Review

The DREAM–DRE interaction: key nucleotides and dominant negative mutants

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Abstract

Transcriptional repressor DREAM, an EF-hand containing calcium-binding protein, blocks basal expression of target genes through specific interaction with DRE sites in the DNA. The sequence GTCA forms the central core of the DRE site, whereas flanking nucleotides contribute notably to the affinity for DREAM. Release of binding of DREAM from the DRE results in derepression, a process that is regulated by Ca$^{2+}$. Change of two amino acids within an EF-hand in DREAM blocks Ca$^{2+}$-induced derepression and results in potent dominant negative mutants of endogenous DREAM. © 2000 Elsevier Science B.V. All rights reserved.

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1. Mechanisms of Ca$^{2+}$-regulated gene expression

The increase in intracellular calcium as a consequence of membrane depolarization or mobilization from intracellular stores elicits profound changes in gene expression [1,2]. The study of the molecular mechanisms by which Ca$^{2+}$ governs gene expression was facilitated by the discovery of the Ca$^{2+}$-dependent inducibility of immediate early genes, since this provided a direct link between the calcium signal and the effect on transcription [3]. The use of this model helped to define the existence of several cellular cascades of kinases and phosphatases that transduce the calcium signal into changes in the phosphorylation state of key transcription factors, eventually responsible for changes in gene expression [4–6]. Furthermore, it was also possible to show that the source, location and extent of the calcium increase influence phosphorylation-dependent gene activation by Ca$^{2+}$. Thus, rise in cytosolic Ca$^{2+}$ levels following activation of voltage-sensitive calcium channels, specifically regulates MAPK-mediated TCF-dependent transcription at the SRE site in the c-fos promoter [7–9]. In contrast, CREB-mediated c-fos activation at the CRE site requires a substantial increase in nuclear calcium [8]. Moreover, it has been recently shown that the spike frequency of Ca$^{2+}$ waves dramatically determines not only the extent of the inductive effect, but also the set of target genes activated. Rapid oscillations stimulate the proinflammatory transcription factors NF-AT, Oct/OAP and NF-κB, whereas infrequent oscillations activate only NF-κB [10]. This most likely has important
physiological consequences since downstream target genes, e.g., interleukin-2 and interleukin-8, will then be differentially regulated [10].

More recently, two new mechanisms not directly dependent of kinase and phosphatase activation have been proposed to participate in Ca$^{2+}$-dependent gene expression. One relies on a direct interaction between Ca$^{2+}$-loaded calmodulin or S-100 Ca$^{2+}$-binding proteins and transcription factors of the basic helix-loop-helix (bHLH) class [11,12]. Upon binding to these Ca$^{2+}$-loaded complexes, bHLH transcription factors fail to bind their targets in DNA and bHLH-dependent transcription is impaired [11–14]. Since bHLH proteins are important determinants of cell fate during development and cell cycle progression, Ca$^{2+}$-mediated repression of bHLH may have a functional role during these processes [15]. Finally, the other new mechanism of Ca$^{2+}$-induced gene expression involves the newly reported DREAM protein [16].

2. The DREAM protein

DREAM is a Ca$^{2+}$-binding protein belonging to the recoverin subfamily of Ca$^{2+}$-binding proteins [16]. It contains four EF-hands, three of which conform to the EF-hand consensus sequence [17], and binds calcium in vitro shifting the fluorescence emission spectra due to tryptophan residues. The most exciting feature of this new calcium-binding protein is its capacity to bind to a specific sequence in the DNA, the downstream regulatory element (DRE) in a Ca$^{2+}$-dependent manner [16,18]. DREAM was cloned in the search for nuclear proteins that interact with an oligonucleotide containing the DRE sequence from the human prodynorphin gene [16]. Binding of DREAM to the DRE in basal conditions results in transcriptional repression. Upon Ca$^{2+}$ stimulation, DREAM detaches from the DRE and transcription is derepressed [16]. This provides a new mechanism of Ca$^{2+}$-dependent gene expression in which a calcium sensor directly binds to DNA and regulates the transcription process as a function of the nuclear calcium concentration. Furthermore, recent studies have shown that the binding of DREAM to DRE sites is regulated also by specific protein–protein interactions with nuclear effectors of the cAMP pathway [19]. Interestingly, these interactions are independent of the levels of nuclear Ca$^{2+}$, indicating that transcriptional derepression at DRE sites can be separately achieved through at least two distinct signaling pathways; calcium and cAMP activation. Since cellular stimulation by hormones or activation of membrane receptors is often followed by a concomitant elevation in intracellular calcium and cAMP, both mechanisms can cooperatively derepress DRE-dependent transcription. Future studies employing transgenic mice overexpressing DREAM mutants insensitive to Ca$^{2+}$, to cAMP or to both stimuli will help to define the relative weight of each pathway in DREAM-dependent transcriptional derepression in vivo.

