The cAMP response element binding protein, CREB, is a potent inhibitor of diverse transcriptional activators

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ABSTRACT

Cyclic AMP response element binding protein (CREB) activates transcription of cAMP response element (CRE)-containing promoters following an elevation of intracellular cAMP. Here we show that CREB and the highly related protein ATF-1 are also potent transcription inhibitors. Strikingly, CREB inhibits transcription of multiple activators, whose DNA-binding domains and activation regions are unrelated to one another. Inhibition requires that the CREB dimerization and DNA-binding domains are intact. However, inhibition is not dependent upon the presence of a CRE in the promoter, and does not involve heterodimer formation between CREB and the activator. The ability of an activator protein to inhibit transcription in such a promiscuous fashion has not been previously reported.

INTRODUCTION

Transcription in eukaryotes is under negative as well as positive control. Several mechanisms for negative regulation (transcriptional repression) have been proposed (reviewed in 1, 2) and include: competition between the inhibitor and the activator for a common promoter binding site (steric occlusion); titration by the inhibitor of the activator's target (e.g. squelching); formation of an inactive activator/inhibitor complex; inhibition of DNA-bound activator by a DNA-bound repressor. These previously described inhibitory mechanisms are dependent upon specific features of the activators, specific elements in the target promoter, or both.

The cAMP response element binding protein CREB (3) is a member of the ATF/CREB family of transcription factors. These proteins bind to highly related DNA sites and possess homologous 'bZIP' domains which consist of a DNA-binding region rich in basic amino acids and a leucine zipper dimerization domain (4, 5). CREB activates transcription of cAMP response element (CRE)-containing genes following an elevation of intracellular cAMP (reviewed in 6). CREB is a substrate for protein kinase A (PKA) and phosphorylation of serine 133 is required for activation (7). CREB can also inhibit transcription of specific promoters. In these instances repression requires specific promoter elements and is relieved by PKA phosphorylation (8, 9).

Here we show that CREB can inhibit transcription of unrelated activators in a manner that does not require specific promoter elements and that does not involve a PKA-dependent pathway.

MATERIALS AND METHODS

Cell cultures and transfection

Monkey kidney CV-1 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum. For transfection experiments, we followed the calcium-phosphate co-precipitation procedure. Subconfluent cells were split 1:8 and plated on 60 mm dishes 24 hrs before transfection. Transfection was performed with 2 μg of reporter plasmid and various amounts of activator coding plasmids as indicated in the figure legends. The total amount of DNA was adjusted to 8 μg with pGEM-3 (Pharmacia). The cells were exposed for 11—12 hrs to the precipitate, washed and further incubated with medium for 24 hrs before harvest. The transfections were performed at least four times with at least two different plasmid preparations for each plasmid.

Plasmids

p(GAL4)2-E1b-CAT (10), pL6EC (10), pGAL4-VP16 (11), pLexA-VP16 (10) and pECE-ATF2 (12) are described. The constructs coding for CREB and CREB mutants are described in (7, 13, 14). pGAL4-ATF4 was constructed by amplifying the full-length ATF-4 cDNA by the polymerase chain reaction and cloning the fragment in the BamHI and XbaI sites of pSG424 (15). pGAL4-ATF4(1—56) was made by deleting a BamHI-BamHI fragment from pGAL4-ATF4. pGAL4-El A(140—182) contains the coding sequences for amino acids 140 to 182 of the adenovirus 5 El A protein inserted in the EcoRI site of pSG424. pGAL4-Pro codes for amino acids 1 to 94 of GAL4 upstream of the amino acids 399 to 499 of CTF/NF-1 and was constructed by inserting a XhoI—XbaI fragment of pGAL4-Pro (16).
CV-1 cells were transfected in duplicate. When harvesting the cells, the duplicates were mixed, then split again in two. One half of the cells were tested for chloramphenicol acetyl transferase (CAT) activity, the other half was resuspended in loading dye and boiled for 10 minutes before loading on a 10% SDS-polyacrylamide gel. The proteins were transferred to a nylon membrane by semi-dry blotting using the Semi-Phor (Hoefer) blotter and detected with an antibody directed against GAL4(1 – 147).

RESULTS

Our initial observations were based upon experiments with the protein ATF-4. ATF-4 is a member of the ATF/CREB family and contains a bZIP DNA-binding domain. Using a partial ATF-4 cDNA clone (4) we isolated a full length ATF-4 cDNA from a human placenta λgt11 library. The full-length ATF-4 sequence is identical to a previously described ATF protein, designated TAXREB67 (17).

