK peptide, a one-turn longer helix was found to interact with CaM (Fig. 3B). This 1-14 type, involving Trp 800 and Leu 813, was initially thought to be a prototype for CaM-target interactions. The 1-16 type, the most recently described CaM-binding mode, was found in the structure of CaM in complex with a CaMKK peptide (Fig. 3C). This recognition mode involves two hydrophobic residues 16 residues apart, Leu 337 and Phe 352, the longest distance so far identified. The CaM-binding region of CaMKK consists of an α-helix harboring these two anchoring residues as well as a short hairpin turn at the C terminus. Interestingly, the orientation of the α-helix of CaMKK is opposite to that of MLCK or CaMKII with respect to the two domains of CaM. The significance of the peptide polarity in the activation of CaMKK has been studied using chimera mutants in which the regulatory domain was replaced with that of MLCK or CaMKII. The results indicated that the exogenous regulatory sequences can function nearly as well as the original sequence of CaMKK. Further work is needed to refine the functional significance of various CaM-binding modes.

**Calcineurin/NF-AT signaling pathway**

Calcineurin, also known as protein phosphatase 2B, is a serine/threonine phosphatase controlled by Ca^{2+}/CaM, which is abundant in brain. This phosphatase has been implicated in many biological processes including lymphocyte activation, neuronal and muscle development, neurite outgrowth, and morphogenesis of vertebrate heart valves. Since cyclopomine A and FK506 were found to be high-affinity inhibitors of calcineurin, this phosphatase has gained broader interests in various signaling fields. In particular, the link between the Ca^{2+}/calcineurin pathway and the NF-AT signaling pathway has been a major interest of recent research, as it is one of the first signaling pathways that bridges the cell membrane with the nucleus.

NF-AT1 is a transcription complex consisting of nuclear (NF-ATn) and cytoplasmic components (NF-ATc) which is a family of four transcription factors (NF-ATc1-4). NF-ATc undergoes cytoplasmic-to-nuclear translocation upon dephosphorylation by calcineurin only the nonphosphorylated NF-ATc moves into the nucleus and binds DNA in association with partner proteins such as AP-1. This nuclear import of the NF-ATc complex is blocked by the drugs cyclopomine A and FK506, underscoring the importance of calcineurin in this process. The outcome of NF-AT1-dependent transcription activation differs from one cell type to another. In lymphocytes, the genes activated include IL-2, IL-3, IL-4, CD40L, FasL, and granulocyte-macrophage colony-stimulating factor (GMCSF). In cardiac endocardial cells, the calcineurin/NF-AT1 pathway activates the expression of genes essential for heart valve morphogenesis. In hippocampal cells, this pathway regulates the expression of the inositol 1,4,5-triphosphate (IP_3) receptor type 1.

Calcineurin is central in this relatively simple mechanism of Ca^{2+}-dependent transcriptional regulation and uses an unconventional method to regulate the NF-ATc transcription complex. Surprisingly, this phosphatase binds directly to the NF-AT1 proteins through a conserved motif in the N terminus of NF-ATc, which enables calcineurin to dephosphorylate serines within the serine-proline (SP) repeats and the serine-rich region of NF-ATc family members. This leads to the release of the SP repeats and prohibits interaction with the two nuclear localization sequences required for nuclear import.
Figure 2.

(A) N-terminal ATP-binding domain

CaM-binding region

Autoinhibitory region

C-terminal substrate-binding domain

inactive

(B) 

Ca\(^{2+}\)/Calmodulin

Substrate

Catalytic cleft

active

Figure 2.
Although it has been thought that the basic nuclear localization sequence forms salt bridges with the acidic phosphoserines of NF-ATc family members, details of the mechanism are not presently understood.

The activation of calcineurin by Ca\(^{2+}\) is mediated by two Ca\(^{2+}\)-binding proteins, the integral subunit calcineurin B (CnB) and CaM. CnB and CaM bind to separate but adjacent sites following the catalytic domain of calcineurin A (CnA).(11,44) In the inactive state, the C-terminal autoinhibitory domain of CnA masks the active site within the catalytic domain (Fig. 4A). This autoinhibition is released when Ca\(^{2+}\) ions bind to CnB and CaM. CnB is a myristoylated EF-hand Ca\(^{2+}\)-binding protein that has a structure very similar to CaM.(45) However, the crystal structures of calcineurin alone and in complex with FK506 and the cytoplasmic protein FKBP12(46,47) shows that the binding mode of CnB to CnA differs significantly from that of CaM to protein kinases (Fig. 4B). Two domains of CnB reside side-by-side on the same surface of the helical portion of CnA, unlike the corresponding domains of CaM commonly wrapping around the helical region of the target protein. Unfortunately, CaM has not been present in any of the crystal structures of calcineurin so far determined. Recent work using proteolytic digestion(48) has indicated that Ca\(^{2+}\)-binding to the C-terminal low-affinity sites of CnB induces a conformational change in CnA involving the exposure of the CaM-binding domain, such that CaM can interact with CnA. This stepwise mechanism for Ca\(^{2+}\)-dependent activation of calcineurin explains how CnB and CaM work together in a concerted manner following an elevation in Ca\(^{2+}\) levels within the cell. In addition, the crystal structure of the calcineurin/FK506/FKBP12 complex revealed how the immunosuppressor agent inactivates the protein phosphatase, providing the structural basis for the action of FK506 (Fig. 4B).