Expression of DREAM mRNA has been observed in the CNS, thyroid gland, immune organs and testis [16]. Surprisingly, when we analyzed its subcellular distribution we found important levels of DREAM-binding activity in nuclear-free cytosolic extracts from HEK 293 cells stably transfected with a DREAM expression vector [16] or from NB69 cells, a human neuroblastoma that has endogenous expression of DREAM [18]. These results suggested that DREAM might have other functions in the cytosol. More recently, other groups have confirmed the cytosolic location of DREAM, disclosing at the same time new functions for DREAM outside the nucleus [20,21]. Using a yeast two-hybrid screening a protein named calsenilin, which is identical to DREAM, was found to interact with the carboxy terminal region of presenilin-2 [20]. However, mutants of calsenilin were not described in this study and a possible regulation of the interaction with presenilin-2 by calcium or cAMP activation remains to be analyzed. Moreover, three new proteins related to DREAM, named KchIP (potassium channel interacting protein)−1 to -3, were found also by a two-hybrid screening to interact with the amino terminal domain of Kv4.2 potassium channels [21]. One of them, KchIP-3, is identical to DREAM and the interaction with the Kv4 potassium channels modulates A-type potassium currents in a Ca$^{2+}$-dependent manner [21]. Notably, the interaction with the potassium channel occurs whether calcium is present or not. However, the change in KchIP-3/DREAM conformation that follows binding to Ca$^{2+}$ profoundly affects channel properties [21]. Noteworthy, A-type potassium cur-
rents are also modulated by cAMP although the mechanism has remained elusive [22]. Taken together, these results indicate that DREAM/KchIP-3/cal- senilin might have pleiotropic functions through the interaction with specific DNA sequences and/or with proteins in different cell compartments [16,19–21]. Dual location, nuclear and cytosolic, and multifunctionality, with specific functions in each compartment, are properties previously described for calmodulin, another Ca\(^{2+}\)-binding protein. It has been shown that calmodulin interacts with slow-desensitizing voltage-dependent potassium channels (SK) in the cell membrane and modulates their permeability [23], controls the activity of many cytosolic enzymes (reviewed in [24,25]) and regulates transcription upon binding to bHLH nucleoproteins [11] and CaM-dependent kinases in the nucleus [4–6, 26]. However, unlike DREAM, calmodulin does not directly regulate transcription since calmodulin binding to DNA has not been demonstrated.

The capacity to bind DRE sites in the DNA makes DREAM so far unique and opens the possibility that there exist other DNA targets for interaction with EF-hand containing proteins. In addition, the search for additional genes containing the DRE sequence that could be targets for DREAM repression/derepression following changes in Ca\(^{2+}\) levels awaits a more definite DRE consensus sequence.

