Ca²⁺-dependent block of CREB–CBP transcription by repressor DREAM

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The calcium-binding protein DREAM binds specifically to DRE sites in the DNA and represses transcription of target genes. Derepression at DRE sites following PKA activation depends on a specific interaction between α CREM and DREAM. Two leucine-charged residue-rich domains (LCD) located in the kinase-inducible domain (KID) and in the leucine zipper of α CREM and two LCDs in DREAM participate in a two-site interaction that results in the loss of DREAM binding to DRE sites and derepression. Since the LCD motif located within the KID in CREM is also present in CREB, and maps in a region critical for the recruitment of CBP, we investigated whether DREAM may affect CRE-dependent transcription. Here we show that in the absence of Ca²⁺ DREAM binds to the LCD in the KID of CREB. As a result, DREAM impairs recruitment of CBP by phospho-CREB and blocks CBP-mediated transactivation at CRE sites in a Ca²⁺-dependent manner. Thus, Ca²⁺-dependent interactions between DREAM and CREB represent a novel point of cross-talk between cAMP and Ca²⁺ signalling pathways in the nucleus.

Keywords: CRE/derepression/DRE/KID/KIX

Introduction

Transcriptional activity of the repressor DREAM depends on its high affinity binding to DRE sites in target genes (Carrión *et al.*, 1999; Osawa *et al.*, 2001). The process is controlled by the levels of nuclear calcium (Carrión *et al.*, 1999; Osawa *et al.*, 2001; Craig *et al.*, 2002), the PI3 kinase pathway (Sanz *et al.*, 2001) and the formation of DREAM– α CREM heteromers (Ledo *et al.*, 2000a). Three functional EF-hands in the DREAM protein sense the intracellular concentration of Ca²⁺ and EF-hand occupancy by Ca²⁺ blocks binding of DREAM to DRE sites. Thus, increased levels of intracellular Ca²⁺ following membrane depolarization or release from intracellular stores result in DREAM-mediated transcriptional derepression (Carrión *et al.*, 1999). Site-directed mutagenesis of two residues within any of the functional EF-hands in DREAM produces mutant proteins (EFmutDREAM) that remain bound to DNA in the presence of elevated Ca²⁺ concentrations. In a background of wild-type DREAM, EFmutDREAMs behave as dominant-negative mutants and block Ca²⁺-dependent derepression (Carrión *et al.*, 1999; Ledo *et al.*, 2000b). In addition to calcium, we have

demonstrated recently that in hematopoietic progenitors binding of DREAM to DRE sites depends on PI3 kinase activation (Sanz *et al.*, 2001). The residues in DREAM and the downstream kinases in the PI3 kinase pathway responsible for this are presently unknown.

Transcriptional derepression at DRE sites following PKA activation depends on a specific protein–protein interaction between DREAM and α CREM that blocks binding of DREAM to the DRE site (Carrión *et al.*, 1998; Ledo *et al.*, 2000a). The transcriptional repressor α CREM modulates CRE-dependent gene expression (Foulkes *et al.*, 1991) and does not bind directly to DRE sites (Ledo *et al.*, 2000a). The DREAM– α CREM interaction involves two leucine-charged residue rich domains (LCDs) located in DREAM at positions 47 and 155, and two LCDs in α CREM located in the kinase-inducible domain (KID) and in the leucine zipper (LZ) (Ledo *et al.*, 2000a). The LCD motif was first described in nuclear coactivators (NCoA-1, p/CIP) and corepressors (N-CoR, SMRT) and has been implicated in protein–protein interactions with nuclear hormone receptors and CBP (LeDouarin *et al.*, 1996; Heery *et al.*, 1997; Torchia *et al.*, 1997; Hu and Lazar, 1999). Moreover, LCDs in the N- and C-terminals of CBP mediate the interaction with nuclear receptors and p/CIP, respectively (Torchia *et al.*, 1997). Two classes of LCDs have been defined; the NR box and the CoRNR ‘corner box’ whose consensus sequence are LxxLL and L/IxxV/II, respectively, where x denotes any amino acid (LeDouarin *et al.*, 1996; Hu and Lazar, 1999). Interestingly, both LCDs in α CREM (ILNEL and LIEEL) have an anti-parallel orientation compared with the NR consensus box, and define a third type of LCD (Ledo *et al.*, 2000a). Among the different isoforms derived from the CREM gene (Foulkes *et al.*, 1991), only α and ϵ CREM displace DREAM from DRE sites (Ledo *et al.*, 2000a). Short ICER–CREM isoforms lack the N-terminal LCD while isoforms containing the second DNA-binding domain (β CREM) have a non-functional LCD at the C-terminal (Ledo *et al.*, 2000a). Moreover, the spacing between the two LCDs in α or ϵ CREM is important since τ_1 CREM or CREB, two proteins that contain the same or very similar LCDs in the KID and LZ domains, but have the Q₂ transactivation domain in between, do not displace the binding of DREAM from DRE sites. Accordingly, a two-site model of interaction was proposed to stabilize the DREAM– α CREM interaction that prevents DREAM binding to the DRE (Ledo *et al.*, 2000a). Site-directed mutagenesis of one residue in any of the LCDs of DREAM blocks the two-site interaction with α CREM and produces DREAM mutants insensitive to PKA-dependent derepression (Ledo *et al.*, 2000a).

Cyclic AMP-dependent gene expression is controlled at the transcriptional level by several bZIP transcription factors, including CREB, CREM and ATF proteins

Fig. 1. DREAM blocks CRE-retarded bands formed by recombinant CREM and CREB proteins. **(A)** EMSA shows the lack of binding of DREAM to the CRE probe (lane 1) and the block by DREAM of the CRE-retarded bands (lanes 3, 5 and 9). Only the migration of the CRE-retarded band obtained with ICER-1 is not modified by DREAM (lane 7). Note the different mobility of the CRE-retarded bands for the different recombinant proteins. **(B)** Excess of cold DRE oligonucleotide or pre-incubation with a DREAM antibody does not modify CRE-retarded bands, but blocks the effect of DREAM on αCREM or CREB–CRE-retarded bands.

(Sassone-Corsi 1995). They bind to CRE sites in target genes as homo- or heterodimers. Dimerization is achieved by the LZ located next to the basic DNA-binding domain in the C-terminal of the protein. Transcriptional activity by these dimers follows after phosphorylation in their KIDs (Yamamoto *et al.*, 1988; Sheng *et al.*, 1991) and the recruitment of the transcriptional cofactor CREB-binding protein, CBP (Nichols *et al.*, 1992; Parker *et al.*, 1996; Goodman and Smolik, 2000; Vo and Goodman, 2001). Importantly, the LCD within the KID domain in αCREM, common to all CREM isoforms and almost identical in CREB, is located within a region important for the interaction with the CREB-interacting domain of CBP (amino acids 455–679) known as the KIX domain (Parker *et al.*, 1996; Shih *et al.*, 1996). In this study, we show that there exists a Ca^{2+} -dependent protein–protein interaction between DREAM and CREB through the LCD located in the KID domain of CREB. As a result of this interaction, DREAM prevents the recruitment of CBP and represses CRE-dependent transcription.

