Three Distinct Regions within the Constitutive Activation Domain of cAMP Regulatory Element-binding Protein (CREB) Are Required for Transcription Activation*

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The transcription factor cAMP regulatory element-binding protein (CREB) mediates both constitutive and cAMP-induced gene expression through distinct, independently acting domains. The constitutive activation domain (CAD) (amino acids (aa) 165–252) encompasses and overlaps exon 9 of the CREB gene (E9, aa 180–243). In the present study, deletion of either the CAD or exon 9 from CREB-GAL4 (CRG) reduced constitutive activity to less than 2-fold, without affecting kinase inducible activity. However, fusion of the CAD to the GAL4 DNA binding domain (CAD-G4) stimulated transcription, whereas fusion of exon 9 sequences did not. Deletion of the amino-terminal flanking region of exon 9 (aa 165–180), but not COOH-terminal flanking sequences (aa 243–252), decreased constitutive activation in either the CAD-G4 or CRG background. Deletion of the previously characterized glutamine-rich region (Q3, aa 218–252) or of a region containing a hydrophobic cluster of amino acids (HC, aa 180–218) also reduced constitutive activation by either CAD-G4 or CRG. No single mutation of hydrophobic residues within HC impaired activity of the CAD, but double and triple mutations did, suggesting that multiple weak interactions are involved in function of the HC region. Thus, exon 9 of the CREB gene is necessary but not sufficient for constitutive activation. The CAD requires three distinct regions for function, suggesting that CREB may interact with multiple targets in the RNA polymerase II complex.

The CREs are binding sites for transcription factors of the CRE-binding protein (CREB)/activator transcription factor family (13–15). Mutation of the CRE reduces constitutive expression of several genes in addition to abolishing inducibility by cAMP (4, 16). CREB is constitutively bound to the CRE of the phosphoenolpyruvate carboxykinase gene and others where it activates basal transcription (17–19). Hormone-induced activation of transcription by CREB is triggered by phosphorylation of Ser-133 in CREB by several protein kinases (cAMP; protein kinase A (12, 20); Ca2+; calmodulin (7); nerve growth factor; Ras-dependent protein kinase (8)) in different cells. The constitutive activation domain of CREB not only establishes the basal level of phosphoenolpyruvate carboxykinase gene transcription, but also influences glucocorticoid induction, both through its influence on basal activation (21) and by direct interaction with glucocorticoid receptor (22). We and others have shown that CREB contains independent domains that mediate constitutive and kinase-inducible transcription (9, 23–25).

The CREB gene contains 11 exons spread over more than 40 kilobases of DNA and is expressed ubiquitously (26, 27). A duplicate locus encoding CREM (cyclic AMP-responsive element modulator) encodes both activators and repressors of transcription due to alternate splicing of its mRNA (28). In contrast to the ubiquitous expression of the CREB gene, expression of CREM is restricted by cell type and developmental stage, acting primarily in the gonads and the pineal gland, and is acutely inducible by cAMP (22, 29). Exon 9 of CREM (roughly equivalent to exon 9 of the CREB gene) codes for a glutamine-rich domain, whose insertion into CREM mRNA by alternate splicing switches the CREM protein from acting as a repressor to an activator during spermatogenesis (29, 30). There is no evidence for regulation by induction or by alternative splicing of exon 9 in the CREB mRNA, but the exon 9 sequences of the CREB and CREM genes are highly homologous (27).

We have identified a constitutive activation domain (CAD) in the COOH-terminal region of CREB that mediates the majority of basal activation by CREB and functions autonomously when fused to the GAL4 DNA binding domain (9). The CAD of CREB (aa 165–252) overlaps exon 9 sequences of the CREB gene (aa 180–243) by 15 amino acids at its amino terminus and by 9 amino acids at its carboxyl terminus. The CAD functionally defined in our previous study contains two subdomains: Y, previously uncharacterized, and Q3, a glutamine (Q)-rich region. Each of these subdomains provided weak activation when fused to the GAL4 DNA binding domain, but together they acted synergistically and restored the majority of CREB constitutive activity. A 15-amino acid serine- and threonine-rich region (ST3) flanks exon 9 at its 5' boundary, and 9 amino acids of Q3 flank the 3' boundary of exon 9. One goal of the present studies was to determine whether exon 9 was necessary and sufficient to encode the CREB CAD.