**S100B-dependent p53 regulation**

S100B belongs to the S100 subfamily of the EF-hand superfamily, which exists as a stable homo- or heterodimer (~ 10 kDa/monomer) in solution.(14) The S100 proteins are overexpressed...
in many tumor cells and have been used as a marker for the classification of tumors. Previous studies have shown that S100B interacts with the tumor suppressor p53 to regulate its transcriptional activity in a Ca\textsuperscript{2+}-dependent manner. The p53 protein is a transcription factor involved in approximately 50% of all human cancers. The p53 protein forms a tetrameric complex, in which each subunit is composed of four structural domains with specific functions: from the N to C terminus, a transactivation domain, a central DNA-binding domain, a tetramerization domain, and a regulatory domain. The S100B protein interacts with the C-terminal portion of p53 and inhibits both p53 tetramerization and phosphorylation by PKC. Recently, more evidence has indicated that S100B inhibits p53 tetramer formation and decreases p53 DNA binding and transcriptional activity. These findings provide a direct link between the Ca\textsuperscript{2+}-binding protein, S100B and transcription factor p53, underscoring the unique involvement of the Ca\textsuperscript{2+}-signaling mechanism in cancer.

A Ca\textsuperscript{2+}-sensitive transcriptional repressor, DREAM

DREAM is a multifunctional Ca\textsuperscript{2+}-binding protein

The 256-residue protein variously named calssenilin, DREAM, and KChIP3 is expressed in the mammalian brain and belongs to the recoverin sub-branch of the EF-hand superfamily (Fig. 5). The DREAM protein, identified in three different biological systems, was first discovered, in a two-hybrid screen, to interact with the C-terminal 40 residues of presenilin-2 and was originally named, calssenilin. Shortly after the discovery of calssenilin, a nearly identical protein was identified in nuclear extracts from human brain that was shown to bind to the downstream regulatory element (DRE) of the prodynorphin and c-fos genes and, in turn, block transcription of these genes only at low Ca\textsuperscript{2+} levels. This Ca\textsuperscript{2+}-sensitive transcriptional repressor protein was named DREAM (Downstream Regulatory Element Antagonist Modulator) and was originally reported to contain 284 residues in which the

**Figure 5.** Amino acid sequence of human DREAM aligned with various members of the recoverin family. The Ca\textsuperscript{2+}-binding loops of the four EF-hands are underlined. Residues highlighted in bold in EF-1 prevent Ca\textsuperscript{2+} binding.
C-terminal 256 residues are identical to calsenilin. Later it was shown that the C-terminal 256 residues of DREAM are sufficient for Ca\(^{2+}\)-regulated transcriptional repression.\(^{66,67}\) The precise length of the endogenous DREAM protein (284 versus 256), however, is still unclear. More recently, in a two-hybrid screen, the 256-residue calasin/DREAM protein was also found to interact with the N-terminal 180 residues of A-type voltage-gated potassium channels. It was also shown to modulate the activity of potassium channels in vivo in a Ca\(^{2+}\)-dependent fashion.\(^{58}\) Hence the calasin/DREAM protein has also been named KChIP3 (potassium channel interacting protein-3).

The C-terminal 190 residues of DREAM are about 40% identical in amino acid sequence to recoverin. The recoverin subgroup includes neuronal Ca\(^{2+}\) sensors, such as recoverin,\(^{59}\) neurocalcin\(^{60}\) and frequenin,\(^{61}\) which all possess N-terminal myristoylation, four EF-hand Ca\(^{2+}\)-binding motifs, and a functional Ca\(^{2+}\)-myristoyl switch. However, unlike recoverin, the DREAM protein contains an extra stretch of residues in its N-terminal region (residues 1-65) and lacks a myristoylation consensus sequence at the N terminus (MGXXXS). Therefore, DREAM does not contain N-terminal myristoylation and does not exhibit a Ca\(^{2+}\)-myristoyl switch.