Results

DREAM blocks binding of CREM or CREB proteins to CRE

To check whether the DREAM–αCREM interaction affects the function of αCREM on CRE-dependent transcription, we first analysed whether DREAM affects binding to CRE sites. For this, we performed gel mobility-shift experiments using recombinant proteins and a CRE

Fig. 2. Effect of calcium and PKA phosphorylation on the interaction between DREAM and CRE-binding proteins. *In vitro* phosphorylation of αCREM **(A)** or CREB **(B)** does not affect the blockade by DREAM of the CRE-retarded bands. Addition of 10 μM Ca^{2+} to the incubation completely prevents the block of DREAM on CRE-retarded bands formed with αCREM **(A)** or CREB **(B)**. The EFmDREAM, insensitive to Ca^{2+} , still blocks the CRE-retarded bands in the presence of 10 μM Ca^{2+} .

as probe. No CRE-retarded band was observed after incubation of DREAM with the CRE probe (Figure 1A, lane 1). However, DREAM blocked the appearance of the αCREM–CRE-retarded band (Figure 1A, lane 3). As a control, we analysed the migration of CRE-retarded bands formed with other CREM isoforms, ICER or CREB, shown previously to be unable to displace DREAM–DRE binding (Ledo *et al.*, 2000a). Unexpectedly, the presence of DREAM also blocked these CRE-retarded bands (Figure 1A, lanes 5 and 9), with the exception of the CRE-retarded bands obtained with ICER proteins that lacked the N-terminal part, including the KID with its LCD (Figure 1A, lane 7; data not shown). The block of the CRE band in the presence of DREAM was specifically competed with an excess of cold DRE (Figure 1B). Moreover, incubation with an antibody against DREAM that did not modify CREM- or CREB–CRE-retarded bands reduced the block of DREAM on CRE-retarded bands (Figure 1B). Taken together, these results disclose a new type of specific interaction between DREAM and CRE-binding proteins that prevent their binding to the CRE probe.

Ca^{2+} -dependent effect of DREAM on CRE-retarded bands

We have shown previously that phosphorylation at Ser68 increases the capacity of αCREM to interact with DREAM and to disrupt DREAM–DRE binding (Ledo *et al.*, 2000a). Thus, we wanted to check whether phosphorylation of CREB or CREM proteins modifies the effect of DREAM on CRE-retarded bands. Interestingly, *in vitro* phosphorylation of recombinant CREB or αCREM with PKA did not prevent the block by DREAM (Figure 2). Moreover, in titration experiments using different amounts of recombinant DREAM, the *in vitro* phosphorylation of CREM or CREB did not increase the capability of DREAM to displace the CRE band (data not shown). These results indicate that the DREAM–CREM or DREAM–CREB

interaction that prevents binding to CRE sites is not dependent on and is not affected by phosphorylation in the KID domain of CRE-binding proteins.

Binding of calcium to the EF-hands of DREAM modifies its conformation, blocking its capacity to bind to the DRE sequence (Carrión *et al.*, 1999; Craig *et al.*, 2002). Thus, we checked whether calcium has an effect on the interaction between DREAM and CREB, or on CREM proteins that modify CRE-retarded bands. Addition of calcium to the incubation reaction did not alter CREM- or CREB-derived CRE-retarded bands, but did neutralize the capacity of DREAM to block these bands (Figure 2). Importantly, the effect of calcium was specific since it was totally absent when EFmDREAM, a dominant-negative mutant of DREAM that still interacts with CREM and CREB, but does not respond to calcium (Carrión *et al.*, 1999), was used (Figure 2). Taken together these results suggest that upon stimulation, the rise in Ca^{2+} levels, but not the phosphorylation in the KID of CREM or CREB, impairs the interaction between repressor DREAM and these CRE-binding proteins.

An LCD-based one-site interaction is responsible for the block of the CRE-retarded band

As mentioned above, the LCD sequence ILNDL, located in the KID of α CREM, participates in a two-site interaction with DREAM, although it is not sufficient to disrupt the DREAM–DRE-retarded band by itself (Ledo *et al.*, 2000a). This sequence is present in all CREM isoforms, is nearly conserved in the KID of CREB, ILNEL, but is absent in ICER proteins whose binding to CRE is not affected by DREAM. Thus, we checked whether this domain is responsible for the interaction between DREAM and CREM or CREB proteins, which blocks the CRE-retarded band. To test this possibility, we used the α CREML73,76V mutant whose LCD sequence in the KID is disrupted (Ledo *et al.*, 2000a). The α CREML73,76V mutant produced a CRE-retarded band that was no different from the CRE-retarded band obtained with wild-type α CREM (Figure 3A, compare lanes 9 and 13). However, in the presence of DREAM, the CRE-retarded band formed with mutant α CREML73,76V was not blocked (Figure 3A, compare lanes 10 and 14). Similarly, an equivalent mutation of the LCD in the KID of CREB, CREBL138,141V, did not modify binding to the CRE probe, but the CRE-retarded band was insensitive to the presence of DREAM (Figure 3A, lane 6). These results confirm that the LCD in the KID of CRE-binding proteins participates in the interaction with DREAM that blocks the CRE-retarded band. To check the corresponding domain(s) in DREAM, we performed similar experiments using DREAM LCD mutants (DREAML47,52V and DREAML155V) shown previously to block the two-site interaction with α CREM (Ledo *et al.*, 2000a). Interestingly, only the mutant DREAML47,52V failed to block the CRE band obtained either with α CREM or with CREB (Figure 3A, lanes 3 and 11). Taken together, these results support a model in which a one-site interaction between DREAM and CRE-binding proteins is stabilized by the LCD-47 of DREAM and the LCD in the KID of CREB or CREM proteins.