We analyzed the transcriptional activity of ATF-4 in cotransfection experiments. Because all cell lines examined to date contain high levels of ATF proteins, we reprogrammed the DNA-binding specificity of ATF-4 by fusing its full-length cDNA in-frame to the coding sequences of the yeast GAL4 DNA-binding domain (amino acids 1 to 147). A plasmid expressing GAL4-ATF4 was cotransfected into CV-1 cells with a reporter plasmid containing the CAT gene downstream of a synthetic promoter containing a consensus TATA box (Adenovirus E1b) and two GAL4 binding sites (see Fig.2B). Fig.1A shows that GAL4-ATF4 activated transcription (lane 2), indicating that it contains a constitutive activation region. To our knowledge ATF-4 is the first example of an ATF protein with a constitutive activation domain.

We next sought to determine whether other ATF proteins can affect the activity of ATF-4. Fig.1A shows unexpectedly that transcriptional stimulation by GAL4-ATF4 was strongly inhibited (> 30-fold) by cotransfection of a plasmid expressing either CREB (3) (lane 3) or the highly related protein ATF-1 (4) (lane 4). Significantly, the amount of transfected CREB expression plasmid required for inhibition is comparable to that required for activation of a CRE-containing promoter (see also Fig.6 and data not shown). In contrast, inhibition was not observed upon cotransfection of an ATF-2 expressing plasmid (18) (Fig.1A, lane 5), indicating that not all ATF/CREB proteins have inhibitory activity. Comparable inhibition was observed when lower amounts of reporter plasmid were transfected (data now shown).

One potential class of inhibitory mechanisms involve heterodimerization: ATF proteins, such as ATF-4 and CREB, contain leucine zipper dimerization motifs (4). Conceivably, some ATF heterodimer combinations are transcriptionally inactive. To address this possibility we asked whether CREB would inhibit a GAL4-ATF4 derivative that could not heterodimerize. The N-terminus of ATF-4 contains multiple acidic amino acids (Fig.1B, bottom) and in particular the region 25 to 45 is acidic and contains proline residues.

Figure 1. CREB and ATF-1 can function as transcription inhibitors. (A) Activity of GAL4-ATF4 in the presence ATF proteins. The presence (+) or absence (−) of the GAL4-ATF4 expression plasmid is shown below each lane. For a schematic representation of the reporter plasmid, see Fig.2B. (B) Activity of GAL4-ATF4(1 – 56) in the presence of CREB. As in part A. Below the autoradiogram is shown ATF-4 amino acids 1 – 56 with acidic residues underlined. Transfection was performed with 2 μg of pGAL4-ATF4 or pGAL4-ATF4(1 – 56) and 2 μg pECE-ATF1, pECE-ATF2 or pRSV-CREB.

Figure 2. CREB inhibits activators containing unrelated transcriptional activation and DNA-binding regions. (A) Transfection of GAL4-VP16, GAL4-E1A, GAL4-Pro and LexA-VP16 constructs in the presence of CREB. The presence (+) or absence (−) of the CREB expression plasmid is indicated below the autoradiogram. The cotransfected activator expression plasmid and reporter plasmid is indicated below the autoradiogram. (B) Structure of the reporters and activator proteins. Transfections were performed as in Fig.1 with 1 ng of pGAL4-VP16, 1 ng pLex-VP16, 50 ng of pGAL4-E1A(140 – 182) or 2 μg of pGAL4-Pro, and 2 μg of pGAL4(2-Elb)-CAT reporter or pL6EC, in the presence or absence of 2 μg of pRSV-CREB.
a short potential helical structure reminiscent of some acidic activation domains (19). Indeed, a GAL4 fusion protein containing ATF-4 residues 1–56, GAL4-ATF4(1–56), activated transcription (Fig.1B, lane 1). Significantly, cotransfection of a CREB expression plasmid also inhibited GAL4-ATF4(1–56) (Fig.1B, lane 3). This result strongly argues that inhibition does not involve heterodimerization through their leucine zipper motif: GAL4-ATF4(1–56) lacks the leucine zipper and instead dimerizes through a region in GAL4(1–147).