Within the CAD, the Y subdomain contains a hydrophobic cluster of amino acids (HC, aa 187–209) of a similar composi-
tion and distribution to a hydrophobic cluster crucial for transcription activation by the strong, constitutive viral activator VP16 (31). In VP16, mutation of a single phenylalanine (Phe-442) abolished transactivation and mutation of adjacent hydrophobic leucine residues reduced transactivation (31, 32). It was proposed that charged amino acids in the VP16 activation domain mediate long range interactions with the basal transcription factors and that stable, tight interactions with the basal factors are formed by the hydrophobic residues (31). Mutation of similar hydrophobic residues in the activation domain of the Rta protein of Epstein-Barr virus reduced activation by 85% (33). Several other transcription activators, such as CREB, SP1, and GAL4, have similar patterns of hydrophobic residues. In the present study, we assessed the relative importance of the hydrophobic residues within the CREB CAD by determining the effects of single and multiple mutations of these amino acids on constitutive and inducible transcription activation.

MATERIALS AND METHODS

Cells, Transfections, and CAT Assays—JEG3 human choriocarcinoma cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Cells at 50–70% confluence were transfected using the calcium phosphate precipitation method (34). G4tk-CAT has already been described (9) and contains four GAL4 binding sites upstream from the herpes thymidine kinase (tk) promoter (34). CRG expression vectors contain the entire activation domain of CREB (aa 1–277) fused to the GAL4 DNA binding domain. CAD-G4 vector expression vectors contain the sequence encoding the constitutive activation domain of CREB (amino acid 165–252) fused to the GAL4DBD. Cells were cotransfected with 10 μg of G4tk-CAT, 1 μg of CRG or CAD-G4, 2 μg of pCH110 (β-galactosidase reference plasmid), and, when indicated, 1 μg of PKA. After 5 h, the medium was removed and cells were washed with phosphate-buffered saline. Cells were harvested 40 h post-transfection. CAT and β-galactosidase assays were carried out as described (9). CAT activity was normalized to β-galactosidase activity for constitutive expression. PKA-inducible activity is derived from the ratio of CAT in cells cotransfected with an expression vector encoding the catalytic subunit of PKA, kindly provided by R. Maurer, Vollum Institute (35), to CAT in cells not cotransfected with PKA (CAT woes CAT-). The results shown represent the mean ± S.E. from 5–27 independent transfections.

Determination of Wild Type and Mutant Protein Stability—JEG3 cells were transfected with 20 μg of wild type or mutant CREB as described above. 48 h after transfection, nuclear extracts were prepared using the Nonidet P-40 protocol (36) and subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with an antibody to the GAL4 DNA binding domain, kindly provided by M. Ptashne, Harvard Medical School (37). All CREB-GAL4 fusion proteins were expressed at similar levels.

In Vitro Mutagenesis—CREB deletion mutants were constructed by asymmetric polymerase chain reaction (38) with a specific mutagenic primer that introduces an Spel site at the borders of a desired deletion and two universal sequencing primers flanking the region to be mutagenized upstream or downstream. The primers used for PCR reactions are as follows.

**Set A**

Primer 1 (sense, 5’-flanking): 5′-GCCATTACCCAGGAGGACCA-3′

Primer 2 (antisense, 3’-flanking): 3′-CGCCTCTTATAGCCTACG-5′

Primer 3 (antisense, Spel site at 5’ boundary of exon 9): 5′-GTTAAGGCAAATCGACCTCCACCGTCA-3′

Primer 4 (antisense, Spel site at 3’ boundary of exon 9): 5′-GTACATCACCACCTAGTGGTCGCAAC-3′