Importantly, mutations involving L138 and L141 in the KID of CREB have been described to preclude binding to

Fig. 3. An LCD in the KID of CREB mediates the interactions with DREAM or with CBP–KIX. **(A)** The interaction between DREAM and CRE-binding proteins involves specific LCDs in both proteins. Mutation in the LCD of the KID of CREB (CREBL138,141V) or α CREM (α CREML73,76V) prevents the block by DREAM (compare lanes 2 and 6 or 10 and 14, respectively). Mutation in the N-terminal LCD of DREAM (DREAML47,52V) specifically cancels the interaction with CREB or α CREM (lanes 2 and 3 or 10 and 11, respectively), while the C-terminal LCD mutant DREAM (DREAML155V) behaves as wild-type DREAM (lanes 2 and 4 or 10 and 12, respectively). **(B)** The KID–KIX interaction is mediated by LCDs in both peptides. The supershifted CRE band obtained with phosphoCREB and CBP–KIX (lanes 2 and 3) is not observed with the LCD mutant CBP–KIXL603,607V (lanes 4 and 5). Conversely, CBP–KIX does not supershift the CRE band obtained with phosphoCREBL138,141V (lanes 13 and 14). Addition of DREAM blocks the supershifted CRE band in a Ca^{2+} -dependent manner. The EFmDREAM, insensitive to Ca^{2+} , still blocks the supershifted CRE-retarded bands in the presence of 10 μ M Ca^{2+} .

the KIX in CBP (Parker *et al.*, 1996; Shih *et al.*, 1996). Furthermore, solution structure analysis of the KID–KIX interaction pointed to the involvement of an α -helical domain in KIX that contains a putative LCD motif (HLVHKLTV) (Radhakrishnan *et al.*, 1997). Wild-type KIX interacts with phosphoCREB and supershifts the CRE-retarded band, while a mutation of the putative LCD in the KIX, KIXL603,607V, prevented the supershift (Figure 3B, compare lanes 2 and 4). Similarly, phosphoCREBL138,141V failed to generate a supershift in the presence of wild-type CBP–KIX (Figure 3B, lane 13). Thus, these results suggest that two LCDs mediate the KID–KIX interaction and enable the recruitment of CBP by phosphoCREB. Furthermore, inclusion of DREAM in the reaction also displaced the appearance of the supershifted CRE band in a Ca^{2+} -dependent manner (Figure 3B, compare lanes 7 and 8). These results suggest that DREAM and CBP might compete for binding to the same LCD in the KID of CREB. If so, it could be hypothesized that DREAM may affect phosphoCREB-dependent CBP recruitment and CREB/CBP-mediated transcription.

Fig. 4. DREAM blocks the CREB–CBP interaction without interacting with CBP. (A) Pull-down experiment showing the interaction between *in vitro* phosphorylated CREB and GST–KIX (lane 4) and the blockade of the interaction by recombinant DREAM (lane 7). LCD DREAM mutant DREAML47,51V does not block the pull-down (lane 6), while empty GST vector (GST-0) or phosphoCREB mutant CREBS133A does not show a pull-down band (lanes 3 and 5, respectively). (B) GST–KIX does not interact with DREAM.

Recombinant DREAM prevents binding of CBP to phosphoCREB

To obtain the first evidence for an interaction between DREAM and the KID of CREB that might affect the recruitment of CBP, we performed pull-down experiments using recombinant proteins. First, we analysed the KID–KIX interaction by pull-down experiments. Incubation of a GST fusion protein containing the CREB-binding domain of CBP (GST–KIX; Parker *et al.*, 1996) efficiently pulled down phosphoCREB (Figure 4A, lane 4). To control for the specificity, we showed that recombinant GST alone (GST-0) did not pull-down phosphoCREB (Figure 4A, lane 3). Moreover, GST–KIX failed to pull-down the phospho mutant CREBS133A (Figure 4A, lane 5), in keeping with previous reports (Parker *et al.*, 1996; Shih *et al.*, 1996; Radhakrishnan *et al.*, 1997). Then, we studied the effect of DREAM on the KID–KIX interaction. Addition of recombinant DREAM to the incubation totally blocked the ability of GST–KIX to pull-down phosphoCREB, while DREAML47,52V failed to inhibit the GST–KIX/phosphoCREB interaction (Figure 4A, compare lanes 7 and 6). These results confirm the data from band-shift assays, and furthermore, indicate that the interaction between DREAM and CREB is not dependent on CREB bound to DNA. Taken together, these results indicate that the binding of DREAM to the LCD in the KID of CREB precludes the interaction with the LCD in the KIX of CBP and that this interaction might block CBP-dependent transactivation at CRE sites. However, these data do not rule out an additional interaction between DREAM and CBP–KIX. We checked this possibility in pull-down experiments but were not able to observe a direct interaction between GST–KIX and DREAM (Figure 4B). Moreover, in gel-shift experiments recombinant CBP–KIX did not affect the binding of DREAM to a DRE probe (data not shown).

DREAM mutants impair CBP-dependent coactivation of Gal4–CREB

Transcriptional activity of phosphorylated CREB depends on its ability to recruit coactivator CBP (Chivria *et al.*,

Fig. 5. Ca²⁺-dependent blockage by DREAM of the recruitment of CBP by GAL4–CREB. (A) Transactivation of the pG5CAT reporter by GAL4–CREBΔLZ and coactivator CBP in basal conditions (open bars) or after Ca²⁺ stimulation (black bars) is blocked in the presence of EFmDREAM, but not by EFmDREAML47,52V. LCD CREB or CBP mutants (GAL4–CREBΔLZ L138,141V or CBPL603,607V) failed to transactivate the pG5CAT reporter. (B) Lack of EFmDREAM effect to block the CBP-dependent transactivation by pGAL4–cJunΔLZ of the pG5CAT reporter in basal conditions (open bars) or after Ca²⁺-stimulation (black bars). For each panel, values are normalized with respect to pcDNA3-transfected cultures in the absence of transfected CBP and without caffeine treatment. Error bars show standard deviations. Data are from four independent experiments performed in triplicate.

1993; Kwok *et al.*, 1994). Since the *in vitro* results described above suggest that DREAM binds to the LCD in the KID of CREB where CBP binds, we wondered whether this is reflected in a lower capacity of CBP to activate CRE-dependent transcription when phosphoCREB is complexed to DREAM. To investigate this possibility, we cotransfected the pG5CAT reporter, containing five GAL4-binding sites, together with the GAL4–CREBΔLZ fusion protein and transcriptional coactivator CBP in HEK293 cells. We then compared the effect of an increase in intracellular calcium and cAMP levels by caffeine (Hernandez-Cruz *et al.*, 1990; Carrión *et al.*, 1999) in the presence of DREAM, the dominant-negative mutant EFmDREAM or the double dominant-negative mutant EFmDREAML47,52V. The use of the GAL4–CREBΔLZ fusion protein, lacking the bZip DNA/dimerization domain (ΔLZ), eliminates the possibility of dimerization with endogenous CREB protein to transactivate the pG5CAT reporter. Cotransfection of GAL4–CREBΔLZ and CBP resulted in a 35-fold transactivation of the pG5CAT reporter after caffeine treatment (Figure 5A).