A characteristic feature of DREAM and the recoverin family is the sequence CPXG that prevents binding of Ca\(^{2+}\) to the first EF-hand (EF-1) as seen in the crystal structures of recoverin,\(^{62}\) neurocalcin\(^{63}\) and frequenin.\(^{64}\) Correspondingly, the DREAM protein, like other members of the recoverin family, has been shown to bind three Ca\(^{2+}\) ions at saturation (in the absence of Mg\(^{2+}\)) to EF-2, EF-3 and EF-4.\(^{65}\) Interestingly, EF-2 binds Ca\(^{2+}\) with relatively low affinity (K\(_{\text{EF2}}\) ~ 50 μM) compared with that of EF-3 and EF-4 (K\(_{\text{EF3}}\) = K\(_{\text{EF4}}\) = 5 μM), and EF-2 contains an aspartate residue instead of glutamate at the 12-position of the EF-hand-binding loop (Fig. 5).\(^{66}\) EF-2 shows only modest discrimination for the binding of Ca\(^{2+}\) over magnesium (Mg\(^{2+}\)), and under physiological Mg\(^{2+}\) concentrations (1 mM), Mg\(^{2+}\) might occupy the second EF-hand site. The bound Mg\(^{2+}\) may be physiologically significant and might facilitate and/or bridge the binding of DNA.

In summary, the neuronal Ca\(^{2+}\)-binding protein named calsenilin/DREAM/KChIP3 possesses multiple functions in different compartments of the cell by regulating different physiologic targets. The multifunctionality of DREAM underscores its biological importance and is reminiscent of other multifunctional transcription factors in the brain such as β-catenin and p53.\(^{67}\) In this section of the review, we focus on the Ca\(^{2+}\)-sensitive transcriptional repressor function of DREAM and emphasize our recent structural characterization of the DREAM protein and its Ca\(^{2+}\)-regulated binding to DNA.

**DREAM controls pain sensation in transgenic mice**
Recent studies on transgenic mice that lack the expression of DREAM indicate that the native DREAM protein is critically important for sensing pain.\(^{68}\) The DREAM knockout mice exhibit a marked attenuation in pain response regardless of stimuli type or tissue affected. Loss of DREAM also leads to increased basal expression of prodynorphin in the spinal cord and in cultured neurons. The reduced pain responses in the knockout mice are, therefore, likely caused by the constitutive activation of the dynorphin-selective κ-opiate receptor. Interestingly, the DREAM knockout mice do not exhibit any physical dependence to endogenous opioids. The DREAM knockout mice also do not display any detectable defects in presenilin processing and/or potassium channel functioning, suggesting that the calsenilin and KChIP3 functions identified in the human brain may not be essential in mice. Perhaps homologs of DREAM (e.g., KChIP1 and KChIP2, Ref. 58) may exist in mice that could functionally substitute for calsenilin and KChIP3.

**Ca\(^{2+}\)-regulated oligomerization of DREAM**
The first indication of protein oligomerization by DREAM was observed in Southwestern analyses probing DREAM binding to the downstream regulatory element (DRE) of the prodynorphin gene.\(^{65,69}\) Three discrete protein bands were present in the Southwestern blot with apparent molecular masses of 30, 60, and 110 kDa, presumably corresponding to monomer, dimer and tetramer species of DREAM. A more rigorous analysis of the oligomerization state of recombinant DREAM was recently performed based on hydrodynamic measurements of DREAM in solution.\(^{65}\) Dynamic light scattering and size-exclusion chromatography studies revealed that the Ca\(^{2+}\)-free DREAM protein in solution exists as a tetramer at protein concentrations higher than 20 μM. In contrast, the Ca\(^{2+}\)-bound DREAM forms a stable dimer in solution at low protein concentrations and forms a tetramer at protein concentrations higher than 200 μM. The dramatically different hydrodynamic properties for the Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms of DREAM may be due, in part, to possible Ca\(^{2+}\)-induced changes in molecular shape. The sharp dependence of the hydrodynamic properties on protein concentration, however, indicates that these changes are mainly the result of protein oligomerization. Very similar Ca\(^{2+}\)-dependent oligomerization properties were observed for the N-terminal deletion mutant (residues 65–256) containing the four EF-hands. Hence, Ca\(^{2+}\)-induced structural changes in the C-terminal EF-hand region may be crucial for the alteration of the oligomerization state. Other recoverin-like proteins are also known to exhibit Ca\(^{2+}\)-regulated dimerization. Neurocalcin, for example, forms a dimer in the Ca\(^{2+}\)-bound state, whereas Ca\(^{2+}\)-free neurocalcin is monomeric.\(^{70}\) Conversely, GCAP-2 forms a dimer only in the Ca\(^{2+}\)-free state and is monomeric in the Ca\(^{2+}\)-bound state.\(^{70}\)