The first PCR reaction was performed with 10 ng of CREB plasmid in 10 μl containing: 10 μl PCR buffer (10 μl), dNTP (0.4 μM), MgSO4 (4 μM), 5’-flanking primer (5 pmol), antisense mutant primer (0.5 pmol), and 1 unit of Vent polymerase (New England Biolabs). Asymmetric amplification was carried out for 30 cycles: 1 min at 95 °C for denaturation, 1 min at 37 °C for annealing, and 45 s at 72 °C for synthesis. The mutated PCR fragment was carried out in this reaction was purified by gel electrophoresis (3% Nusieve gel) with Mermaid (Bio 101 catalog no. 3100), as recommended by the manufacturer. The second amplification was performed by using 3’-flanking primer (5 pmol) and the mutated fragment purified from the first reaction. 30 cycles were carried out: 1 min at 95 °C, 1 min at 55 °C, and 45 s at 72 °C. Vent polymerase, which has a proofreading function, was used in all PCR reactions to reduce the error rate during synthesis. Fragments containing the mutation of interest were isolated from 3% Nusieve gel and cloned back to either the CRG or CAD-G4 vector (a StuI/PstI fragment for amplifications done with primers 1, 3, and 2; a PstI/SpeI fragment from amplifications done with primers 1, 4, and 2). The presence of the expected mutation and the absence of unwanted mutations were verified by dideoxy DNA sequencing of the final product used for experiments.

CREB hydrophobic amino acid mutants were made by reiterated amplification of CREB using mutated primers. In order to obtain different mutations using a single synthetic oligonucleotide preparation, a mixed population of mutagenic oligonucleotides were primers were produced by incorporating a set ratio of wild type/mutant nucleotide in codons targeted for mutagenesis during oligonucleotide synthesis. The ratio of wt/mut sequence was set to favor the synthesis of oligonucleotides containing multiple mutations. For primer 2 in set A below, the ratio of wt/mut nucleotide was set at 0.3 to produce 9% wt, 42% singly mutated, and 49% doubly mutated products. For primer 4 in set B, the ratio of wt/mut nucleotide was set at 0.7 to produce 24% wt, 41% singly mutated, 27% doubly mutated products, and 8% each with 3 or 4 mutations. Two sets of primers used in this section are as follows.

**Set A**

Primer 1 (sense, 5’-flanking): 5′-GCCATTACCCAGGAGGACCA-3′

Primer 2 (antisense, G188R, L193Q): 3′-GGGGACCTGTTACAACTCGT/AGACCTTACGAGGAGG/AGACCTATACGCAGGCA-3′

Amplifications were performed for 1 min at 95 °C denaturation, 1 min at 62 °C for annealing, and 1 min at 72 °C for extension. A StuI/PstI fragment containing G188R and/or L193Q mutations or KpnII/HpaI fragment containing different combinations of V201G, L204Q, L207S, and M209T mutations were subcloned back into a shuttle vector. All of the amplified sequences were analyzed by DNA sequencing to identify the mutations introduced and to verify that no unwanted mutations had been introduced. Then, the desired Spel fragments containing the desired mutations were cloned back into both the CRG and CAD-G4 vectors.

RESULTS AND DISCUSSION

We have shown previously that deletion of either the Y or Q3 regions reduces constitutive activity of CRG and that deletion of both abolishes constitutive activity (9). Similarly, the fusion of either Y or Q3 alone to the GAL4DBD provided little constitutive activity but fusion of both Y and Q3 to the GAL4DBD restored the majority of basal activity. This bipartite constitutive activation domain includes and overlaps exon 9 of the CREB gene. We now recognize that subdomain Y includes a cluster of hydrophobic amino acids that are similar in composition and distribution to a hydrophobic cluster required for activation by VP16 (31). In order to examine the requirements for CREB CAD function, we constructed vectors containing:

1) single or multiple mutations of sequences within the CAD but outside of exon 9, or 2) point mutations in amino acids within the hydrophobic cluster. These mutations were made and analyzed in the context of CAD-G4 (an 165–252 fused to the GAL4DBD) to determine whether they affected autonomous function of the CAD. The same mutations were made and analyzed in the context of the entire CREB activation domain (an 1–277) in CRG to determine their effects on the native CREB activation.
The structure of wild type and mutated forms of CREB expression plasmids are shown on the left. JEG3 cells were cotransfected with 10 μg of G4tk-CAT, 2 μg of PCH110, and 1 μg of the indicated CRG plasmid, in the absence or presence of 1 μg of RSV-PKA expression plasmid. Constitutive CAT activity represents the CAT activity corrected for β-galactosidase activity. PKA-induced CAT activity is the CAT activity obtained in the presence of PKA divided by that obtained in the absence of PKA. The results shown are the mean ± S.E. from a minimum of five independent experiments.