Fig. 6. Ca^{2+} -dependent regulation by DREAM of CRE-dependent transcription. (A) Western blot analysis of DREAM expression in PC12 cells using the monoclonal anti-DREAM antibody 1B1. Lack of cross-reactivity of the 1B1 monoclonal with DREAM-related proteins KchIP-1 and -2 after overexpression in HEK293 cells. (B) Specific repression by the EFmDREAM mutant of caffeine-induced transactivation of pSomCAT after transient cotransfection in PC12 cells. (C and D) Effect of EFmDREAM on the transactivation of pSomCAT after the activation of different signalling pathways in wild-type and PKA-deficient PC12 cells (line A126-1B2). Values are expressed as fold-induction in CAT activity with respect to untreated cultures transfected with empty expression vector pcDNA3. Open bars represent control values and filled bars values after stimulation with the different drugs. Error bars show standard deviations. Data are from four independent experiments performed in triplicate.

A similar induction following caffeine treatment was observed after cotransfection of GAL4-CREBALZ, CBP and DREAM (Figure 5A). However, in cells cotransfected with the dominant-negative mutant EFmDREAM, transactivation of the pG5CAT reporter by GAL4-CREBALZ and CBP after caffeine was dramatically reduced (Figure 5A). Importantly, this blockage was not observed after cotransfection with the double mutant EFmDREAML47,52V, as it was unable to bind to CREB and to block the interaction of phosphoCREB with CBP-KIX *in vitro* (Figure 5A). Activation by caffeine of endogenously expressed CBP or its homologue p300, in HEK293 cells showed essentially similar repression by EFmDREAM and no repression by EFmDREAML47,52V, although the levels of induction were lower (Figure 5A). Moreover, mutation of the LCD in the KID of CREB in construct pGAL4-CREBALZL138,141V completely abolished transactivation of the pG5CAT reporter following caffeine, even after cotransfection with CBP, in keeping with the *in vitro* results (Figure 5A). Similarly, mutation of the LCD in the KIX of CBP in construct CBPL603,607V blocked its transactivating effect on the pG5CAT reporter (Figure 5A). Control experiments using the empty vector pGAL4 did not result in significant transactivations of the pG5CAT reporter after caffeine treatment and/or cotransfection with the different expression vectors (data not shown). Importantly, in these experiments, we optimized the

transfection conditions to study the block by DREAM mutants following Ca^{2+} stimulation. Because of the low amount of reporter and the short time between transfection and harvest of the cells, the basal acetylation activity was very low and did not allow observation of any noticeable effect on basal activity after DREAM or EFmDREAM overexpression (Figure 5A). Together, these results confirm a Ca^{2+} -dependent DREAM-CREB interaction that can block the ability of phosphoCREB to recruit CBP and thus block the transactivation by CBP of CREB-dependent transcription. Essentially similar results were obtained when we used a full-length GAL4-CREB fusion construct, confirming that the LCD within the dimerization domain in the C-terminal part of CREB does not play a role in the interaction between CREB and DREAM. Moreover, experiments using the GAL4-CREBΔLZS142,143A mutant gave essentially the same results (data not shown) suggesting that the Ca^{2+} -dependent unbinding of DREAM from CREB relies exclusively on the ability of DREAM to bind Ca^{2+} and is not due to the Ca^{2+} -dependent phosphorylation of CREB at Ser142,143 (Matthews *et al.*, 1994; Sun *et al.*, 1994).

Interaction between the N-terminal domain of c-Jun and the KIX domain of CBP participates in the transactivation by CBP at AP-1 sites (Arias *et al.*, 1994; Kwok *et al.*, 1994). As a control for the specificity of the DREAM blockage of the KID-KIX interaction, we performed additional experiments using the pG5CAT reporter and an expression vector for the GAL4-cJunΔLZ fusion protein. Treatment with caffeine increased transactivation by GAL4-cJunΔLZ of the pG5CAT reporter 5- and 12-fold in the absence and in the presence of exogenously expressed CBP, respectively (Figure 5B). Importantly, cotransfection of EFmDREAM had no effect (Figure 5B). Furthermore, use of the LCD mutant in the KIX domain, CBPL603,607V, had essentially the same transactivating activity as CBP on the GAL4-cJunΔLZ-pG5CAT system (data not shown). These results strengthen the notion that the Ca^{2+} -dependent repression of CBP-mediated transactivation by DREAM is specific and exclusively due to the ability of DREAM to interact with CREB.

DREAM interferes with CRE-dependent transcription

To investigate whether the interaction between DREAM and CREB could affect CRE-dependent transcription, we performed transfection experiments using pSomCAT, a reporter plasmid that contains the canonical CRE from the somatostatin promoter (Montminy *et al.*, 1986), and PC12 cells, a cell line with endogenous expression of DREAM (Figure 6A). Increased levels of intracellular Ca^{2+} and cAMP after caffeine treatment (Hernandez-Cruz *et al.*, 1990) resulted in a 12-fold transactivation of pSomCAT in PC12 cells (Figure 6B). Cotransfection with DREAM did not modify the induction of pSomCAT by caffeine (Figure 6B). However, coexpression with EFmDREAM, insensitive to calcium, significantly reduced the transactivation of the CRE reporter by caffeine (Figure 6A). Coexpression with the double mutant EFmDREAML47,52V, unable to respond to calcium and to interact with CREB, did not modify pSomCAT transactivation after caffeine (Figure 6B). Meanwhile, the double mutant EFmDREAML155V, which is still able

to interact with CREB, behaved as EFmDREAM (Figure 6B). Since caffeine affects at least two major signalling pathways, Ca^{2+} and cAMP, in the next experiments, we tried to dissociate these two pathways to better understand the regulation of the DREAM–CREB interaction. Overexpression of the catalytic subunit of PKA, to mimic the effect of an increase in cAMP levels, led to a 14-fold transactivation of the pSomCAT reporter in PC12 cells (Figure 6C) that was reduced by coexpression with EFmDREAM (Figure 6C). Alternatively, Ca^{2+} mobilization by caffeine in A126–1B2 cells, a clone of PC12 cells deficient in the catalytic subunit of PKA (Van Buskirk *et al.*, 1985) increased the transactivation of the pSomCAT reporter 12.5-fold and this effect was blocked by EFmDREAM (Figure 6C). Furthermore, several other stimuli are able to selectively phosphorylate CREB at different serine residues within the KID region and differentially regulate CRE-dependent transcription in PC12 cells (Fiol *et al.*, 1994; Matthews *et al.*, 1994; Sun *et al.*, 1994; Mayr *et al.*, 2001). Thus, we next studied the effect of DREAM on CRE-dependent transcription upon these various stimuli. Entrance of extracellular calcium through voltage-dependent calcium channels upon depolarizing concentrations of KCl, PKC activation by phorbol esters or activation of the Erk-MAP-kinase pathway after exposure to EGF or NGF lead to CREB phosphorylation (Mayr *et al.*, 2001; West *et al.*, 2001). Treatment of PC12 cultures with these agents increased the expression of the pSomCAT reporter 2- to 4-fold (Figure 6D). Importantly, cotransfection of the EFmDREAM mutant blocked the transactivation of the pSomCAT reporter in all cases (Figure 6D), while cotransfection with wild-type DREAM or the double mutant EFmDREAML47,52V did not affect the increase in CRE transcription elicited by the different stimuli (data not shown). The lower transactivating activity of these stimuli on CRE transcription is in agreement with their low capability to induce the interaction between phosphoCREB and CBP in the nucleus, as assayed by fluorescence resonance energy transfer (Mayr *et al.* 2001). Taken together, these results further strengthen the *in vitro* data, and propose a central role for Ca^{2+} regulating the unbinding of DREAM from CREB in CRE-dependent transcription activated by different signalling pathways. In control experiments using pTRETTCAT, a reporter plasmid containing the AP-1 site from the collagenase promoter, neither DREAM nor EFmDREAM modified its basal or caffeine-induced transactivation (data not shown), even when cotransfected at DNA concentrations up to four times higher than those used in the pSomCAT experiments. These results indicate the specificity of the DREAM-mediated Ca^{2+} -dependent repression at CRE sites.