To determine whether inhibition was specific to GAL4-ATF4, we analyzed GAL4 fusion proteins containing well characterized acidic and non-acidic activation regions. GAL4 fusion proteins containing the acidic activation domain of the HSV1 VP16 protein (amino acids 412 to 490), the metal-binding activation domain of the adenovirus 5 E1A protein (amino acids 140 to 182) or the proline-rich activating region of CTF/NF-1 (amino acids 399 to 499), all stimulated transcription to varying extents (Fig.2). Significantly, CREB inhibited transcription directed by all three activators. Fig.2 also shows that CREB inhibited LexA-VP16, indicating that inhibition did not require the GAL4 DNA-binding domain. Inhibition by CREB occurred in several unrelated cell lines (data not shown), indicating that no species- or tissue-specific factor was involved.

One way by which CREB could inhibit transcription in this assay is to block expression of the activator. To address this possibility we measured the effect of CREB on the steady-state levels of three of the GAL4 derivatives analyzed in Fig.2. Fig.3 shows in an immunoblot assay that upon cotransfection of the CREB expression plasmid, the levels of GAL4-VP16 and GAL4-E1A were unchanged, while the level of GAL4-Pro was decreased only 2-fold. This modest decrease cannot account for the much stronger (>30-fold) inhibition we observed: transfection and assay conditions were performed in the linear range.

To gain insight into the mechanism of inhibition we analyzed several well characterized CREB derivatives (7, 13, 14) (Fig.4). Previous studies have shown that these derivatives are expressed at comparable levels in transfected cells (7, 13, 14). Fig.4 shows that a CREB derivative with a disrupted leucine zipper (CREB ΔLZ) failed to inhibit GAL4-ATF4 (lane 4). Remarkably, a single amino-acid substitution within the basic region of CREB's bZIP DNA-binding domain (CREB304E), which abolishes DNA-binding (13), also eliminated inhibition (lane 5). We conclude that inhibition by CREB requires intact dimerization and DNA-binding domains. In contrast, two CREB derivatives with impaired transcriptional activation regions inhibited GAL4-ATF4 directed transcription: these included ΔCREB (lane 7), the natural variant of CREB that contains a 14 amino acids deletion of the so-called α-peptide (14), and a point mutation at serine 133 in the PKA motif (CREBM1) (lane 6). Identical results were obtained with these CREB derivatives when the activator was GAL4-VP16, rather than GAL4-ATF4 (data not shown).

To rule out the possibility that inhibition was due to binding of CREB to a putative 'cryptic' CRE adventitiously present in the promoters described above, we tested the ability of CREB to inhibit transcription from two completely unrelated promoters. We have previously shown that a GAL4-Tat fusion protein can activate transcription from a Human Immunodeficiency Virus (HIV) LTR derivative containing GAL4 binding sites (20). Figure 5A shows that activation by GAL4-Tat was completely inhibited by CREB. Figure 5B shows that CREB could also partially inhibit transcription directed by the powerful SV40 early promoter. Significantly, the effect of CREB mutants on inhibition of the SV40 early promoter paralleled the results of Fig.4. Thus, these combined data indicate that CREB could inhibit transcription of several completely unrelated promoters, strongly arguing that inhibition was not due to a cryptic CRE.

Activation of transcription by CREB involves phosphorylation by PKA (7). We therefore tested whether PKA affected CREB's ability to inhibit transcription (Fig.6). Cells were transfected with a constant amount of a plasmid expressing GAL4-VP16 and increasing amounts of the CREB expression plasmid. Twelve hours before harvest cells were treated with forskolin and 3-isobutyl-1-methyl-xantherine (IBMX) to raise intracellular cAMP. Fig.6 shows that increasing the intracellular cAMP concentration did not affect inhibition at any level of CREB. Identical results

**Figure 3.** CREB does not affect the steady-state levels of the activators. The presence (+) or absence (−) of the CREB expression plasmid is shown below each lane. The cotransfected activator expression plasmid is indicated below the autoradiogram. CV-1 cells were transfected as in Fig.1 with 2 μg pGAL4-Tat-E1b-CAT reporter alone (lane 1), reporter and 1 μg pGAL4-VP16 (lanes 2–3), or pGAL4-E1A (lanes 4–5) or pGAL4-Pro (lanes 6–7) in the presence (lanes 3, 5 and 7) or absence (lanes 1, 2, 4 and 6) of 2 μg pRSV-CREB.

**Figure 4.** Transcription inhibitory activity of CREB derivatives. (A) Activity of GAL4-ATF4 in the presence of CREB derivatives. As in Fig.1. (B) Schematic representation of the CREB derivatives. 1 μg of pGAL4-ATF4 was cotransfected with 2 μg pGAL4-Tat-E1b-CAT reporter and 2 μg of each of the CREB coding plasmids as described in Fig.1.
we discuss several possible models for how CREB could inhibit transcription in such a promiscuous fashion.