**DREAM is a Ca\(^{2+}\) sensor that binds DNA and represses transcription**
The native DREAM protein was first discovered to bind to the DRE silencer element of the prodynorphin gene based on...
crosslinking studies, gel-shift assays and Southwestern analysis.\textsuperscript{(55,65)} The DREAM protein was then shown in a CAT assay to suppress the basal transcription of the prodynorphin gene,\textsuperscript{(65)} suggesting that DREAM binds to the DRE silencer element. More rigorous thermodynamic measurements performed on recombinant DREAM indicated that the Ca\textsuperscript{2+}-free, tetrameric DREAM binds to four molecules of DRE at saturation.\textsuperscript{(65)} One DRE binds tightly to the tetrameric protein with a dissociation constant ($K_d$) of 75 nM and the other three bind more weakly ($K_d = 700$ nM). No significant DNA binding was observed for the Ca\textsuperscript{2+}-bound protein. Isothermal titration calorimetry studies revealed that the DRE binding to DREAM is entropically driven ($\Delta H = +25$ kcal/mol), which may result from the desolvation of water molecules and/or conformational changes in DREAM induced by DRE binding. Many other DNA-binding proteins like the TATA-binding protein,\textsuperscript{(71)} nuclear hormone receptors,\textsuperscript{(72)} and the Cro repressor\textsuperscript{(73)} also exhibit similar entropically dominated binding to DNA.

DNA-binding studies performed on a series of deletion mutants of DREAM indicated that the DNA-binding site is delocalized within the DREAM tetramer. An N-terminal protein fragment consisting of residues 1–70 and lacking the EF-hand motifs was shown to bind non-specifically to DNA regardless of the Ca\textsuperscript{2+} level. A C-terminal protein fragment consisting of residues 65–256 containing the four EF-hands exhibited Ca\textsuperscript{2+}-regulated and sequence-specific DNA binding. Therefore, the DNA-binding site in DREAM appears to contain residues from both the N-terminal and C-terminal domains. However, the N-terminal residues, 1–70 are NOT essential for the Ca\textsuperscript{2+}-regulated and sequence-specific aspects of DNA binding. In addition, the C-terminal fragment (residues 65–256) forms a tetramer in the Ca\textsuperscript{2+}-free state that dissociates into dimers in the Ca\textsuperscript{2+}-bound state. Hence, it would seem that the C-terminal residues, 65–256 possess all the essential features to serve as a Ca\textsuperscript{2+}-regulated transcriptional repressor. Correspondingly, DREAM has a caspase-3 cleavage site at residues Asp 61–Asp 64 located at the junction of the N-terminal and C-terminal domains.\textsuperscript{(74)} Therefore, it appears that DREAM may function as a transcriptional repressor even after cleavage by caspase-3.

**Mechanism of Ca\textsuperscript{2+}-regulated transcriptional repression by DREAM**

A schematic mechanism for Ca\textsuperscript{2+}-regulated transcriptional repression by DREAM is shown in Fig. 6. In the basal resting condition, the nuclear Ca\textsuperscript{2+} concentration is quite low (< 200 nM) and tetrameric DREAM in the Ca\textsuperscript{2+}-free state is bound to a DRE silencer element of the prodynorphin gene. A pain stimulus or related neuronal depolarization leads to a rise in the intracellular Ca\textsuperscript{2+} concentration causing an increase in nuclear Ca\textsuperscript{2+} levels. A rise in nuclear Ca\textsuperscript{2+} beyond the dissociation constant of DREAM (> 10 $\mu$M) leads to Ca\textsuperscript{2+} binding by DREAM, inducing conformational changes within the EF-hand motifs that in turn lead to the dissociation of the tetrameric DREAM protein into dimers, causing the Ca\textsuperscript{2+}-bound DREAM to dissociate from the DRE. We suggest that the Ca\textsuperscript{2+}-bound