To further substantiate the role of endogenous DREAM in basal CRE-dependent transcription, we overexpressed an antisense expression vector for DREAM in PC12 cells to transiently knockdown the levels of endogenous DREAM protein (Figure 7A). Reduced levels of DREAM resulted in a significant induction of basal pSomCAT expression (Figure 7B). Basal expression of a reporter containing a DRE site was also increased following the reduction in the levels of repressor protein DREAM, while the activity of a reporter construct containing an AP-1 site was not affected (Figure 7B).

Fig. 7. Endogenous DREAM regulates basal CRE-dependent transcription. (A) Reduced levels of repressor DREAM protein in PC12 cells 48 h after transient transfection with a DREAM antisense expression plasmid (pAS-DREAM). No change in CREB protein levels is also shown by western blotting. (B) Increased basal activity of reporter plasmids pSomCAT (CRE) and pHD3CAT (DRE) after transient knockdown of DREAM protein levels (filled bars). No change in basal expression of pTRETTCAT (AP-1) by pAS-DREAM (filled bar). Error bars show standard deviations. Data are from four independent experiments performed in triplicate.

Importantly, antisense DREAM did not modify the levels of endogenous CREB (Figure 7A), suggesting that the increase in CRE-dependent transcription is a reflection of the derepression of CREB following the reduction in the levels of DREAM in the cell.

DREAM interacts with CREB *in vivo*

To analyse whether the interaction between DREAM and CREB proteins occurs *in vivo*, we performed coimmunoprecipitation experiments using nuclear extracts from brain. Consistent with the results from transfection, pull-down and band-shift experiments, CREB was immunoprecipitated from brain nuclear extracts using an anti-DREAM antibody (Figure 8A, lane 3). Similarly, DREAM was immunoprecipitated from brain nuclear extracts using an anti-CREB antibody (Figure 8B, lane 2). As a result of the Ca^{2+} -dependent interaction between CREB and DREAM, co-immunoprecipitation of DREAM with the anti-CREB antibody was reduced in the presence of Ca^{2+} (Figure 8B, lane 3). To control for the specificity of the co-immunoprecipitations, several controls were included. Pre-adsorption of the anti-DREAM antibody with recombinant DREAM protein completely blocked its ability to immunoprecipitate CREB (Figure 8A, lane 2). DREAM or CREB were immunoprecipitated from brain nuclear extracts using anti-DREAM or anti-CREB antibodies, respectively (Figure 8A and B, lanes 4). Moreover, pre-immune serum or antibodies against several nucleoproteins like p53, C/EBP or thyroid hormone receptor α failed to immunoprecipitate DREAM or CREB, respectively (Figure 8A and B, lanes 5; data not shown). Immunoprecipitation of CREB with the anti-DREAM antibody was not observed with nuclear extracts from

Fig. 8. Coimmunoprecipitation of DREAM and CREB proteins from brain nuclear extracts. **(A)** Detection of the CREB protein by western blotting (WB) after immunoprecipitation (IP) with the indicated polyclonal antibodies. **(B)** Detection of the DREAM protein using the 1B1 monoclonal anti-DREAM antibody after IP with the indicated polyclonal antibodies. Lane 1 represents the input (10%) and the asterisks mark the migration of the 40 kDa CREB band (A) or the 28 kDa band corresponding to monomer DREAM (B).

liver, a tissue with very low endogenous expression of DREAM (Carrión *et al.*, 1999; data not shown).

Taken together, our results indicate that under basal conditions, DREAM tonically modulates the activity of promoters containing CRE sites in a Ca²⁺-dependent manner by the formation of protein complexes with CREB and CREM isoforms. Thus, the interaction between DREAM and CRE-binding transcription factors represents a novel point of cross-talk between the cAMP and Ca²⁺-dependent signalling cascades at the level of gene expression.

Discussion

Here we have identified a novel protein–protein interaction between transcriptional repressor DREAM and CREB that involves α -helical domains of the LCD type present in both proteins and is independent of the phosphorylation state of CREB. Importantly, binding of DREAM prevents recruitment of CBP by phosphoCREB and affects CRE-dependent transcription in this way.

Our data support the existence of an intermediate step between CREB phosphorylation and CBP recruitment that involves the unbinding of DREAM from the KID in CREB. The existence of such an intermediate regulatory step was somehow anticipated by reports from different laboratories showing that CREB phosphorylation is necessary but not sufficient for CBP recruitment and CRE-dependent transcription (Sun and Maurer, 1995; Cardinaux *et al.*, 2000; Shaywitz *et al.*, 2000). Recently, the existence of an inhibitory factor for the KID–KIX interaction that could account for the distinct effects of cAMP and mitogenic signals on CBP recruitment was proposed (Mayr *et al.*, 2001). Whether such a factor corresponds exclusively to DREAM or whether other co-repressors are also acting at this level is presently not known. Conversely, several protein–protein interactions have been described that stabilize the KID–KIX complex and increase CRE-dependent transcription. A positive regulation of CREB–KID and CBP–KIX interaction has been reported for the mixed-lineage leukaemia protein

after its interaction with KIX (Ernst *et al.*, 2001) and for c-Jun through its C-terminal interaction with the C/H2 domain of CBP (Hu *et al.*, 1999). Moreover, a transcription factor-dependent regulation of CBP also controls CBP-dependent transcription (Soutoglou *et al.*, 2001). For instance, it has been shown recently that several transcription factors, including hepatocyte nuclear factor 1a (HNF-1a), HNF-4 and Sp1, increase the histone acetyltransferase activity of CBP and CBP-associated factor (P/CAF) (Soutoglou *et al.*, 2001), and could affect CREB-dependent transcription at specific promoters in this way.