3. The target DRE sequence

The initial DRE sequence was found in the course of our studies on the regulatory mechanisms controlling the expression of the human prodynorphin promoter [18]. By deletion analysis of the human prody-
norphin gene we defined a regulatory element located within the first exon in the 5′ untranslated region that seemed to be critical for basal and PKA-dependent expression of this gene in several human neuroblastoma cell lines [18]. Similarly, Ca\(^{2+}\)-dependent expression of the human prodynorphin promoter was also regulated by the DRE sequence (Fig. 1). The presence of the DRE was necessary for the inducibility of the reporters after intracellular calcium release by caffeine treatment [27] (Fig. 1B), and mutation of the DRE derepressed transcription, increasing basal CAT activity to the level of activity observed after calcium stimulation in the wild-type promoter (Fig. 1C). The derepression of DREAM by caffeine is related to the release of Ca\(^{2+}\) since it was mimicked by ionomycin or elevated extracellular K\(^{+}\) treatments that increase intracellular Ca\(^{2+}\) concentrations, and it was blocked by thapsigargin and ryanodine, drugs that block intracellular Ca\(^{2+}\) release [16,28]. Moreover, derepression by caffeine is not related to phosphodiesterase inhibition and subsequent increase in cAMP since direct PKA activation by forskolin failed to affect the repression of pTKDRE-CAT by DREAM in HEK293 cells [16]. Furthermore, it was observed that the DRE element functions as a position-dependent, orientation-independent regulatory element that repressed transcription even when inserted in heterologous promoters. Importantly, the DRE functions only when located downstream from the TATA box [18], the sequence where the transcription initiation complex assembles to start the transcription of the gene. Because of this, the new regulatory element was named as downstream regulatory element (DRE). To outline a consensus DRE sequence, we performed serial substitutions to define key residues within the human prodynorphin DRE sequence (hDynDRE). First, a series of hDynDRE mutants in which two nucleotides at a time were replaced by two adenosine residues was created. These mutants were then analyzed for their capacity to compete the DRE retarded band obtained with the hDynDRE probe and nuclear extracts from NB69 human neuroblastoma cells [18]. As a result, a central core of the DRE element was proposed containing the GTCA sequence [18]. Moreover, these studies pointed out the importance of the G residue in the DRE core. Recently, to analyze in more detail the DRE consensus sequence, we used recombinant DREAM protein in EMSA using as probes these mutants as well as new single nucleotide mutants. We then compared the intensity of the different DRE retarded bands with the DRE band obtained with the hDynDRE oligonucleotide. Single nucleotide mutations within the GTCA core in hDynDRE mutants 1–5 disrupted to a great extent the binding to the DREAM protein (Table 1), as predicted from previous results [18]. Interestingly, the most striking loss of binding (more than 85%) was observed with mutant oligonucleotide 5 bearing an A to T substitution while an A to C substitution (mutant 4) still retained 28% of activity compared with the wild-type hDynDRE sequence (Table 1). Double substitution within the GTCA core resulting in mutants 6 to 8 showed very poor capacity to bind DREAM (Table 1). Comparison of hDynDRE with the DynDRE from the rat prodynorphin promoter showed an even greater capacity of rDynDRE to bind DREAM than hDynDRE (Table 1). Compared to hDynDRE, the rDynDRE contains numerous substitutions outside the central GTCA core. Thus, its higher affinity for DREAM confirms the flexibility of the DRE sequence in the flanking regions outside the central core, as previously suggested [16,18]. Furthermore, initial search in DNA data bases for the presence of DRE sequences in regulatory regions downstream from the TATA box of genes known to be regulated by calcium ions identified an inverted DRE sequence within the 5′ untranslated region of the human c-fos gene [16]. This inverted c-fos DRE sequence binds recombinant DREAM in vitro and mediates DREAM-dependent repression of the c-fos gene in transient transfection experiments [16].

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<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Relative affinity</th>
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<tbody>
<tr>
<td>hDynDRE</td>
<td>5′-CCGGAGTCAAGGAGGCC-3′</td>
<td>100%</td>
</tr>
<tr>
<td>Mut1 hDynDRE</td>
<td>5′-CCGGAGATCAAGGAGGCC-3′</td>
<td>34%</td>
</tr>
<tr>
<td>Mut2 hDynDRE</td>
<td>5′-CCGGAGATCAAGGAGGCC-3′</td>
<td>27%</td>
</tr>
<tr>
<td>Mut3 hDynDRE</td>
<td>5′-CCGGAGATCAAGGAGGCC-3′</td>
<td>36%</td>
</tr>
<tr>
<td>Mut4 hDynDRE</td>
<td>5′-CCGGAGATCAAGGAGGCC-3′</td>
<td>28%</td>
</tr>
<tr>
<td>Mut5 hDynDRE</td>
<td>5′-CCGGAGATCAAGGAGGCC-3′</td>
<td>14%</td>
</tr>
<tr>
<td>Mut6 hDynDRE</td>
<td>5′-CCGGAGATCAAGGAGGCC-3′</td>
<td>16%</td>
</tr>
<tr>
<td>Mut7 hDynDRE</td>
<td>5′-CCGGAGATCAAGGAGGCC-3′</td>
<td>15%</td>
</tr>
<tr>
<td>Mut8 hDynDRE</td>
<td>5′-CCGGAGATCAAGGAGGCC-3′</td>
<td>10%</td>
</tr>
<tr>
<td>RatDynDRE</td>
<td>5′-CCGGAGTCAAGGAGGCC-3′</td>
<td>130%</td>
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Interestingly, a second inverted DRE sequence was also observed in the P2 promoter of the CREM gene (Link et al., in preparation). The P2 promoter controls the calcium- and cAMP-inducible expression of transcriptional repressor ICER isoforms [29]. In vitro analysis as well as transient transfection experiments confirmed the functionality of the ICER DRE site (Link et al., in preparation). Surprisingly, analysis of these two inverted DRE sequences showed in both cases a greater affinity for the DREAM protein than hDynDRE in band shift experiments (Table 2). However, analysis of an oligonucleotide containing the hDynDRE sequence synthesized in the inverted orientation had an affinity for DREAM similar to that of wild-type hDynDRE (Table 2). These results indicate that the central core 5'-GTCA-3' can function also in the inverted, 5'-ACTG-3', arrangement and that nucleotides flanking the central core are important determinants of the affinity for DREAM. Disclosure of new bona fide target genes for DREAM regulation in vivo using chromatin immunoprecipitation will undoubtedly help to delineate the contribution of nucleotides flanking the DRE core in the DREAM–DRE interaction.