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**Figure 6.** Schematic mechanism of Ca\textsuperscript{2+}-regulated transcriptional repression by DREAM. The DRE sequence (shaded box), RNA polymerase (Pol2, shaded circle) and DREAM polypeptides (ovals) are represented schematically. The Ca\textsuperscript{2+}-unbound (apo-) DREAM tetramer binds to the duplex DNA at the DRE site and blocks transcription of target genes at low Ca\textsuperscript{2+} levels in the resting basal state. Transcription occurs at high Ca\textsuperscript{2+} levels because the Ca\textsuperscript{2+}-bound DREAM protein forms dimers that dissociate from the duplex DNA and may regulate cytosolic target proteins.
DREAM dimer might be free to translocate into the cytoplasm and interact with cytosolic target proteins such as presenillin-2 (PSEN2) and/or voltage-gated potassium channels. The DNA-binding site of the DREAM tetramer consists of residues from both the N-terminal and C-terminal regions of the protein. A 4:1 stoichiometric ratio of DNA bound per DREAM tetramer indicates that there are four distinct DNA-binding sites on each tetramer. However, the observed anti-cooperativity of DNA binding by DREAM results in only one site having high affinity with the other three sites having markedly lower affinities. We suggest that the single site with highest affinity may be biologically most important because the DREAM tetramer concentration in the cell nucleus should be much higher than that of DRE. DRE binding should, therefore, be limited primarily to the high-affinity site on the DREAM tetramer. The tetramer of DREAM bound to only one DNA element, as depicted in Fig. 6, may be structurally unique as a transcriptional regulator complex. Other known transcriptional repressors (such as TrpA and Cro) are homodimers that recognize one promoter sequence. The Met repressor is a tetramer that binds to two adjacent DNA elements and p53 is a tetramer that binds four DNA elements. To our knowledge, no other tetrameric transcriptional repressors or activators are known that bind to only one DNA sequence element.

**Other sequence-specific DNA-binding proteins containing EF-hand motifs**

The eukaryotic parasite Entamoeba histolytica contains a 22 kDa EF-hand Ca$^{2+}$-binding protein that regulates the expression of virulence factor genes. This EF-hand Ca$^{2+}$-binding protein, named URE3-BP, binds specifically to the upstream regulatory element-3 (URE3) of the lectin heavy subunit gene and negatively regulates its expression. The amino acid sequence of URE3-BP contains three EF-hands, no recognizable DNA-binding motif, and bears almost no sequence similarity to DREAM. Like DREAM, however, the URE3-BP protein represses transcription at basal Ca$^{2+}$ levels and exhibits Ca$^{2+}$-regulated and sequence-specific binding to DNA. It seems that URE3-BP and DREAM may belong to a new class of EF-hand Ca$^{2+}$-binding proteins that regulate transcription by their Ca$^{2+}$-regulated binding to specific DNA elements. Future studies looking for other EF-hand Ca$^{2+}$-binding proteins that exhibit both Ca$^{2+}$-regulated and sequence-specific binding to either upstream or downstream DNA-regulatory elements in transcription should prove to be fruitful.

**Conclusions**

It is well established that the fluctuation in cytoplasmic Ca$^{2+}$ concentration stimulates a wide range of signaling cascades, some of which influence gene transcription, but how does a fluctuation in cytoplasmic Ca$^{2+}$ concentration reach the nucleus? Previous studies have shown that the time course of nuclear Ca$^{2+}$ concentration is similar to that of the cytoplasmic Ca$^{2+}$ concentration, making it conceivable that Ca$^{2+}$ signals created by the opening of plasma membrane or endoplasmic reticulum ion channels could stimulate Ca$^{2+}$-binding proteins present in the nucleus. The nuclear Ca$^{2+}$ concentration could also be modulated by specific Ca$^{2+}$-release channels localized on the inner nuclear membrane activated by IP$_3$ or cADP ribose. Following the Ca$^{2+}$ elevation in the nucleus, CaMKIV could also be activated to phosphorylate CREB and CBP/p300 which, in turn, activate transcription of various genes. In addition to CaM, other nuclear Ca$^{2+}$-sensors such as DREAM and S100B could directly regulate transcription through interaction with a promoter DNA element or a transcription factor. Calcineurin, on the contrary, does not translocate into the nucleus, yet it can regulate transcription through the interaction with and the dephosphorylation of the cytoplasmic NF-AT proteins, which in turn translocate into the nucleus, thereby activating target genes. Clearly, there are a variety of ways to regulate transcription through Ca$^{2+}$ signals.

As the number of proteins found in numerous genomes increases, there is no doubt that more Ca$^{2+}$-binding proteins will be identified and found to be responsible for the regulation of gene transcription. Molecular and structural mechanisms for these regulatory processes may differ from one Ca$^{2+}$-binding protein to another as seen in the four presently known cases which all use distinct methods to regulate gene transcription. At present, we may be seeing only the tip of the ‘iceberg’ of transcription machinery dependent on Ca$^{2+}$ signals.

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**REFERENCES**