Signalling-dependent modifications of CBP could also represent a regulatory step prior to CBP recruitment to the transcription complex. Overexpression of coactivator-associated arginine methyltransferase 1 (CARM1) has recently been shown to methylate CBP within the KIX domain (Xu *et al.*, 2001). CARM1-mediated methylation of CBP–KIX occurs at R600, a residue critical for the appropriate folding of KIX to interact with CREB–KID. As a result, methylated KIX does not bind to KID and CREB-mediated gene expression is blocked. While increase in intracellular calcium is shown to efficiently release CREB from DREAM repression, further studies are needed to know the extent of CBP methylation in basal conditions and to identify the mechanism of demethylation that could allow CREB-dependent transcription to occur. Moreover, phosphorylation of CBP by different kinases, including those participating in Ca²⁺- and cAMP-dependent signalling pathways, has been proposed to affect CBP transactivation (Chawla *et al.*, 1998; for a review, see Vo and Goodman, 2001). Validation of these mechanisms has been hampered by the lack of a conclusive identification of the residue that is phosphorylated in each case. However, it has been described recently that activity-induced CaMK-IV-dependent phosphorylation of CBP at serine 301 contributes to CREB/CBP transcription (Impey *et al.*, 2002). Moreover, PI3K-dependent phosphorylation of CBP at serine 436, following growth factor treatment, is required for its recruitment to transcriptional complexes in promoters containing Pit-1 and AP-1 responsive elements (Zanger *et al.*, 2001).

Binding of Ca²⁺ to EF-hands in calcium-binding proteins triggers a conformational change that underlies their Ca²⁺-dependent biological effects. In previous work, we have shown that mutations of one, two or three EF-hands in DREAM result in indistinguishable dominant-negative mutants that remain bound to DNA in the presence of Ca²⁺ ions (Carrión *et al.*, 1999). A recent study using microelectrospray ionization mass spectrometry showed the Ca²⁺-dependent change in the conformation of the DREAM protein (Craig *et al.*, 2002). This change only occurs when DREAM is fully loaded with four mole equivalents of Ca²⁺, confirming that all EF-hands in DREAM are equally required for the unbinding of DREAM from DNA. Future studies should ascertain whether the Ca²⁺-dependent unbinding of DREAM from its interaction with CREB and CREM proteins also needs full occupancy of the EF-hands. Importantly, the dominant-negative mutant EFmDREAM blocks pSomCAT transactivation induced by different stimuli, including the overexpression of the catalytic subunit of PKA. These results suggest a central role for basal Ca²⁺ mobilization in CRE-dependent transcription. We have

also shown that binding of DREAM to DRE sites in the DNA is regulated by the PI3-kinase pathway (Sanz *et al.*, 2001). The molecular basis for this regulation and the role of this growth factor-dependent pathway in DREAM–CREB interaction and CREB-dependent transcription are presently under investigation.

The strength of the KID–KIX interaction determines the magnitude of the CREB-dependent transcriptional response. Phosphorylation of S133 in the KID allows a conformational change from coil to α -helix that permits the interaction with KIX. However, several mutations have been described either in the KID or in the KIX that facilitate the interaction without the need of KID phosphorylation (Cardinaux *et al.*, 2000; Shaywitz *et al.*, 2000). Here we show that the ability of DREAM to interact with CREM and CREB and block their binding to CRE sites is not modulated by the phosphorylation of their KID domains. This result parallels previous work showing that phosphorylation of S68 is not required for the double-site interaction between α CREM and DREAM, that blocks DREAM binding to DNA (Ledo *et al.*, 2000a), although, phosphorylation of S68 potentiates the double-site interaction. Further studies using NMR to solve the solution structure of DREAM, bound and not bound to CRE-binding proteins, are needed to understand the differential effect of phosphorylation in the two-site versus the one-site interaction. Together, our results suggest that the ultimate consequence of an increase in kinase and/or Ca^{2+} signalling upon cellular stimulation is a derepression of DRE- and CRE-dependent transcription.

The interaction between DREAM and CREB and CREM proteins through LCD α -helices is in accordance with the major role of these relatively small protein domains in transcriptional regulation. The LCD-type of protein–protein interactions was first described between nuclear coactivators (NCoA-1, p/CIP) or corepressors (N-CoR, SMRT) and nuclear hormone receptors or CBP (LeDouarin *et al.*, 1996; Heery *et al.*, 1997; Torchia *et al.*, 1997; Hu and Lazar, 1999). Moreover, LCDs located in the N- and C-terminal ends of CBP mediate the interaction with nuclear receptors and p/CIP, respectively (Torchia *et al.*, 1997). In addition, here we have shown that LCD interactions are responsible for the recruitment of CBP by phosphoCREB and its blockade by DREAM. Protein–protein interactions between DREAM and other cellular proteins, including presenilins, and voltage-dependent K^+ channels of the Kv4 class have been reported (Buxbaum *et al.*, 1998; An *et al.*, 2000). In both cases, however, it is not known presently whether the interaction involves the LCDs in DREAM. Moreover, the domains in the presenilins or the Kv4 channels that bind DREAM are not characterized (An *et al.*, 2000; Bähring *et al.*, 2001). Importantly, binding of DREAM to Kv4 channels or to presenilins is not regulated by Ca^{2+} (An *et al.*, 2000; Choi *et al.*, 2001), although DREAM regulates the gating of the Kv4 channel in a Ca^{2+} -dependent manner (An *et al.*, 2000). Thus, in addition to control DRE-dependent gene expression, DREAM binds to a subset of cellular proteins, in and outside the nucleus, and like calmodulin, DREAM controls multiple cellular functions upon conformational changes that are not always exclusively related to changes in Ca^{2+} concentration.

Materials and methods

Cell culture, transfection and CAT assay

Cells were grown in DMEM containing 2 mM Glutamax-1 and 50 $\mu\text{g}/\text{ml}$ gentamicin and supplemented with 10% fetal calf serum (FCS; HEK293) or 10% horse serum plus 5% FCS (PC12 and A126–1B2). Transfections by calcium phosphate precipitation and CAT activity assays were performed as described (Carrion *et al.*, 1998). For stimulation, caffeine (10 mM), K^+ (65 mM), EGF or NGF (25 ng/ml) and PMA (100 nM) were added to the cultures 24 h before harvesting the cells. The reporter plasmids pH3CAT, pSomCAT and pTRETGCAT and the expression vectors for DREAM- and CRE-binding proteins have been described previously (Montminy *et al.*, 1986; Foulkes *et al.*, 1991; Arias *et al.*, 1994; Carrion *et al.*, 1999).

Electrophoretic mobility-shift assay

EMSA using recombinant proteins and *in vitro* phosphorylation by PKA was performed as described (Parker *et al.*, 1996; Ledo *et al.*, 2000a). Where indicated, calcium was added to the incubation reaction at a concentration of 10 μM . Site-directed mutagenesis of DREAM, CREB, CREM and CBP–KIX were performed by the QuikChange method (Stratagene).