4. Targeting endogenous DREAM function with dominant negative mutants

Analysis of DREAM mutants insensitive to calcium stimulation showed that substitution of two amino acids at a single functional EF-hand was enough to prevent Ca$^{2+}$-induced unbinding from the DRE and derepression [16]. Furthermore, the effect of the single EF-hand mutation was maximal and no further effect could be observed when compared with DREAM mutants in which two or even all three functional EF-hands were mutated (Fig. 2). These results suggest that the conformational change of the DREAM protein that prevents binding to the DRE following binding to Ca$^{2+}$ requires the coordinated change at each individual EF-hand. Since cross-linking and southwestern analysis have indicated that DREAM binds to DRE sites as a tetramer [16,18], we wanted to check whether DREAM mutants insensitive to Ca$^{2+}$ could behave as dominant negative mutants for Ca$^{2+}$ stimulation in a background of endogenous DREAM protein. To test this hypothesis we analyzed the DRE-dependent derepression after caffeine stimulation in the presence of different combinations of wild-type DREAM and EF-hand DREAM mutant. The experiments were performed in HEK293 cells, which lack endogenous expression of DREAM, and we used the triple EF-hand DREAM mutant which has substitutions in all three functional EF-hands (234EFmutDREAM). A reporter plasmid containing the minimal promoter of the human prodynorphin gene including the DRE site (pHD3CAT) [18] was used in these experiments. Exposure to caffeine induced a 6-fold increase of the pHD3CAT reporter. Cotransfection with the wild-type DREAM expression vector did not affect the induction after caffeine while cotransfection with mutant DREAM almost completely blocked the induction after caffeine (Fig. 3), in keeping with previous results using other DRE reporters [16].

Table 2

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<tr>
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<tr>
<td>hDynDRE</td>
<td>5'-CCGGAGTCAAGGAGGCC-3'</td>
<td>100%</td>
</tr>
<tr>
<td>c-fosDRE</td>
<td>5'-ACGGAACACTGAGAGAG-3'</td>
<td>157%</td>
</tr>
<tr>
<td>ICERDRE</td>
<td>5'-TATTTTGGACTGCTGTA-3'</td>
<td>166%</td>
</tr>
<tr>
<td>Reverse hDynDRE</td>
<td>5'-CCGGAGGACTGAGGCC-3'</td>
<td>97%</td>
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![Fig. 2. Derepression by calcium stimulation of DRE-dependent transcription is blocked in the presence of EF-hand mutants of DREAM. DREAM (black bar) and single, double or triple DREAM mutants insensitive to calcium (EFmDREAM, hatched bars) repress to a similar extent basal activity of DRE-containing reporter pTKDRECAT in HEK293 human carcinoma cells. Exposure to 10 mM caffeine blocks the repression of pTKDRECAT activity by DREAM (black bar) but does not affect repression by EFmDREAM mutants (hatched bars).](image-url)
transfection with 234EFmutDREAM and wtDREAM in ratios 1:1, 1:2 and 1:3 did not significantly change the blockade observed with 234EF-mutDREAM alone. Interestingly, the effect of caffeine was partially recovered after cotransfection with 234EFmutDREAM and wtDREAM in a ratio 1:6 and the effect of the DREAM mutant was negligible at ratios equal or higher than 1:9 with wtDREAM (Fig. 3). These results support the hypothesis that DREAM binds to the DNA as a multimer and strengthen the notion of a finely tuned regulation of the DREAM–DRE interaction by calcium ions. Furthermore, these results indicate that DREAM mutants insensitive to Ca\(^{2+}\) stimulation are dominant negative mutants of Ca\(^{2+}\)-regulated DREAM function.

5. Concluding remarks

The discovery of DREAM has initiated a new avenue of research for other calcium sensors able to directly link changes in the nuclear concentration of Ca\(^{2+}\) with profound effects on gene expression. Further analysis of the DREAM protein revealing (i) the amino acids that compose the DNA-binding domain, (ii) the region that participates in the oligomerization of DREAM and (iii) the molecular mechanism of the transcriptional repression by DREAM, should promptly clarify several fundamental aspects of DREAM function in the nucleus. Moreover, studies using DREAM as the bait in a yeast two-hybrid screen could disclose new protein–protein interactions, revealing new functions for the DREAM protein in the cytosol. Finally, preparation of transgenic mice targeting the expression of dominant negative mutants of DREAM to neurons, thymus, spermaticides or thyroid gland with tissue-specific promoters should also shed light on the functional role of DREAM in vivo.

References