One possibility is that CREB inhibits transcription by a squelching type of mechanism (21). While difficult to rule out absolutely, for several reasons we do not favor this idea. For example, the activation domain of CREB has been mapped and is separable from the bZIP DNA binding domain (22). We find that a single amino acid substitution in the basic region, and an in-frame deletion of the leucine zipper, eliminated inhibition by CREB; if squelching was the inhibitory mechanism these mutations would be predicted to either have no effect or perhaps even to increase inhibition. Conversely, mutations within the known activation region of CREB did not significantly affect its ability to inhibit transcription.

An alternative model for inhibition would involve direct binding of CREB within the vicinity of the target promoter. We again do not favor this model for several reasons. First, the reporter plasmids used in these experiments contain synthetic promoters, which lack a CRE. Second, inhibition was observed using several different promoters that are completely unrelated to one another. Third, if a cryptic CRE were responsible for inhibition we would expect that relatively high levels of CREB would be required for inhibition. However, the amount of transfected CREB expression plasmid required to inhibit transcription was comparable to that required to activate a consensus CRE-containing promoter. Finally, the possibility that the reporters contain a cryptic CRE would still not explain why CREB inhibited, rather than activated, transcription particularly in the forskolin induction experiment of Figure 6.

A final possible inhibitory mechanism would involve direct interaction between CREB and the activators. This also seems implausible: inhibition was observed with activators containing unrelated DNA-binding domains and activation regions.

The model we favor is premised on the observation that mutations in the basic region and leucine zipper of CREB eliminated inhibition, suggesting that inhibition requires DNA binding. However, as discussed above, in our experiments the repressed promoters lack a CRE, suggesting that the target CRE must be present elsewhere. Based upon these considerations, we suggest a working model in which CREB binds to a CRE in a cellular promoter and down-regulates a gene encoding an essential transcription factor. For example, CREB could block binding of a positively-acting ATF protein, or antagonize a DNA-bound activator required for expression of the putative cellular gene.

This proposal does not imply that CREB will inhibit all promoters equally. For example, we have found that CREB only partially inhibits the strong SV40 early promoter/enhancer (Figure 5B). Differences in the level of inhibition may reflect, for example, variable abilities of promoters to compete for a limiting transcription component. Alternatively, recent studies indicate that promoters may differ in their requirements for some basic transcription factors (23). These possibilities could explain how CREB, a potent transcription activator, avoids repressing itself when activating a CRE-containing promoter. The difference between CREB's ability to activate or inhibit transcription may also be related to phosphorylation. Although inhibition by CREB does not appear to be modulated by PKA, we do not rule out the possible role of other phosphorylation events: CREB contains potential phosphorylation sites for several kinases (22, 24, 25). Finally, the ability of CREB to activate or inhibit transcription may be dependent on promoter context, i.e., the other transcription factors bound to the promoter. For example, CREB can modulate the activity of the somatostatin promoter in a phosphorylation-independent fashion (26).

DISCUSSION

In this paper we show that CREB and ATF-1 can inhibit activation by diverse classes of transcription factors, whose DNA-binding and activating regions are unrelated to one another. This inhibition requires intact CREB dimerization and DNA-binding domains, and does not involve phosphorylation of CREB by PKA. Below we discuss several possible models for how CREB could inhibit transcription in such a promiscuous fashion.
There have been several previous reports that a specific ATF/CREB protein could repress transcription of a particular target promoter (8, 9, 27, 28). However, in these instances repression required a CRE/ATF site in the target promoter (27, 28), or inhibition was relieved by PKA phosphorylation (8, 9). It therefore seems unlikely that the relatively specific repression observed in these previous studies is related to the more general inhibition described here. In any case, it is evident that CREB can either activate or repress transcription dependent upon particular circumstances such as the target promoter. Other transcription regulators, such as the YY1/NF-E1/6 protein (29), steroid receptors and AP-1 (30–33) can also either activate or repress transcription in different promoter contexts.

It is interesting to speculate upon the role of CREB's transcriptional inhibitory activity. Increased intracellular cAMP acts through CREB to preferentially induce a set of proteins, such as neuropeptides, that are expressed from CRE-containing promoters. Repression by CREB may represent the complementary component of this transcriptional induction: by repressing transcription of some cellular genes CREB facilitates the selective expression of cAMP-inducible genes. Similarly, induction of gene expression in response to heat-shock also involves both positive and negative regulation (34).

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