Antibodies, coimmunoprecipitation and western blotting

Monoclonal antibodies against DREAM were prepared as described previously (Galfre *et al.*, 1977) using KLH-conjugated recombinant DREAM. IgGs were affinity purified using protein G–Sephacrose. The 1B1 monoclonal anti-DREAM antibody that recognized DREAM as a 28 kDa band and did not cross react with KchIP-1 and -2 proteins was chosen for western blot assays. For the coimmunoprecipitation experiments, nuclei from adult rat whole brain or liver were isolated as described (Gorski *et al.*, 1986). Nuclei were hypotonically lysed in water by six freeze/thaw cycles and the lysates were centrifuged at 15 000 g for 15 min. The supernatants were diluted 1:1 with 2 $\times$ immunoprecipitation (IP) buffer [1 $\times$ is 10 mM HEPES pH 7.9, 8 mM MgCl_2 , 2 mM EGTA, 1 mM dithiothreitol (DTT), 10% glycerol, supplemented with a protease inhibitor cocktail (Calbiochem)]. For coimmunoprecipitation in the presence of Ca^{2+} , the EGTA was omitted and 2 mM CaCl_2 included. The nuclear extracts were pre-cleared by incubation with protein A–Sephacrose (Pharmacia) for 1 h, and subsequently, cleared lysates were incubated with polyclonal anti-DREAM (FL-214, Santa Cruz) or anti-CREB (9192, New England Biolabs) antibodies overnight to immunoprecipitate CREB and DREAM, respectively. Immuno-complexes were captured with protein A–Sephacrose for 1 h and pellets were washed four times with IP buffer. Protein complexes were eluted by boiling in SDS sample buffer and subjected to western blotting using monoclonal 1B1 or anti-CREB (X-12, Santa Cruz), respectively.

In vitro protein interaction analysis

^{35}S -labelled CREB and DREAM proteins were *in vitro* transcribed/translated using the T7-TNT system (Promega). Two micrograms of purified recombinant CBP–KIX–GST fusion protein (GST–KIX) bound to glutathione–Sephacrose (Pharmacia) were incubated with ^{35}S -labelled proteins, in the presence or absence of recombinant DREAM protein, in interaction buffer [20 mM HEPES, pH 7.9, 10% glycerol, 150 mM KCl, 2 mM MgCl_2 , 0.5 mM EGTA, 1 mM DTT, 0.2% NP40, 0.5% Blotto (Bio-Rad), protease inhibitor cocktail (Calbiochem)]. After five washes in the same buffer, bound proteins were eluted with SDS sample buffer, resolved in SDS–PAGE and detected by fluorography.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

- An, W.F. *et al.* (2000) Modulation of A-type potassium channels by a family of calcium sensors. *Nature*, **403**, 553–556.
- Arias, J., Alberts, A.S., Brindle, P., Claret, F.X., Smeal, T., Karin, M., Feramisco, J. and Montminy, M.R. (1994) Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature*, **370**, 226–229.
- Bähring, R., Dannenberg, J., Peters, H.C., Leicher, T., Pongs, O. and Isbrandt, D. (2001) Conserved Kv4 N-terminal domain critical for effects of Kv channel-interacting protein 2.2 on channel expression and gating. *J. Biol. Chem.*, **276**, 23888–23894.
- 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.
- Cardinaux, J.R., Notis, J.C., Zhang, Q., Vo, N., Craig, J.C., Fass, D.M., Brennan, R.G. and Goodman, R.M. (2000) Recruitment of CREB binding protein is sufficient for CREB-mediated gene activation. *Mol. Cell. Biol.*, **20**, 1546–1552.
- Carrión, A.M., Mellström, B. and Naranjo, J.R. (1998) PKA-dependent derepression of the human prodynorphin gene via differential binding to an intragenic silencer element. *Mol. Cell. Biol.*, **18**, 6921–6929.
- Carrión, A.M., Link, W.A., Ledo, F., Mellström, B. and Naranjo, J.R. (1999) DREAM is a Ca²⁺-regulated transcriptional repressor. *Nature*, **398**, 80–84.
- Chawla, S., Hardingham, G.E., Quinn, D.R. and Bading, H. (1998) CBP: a signal regulated transcriptional coactivator controlled by nuclear calcium and CaM kinase IV. *Science*, **281**, 1505–1509.
- 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's disease-associated C-terminal fragment of presenilin 2. *J. Biol. Chem.*, **276**, 19197–19204.
- Chrivia, J.C., Kwok, R.P.S., Lamb, N., Hagiwara, M., Montminy, M.R. and Goodman, R.H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature*, **365**, 855–859.
- Craig, T.A., Benson, L.M., Venyaminov, S.Y., Klimtchuk, E.S., Bajzer, Z., Prendergast, F.G., Naylor, S. and Kumar, R. (2002) The methal binding properties of DREAM: evidence for calcium mediated changes in DREAM structure. *J. Biol. Chem.*, **277**, 10955–10966.
- Ernst, P., Wang, J., Huang, M., Goodman, R.H. and Korsmeyer, S.J. (2001) MLL and CREB bind cooperatively to the nuclear coactivator CREB-binding protein. *Mol. Cell. Biol.*, **21**, 2249–2258.
- Fiol, C.J., Williams, J.S., Chou, C.H., Wang, Q.M., Roach, P.J. and Andrisani, O.M. (1994) A secondary phosphorylation of CREB at Ser129 is required for the cAMP-mediated control of gene expression. A role for glycogen synthase kinase-3 in the control of gene expression. *J. Biol. Chem.*, **269**, 32187–32193.
- Foulkes, N.S., Borrelli, E. and Sassone-Corsi, P. (1991) CREM gene: use of alternative DNA-binding domains generate multiple antagonists of cAMP-induced transcription. *Cell*, **64**, 739–749.
- Galfre, G., Howe, S.C., Milstein, X.C., Butcher, G.W. and Howard, J.C. (1977) Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature*, **266**, 550–552.
- Goodman, R.M. and Smolik, S. (2000) CBP/p300 in cell growth, transformation and development. *Genes Dev.*, **14**, 1553–1577.
- Gorski, K., Carneiro, M. and Schibler, U. (1986) Tissue specific *in vitro* transcription from the mouse albumin promoter. *Cell*, **47**, 767–776.
- Heery, D.M., Kalkhoven, E., Hoare, S. and Parker, M.G. (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature*, **387**, 733–736.
- Hernández-Cruz, A., Sala, F. and Adams, P.R. (1990) Subcellular calcium transients visualized by confocal microscopy in a voltage-clamped vertebrate neuron. *Science*, **247**, 858–862.
- Hu, P.-P., Harvat, B.L., Hook, S.S., Shen, X., Wang, X.-F. and Means, A.R. (1999) c-Jun enhancement of cyclic adenosine 3'5'-monophosphate response element-dependent transcription induced by transforming growth factor- β is dependent of c-jun binding to DNA. *Mol. Endocrinol.*, **13**, 2039–2048.
- Hu, X. and Lazar, M.A. (1999) The CoNRN motif controls the recruitment of corepressors by nuclear receptors. *Nature*, **402**, 93–96.
- Impey, S. *et al.* (2002) Phosphorylation of CBP mediates transcriptional activation by neural activity and CaM Kinase IV. *Neuron*, **34**, 235–244.
- Kwok, R.P.S., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bächinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R. and Goodman, R.H. (1994) Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature*, **370**, 223–226.
- Ledo, F., Carrión, A.M., Link, W.A., Mellström, B. and Naranjo, J.R. (2000a) DREAM- α CREM interaction via LCD motifs derepresses DRE-dependent transcription. *Mol. Cell. Biol.*, **20**, 9120–9126.
- Ledo, F., Link, W.A., Carrión, A.M., Echeverría, V., Mellström, B. and Naranjo, J.R. (2000b) The DREAM-DRE interaction: key nucleotides and dominant negative mutants. *Biochim. Biophys. Acta*, **1498**, 162–168.
- LeDouarin, B., Nielsen, A.L., Garnier, J.M., Ichinose, H., Jeanmougin, F., Losson, R. and Chambon, P. (1996) A possible involvement of TIF1 α and TIF1 β in the epigenetic control of transcription by nuclear receptors. *EMBO J.*, **15**, 6701–6715.
- Matthews, R.P., Guthrie, C.R., Walles, L.M., Zhao, X., Means, A.R. and McKnight, G.S. (1994) Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. *Mol. Cell. Biol.*, **14**, 6107–6116.
- Mayr, B.M., Canettieri, G. and Montminy, M.R. (2001) Distinct effects of cAMP and mitogenic signals on CREB-binding protein recruitment impart specificity to target gene activation via CREB. *Proc. Natl Acad. Sci. USA*, **98**, 10936–10941.
- Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G. and Goodman, R.H. (1986) Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl Acad. Sci. USA*, **83**, 6682–6686.
- Nichols, M., Weih, F., Schmid, W., DeVack, C., Kowenz-Leutz, E., Luckow, B., Boshart, M. and Schültz, G. (1992) Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP-induced gene transcription. *EMBO J.*, **11**, 3337–3346.
- 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, Calsenilin/DREAM/ KChIP3. *J. Biol. Chem.*, **276**, 41005–41013.
- Parker, D., Ferreri, K., Nakajima, T., LaMorte, V.J., Evans, R., Koerber, S.C., Hoeger, C. and Montminy, M.R. (1996) Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. *Mol. Cell. Biol.*, **16**, 694–703.
- Radhakrishnan, I., Perez-Alvarado, G.C., Parker, D., Dyson, H.J., Montminy, M.R. and Wright, P.E. (1997) Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator: coactivator interactions. *Cell*, **91**, 741–752.
- Sanz, C., Mellström, B., Link, W.A., Naranjo, J.R. and Fdez-Luna, J.L. (2001) IL-3-dependent activation of DREAM is involved in transcriptional silencing of the apoptotic hrk gene in hematopoietic progenitors. *EMBO J.*, **20**, 2286–2292.
- Sassone-Corsi, P. (1995) Transcription factors responsive to cAMP. *Annu. Rev. Cell Dev. Biol.*, **11**, 355–377.
- Shaywitz, A.J., Dove, S.L., Kornhauser, J.M., Hochschild, A. and Greenberg, M.E. (2000) Magnitude of the CREB-dependent transcriptional response is determined by the strength of the interaction between the kinase inducible domain of CREB and the KIX domain of CREB-binding protein. *Mol. Cell. Biol.*, **20**, 9409–9422.
- Sheng, M., Thompson, M.A. and Greenberg, M.E. (1991) CREB: a Ca²⁺-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science*, **252**, 1427–1430.
- Shih, H.-M., Goldman, P.S., DeMaggio, A.J., Hollenberg, S.M., Goodman, R.H. and Hoekstra, M.F. (1996) A positive genetic selection for disrupting protein-protein interactions: identification of CREB mutations that prevent association with the coactivator CBP. *Proc. Natl Acad. Sci. USA*, **93**, 13896–13901.
- Soutoglou, E., Viollet, B., Vaxillaire, M., Yaniv, M., Pontoglio, M. and Talianidis, I. (2001) Transcription factor-dependent regulation of CBP and P/CAF histone acetyltransferase activity. *EMBO J.*, **20**, 1984–1992.
- Sun, P. and Maurer, R.A. (1995) An inactivating point mutation demonstrates that interaction of cAMP response element binding protein (CREB) with the CREB binding protein is not sufficient for transcriptional activation. *J. Biol. Chem.*, **270**, 7041–7044.
- Sun, P., Ensien, H., Myung, P. and Maurer, R.A. (1994) Differential activation of CREB by Ca²⁺/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that is negatively regulated by activity. *Genes Dev.*, **8**, 2527–2539.
- Torchia, J., Rose, D.W., Inostroza, J., Kamei, Y., Westin, S., Glass, C.K. and Rosenfeld, M.G. (1997) The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature*, **387**, 677–684.

- Van Buskirk,R., Corcoran,T. and Wagner,J.A. (1985) Clonal variants of PC12 pheochromocytoma cells with defects in cAMP-dependent protein kinases induce ODC in response to nerve growth factor but not to adenosine agonists. *Mol. Cell. Biol.*, **5**, 1984–1992.
- Vo,N. and Goodman,R.M. (2001) CREB-binding protein and p300 in transcriptional regulation. *J. Biol. Chem.*, **276**, 13505–13508.
- West,A.E., Chen,W.G., Dalva,M.B., Dolmetsch,R.E., Kornhauser,J.M., Shaywitz,A.J., Takasu,M.A., Tao,X. and Greenberg,M.E. (2001) Calcium regulation of neuronal gene expression. *Proc. Natl Acad. Sci. USA*, **98**, 11024–11031.
- Xu,W., Chen,H., Du,K., Asahara,H., Tini,M., Emerson,B.M., Montminy,M.R. and Evans,R.M. (2001) A transcriptional switch mediated by cofactor methylation. *Science*, **294**, 2507–2511.
- Yamamoto,K.K., Gonzalez,G.A., Biggs,W.H.,III and Montminy,M.R. (1988) Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature*, **334**, 494–498.
- Zanger,K., Radovick,S. and Wondisford,F.E. (2001) CREB binding protein recruitment to the transcription complex requires growth factor-dependent phosphorylation of its GF box. *Mol. Cell*, **7**, 551–558.

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