Role of Exon 9 in CREB CAD Function—In some cases, exons completely encode functional domains of proteins. We tested whether this is true of exon 9 of the CREB gene. To determine whether CREB exon 9 is necessary for CAD function, we deleted precisely the amino acids encoded by exon 9 of the CREB gene (aa 180–243). This deletion (CRG-AE9) abolished constitutive activity of CRG without affecting inducible activity (Fig. 1). Deletion of both the Y and Q3 subdomains of the CAD (aa 165–252) also abolished constitutive activity without affecting inducible activity. In contrast, fusion of the Y and Q3 subdomains of CAD to the GAL4 DNA binding domain (CAD-G4) restored the majority of constitutive activity, whereas fusion of exon 9 sequences to the GAL4 DNA binding domain (E9-G4) provided little constitutive activity. Stable proteins were produced by these fusion proteins, as by all fusion proteins and mutants tested in this study, as assessed by Western blotting with an antibody against the GAL4DBD (data not shown). These results demonstrate that exon 9 is necessary but not sufficient for CAD function and does not encode the CREB CAD. However, exon 9 does encode subdomains of importance to CAD function, as expected from our previous studies, in which deletion of either Y (aa 165–218) or Q3 (aa 218–252) severely impaired constitutive function and deletion of both Y and Q3 abolished constitutive activity. Since exon 9 of CREM encodes nearly identical sequences (27, 28), we speculate that neither the CREB nor CREM exon 9 directly encodes a complete constitutive activation domain. An analogous situation is observed with exons 10 and 11 of the CREB gene, each of which encodes a substantial but incomplete portion of the CREB DNA binding domain (27, 39).

Components of the CAD—To determine the contribution of sequences that flank exon 9 to CAD function, deletions of the 5′- and 3′-flanking sequences were made and the function of the proteins encoded by these mutants was analyzed. Deletion of the 3′-flanking sequences (CRG-Δ3′F, Fig. 2) had no effect on constitutive activity. Hence, these sequences are not required for CAD function. In contrast, deletion of the 5′-flanking sequences (CRG-Δ5′F) reduced constitutive activity, indicating that this region (aa 165–180) is required for CAD function. To determine whether the function contributed by the Y subdomain was contained entirely within this region flanking exon 9, the remaining sequences within Y were deleted (CRG-ΔHC; aa 180–218). Constitutive activity was diminished in CRG-ΔHC, indicating that the hydrophobic cluster region within aa 180–218 is required for CAD function. As in our previous studies, deletion of Q3 (CRG-ΔQ3) reduced constitutive activity. None of these mutations affected PKA-inducibility (Fig. 2) or the amount of protein detected by Western blotting with an anti-GAL4DBD antibody, so they do not simply destabilize the proteins made.

To determine whether these regions are essential for autonomous function of the CREB CAD, the same mutations examined above were analyzed in the CAD-G4 background (Fig. 3). As in the CRG background, deletion of the 3′-flanking sequences in CAD-Δ3′F were not necessary for constitutive activity. Also in parallel to the results obtained with CRG plasmids, ST3 (CAD-ΔST3), HC (CAD-ΔHC) and Q3 (CRG-ΔQ3) were all necessary for constitutive activity. Taken together, these data indicate that there are at least three critical regions of the CREB CAD, as illustrated in Fig. 4A, although there could be more. ST3 flanks the 5′ border of exon 9 and is rich in serine and threonine (8 of 15 aa are Ser or Thr). There are two additional ST-rich regions in the amino terminus of CREB. ST1 (aa 39–54) is part of a weak basal activation domain and is adjacent to Q2 (aa 62–87), which is also required for the amino-terminal CAD. ST2 (aa 108–121) is an essential part of the kinase-inducible domain (KID) and is phosphorylated in vivo (24, 40). There is no evidence for phosphorylation of the ST3 region in CAD, so the relevance of the enrichment in serine and threonine to function of this region is unclear. HC contains the hydrophobic cluster related to those important for the function of VP16, the Rta protein and perhaps SP1, and GAL4. HC is discussed in detail below. Q3 is rich in glutamine residues like the activation domains of SP1 and other transcription factors (41). There are two other glutamine-rich regions in CREB: Q1 (aa 23–37), which appears to be dispensable; and Q2 (aa 62–87), that, together with ST1, forms the weak amino-terminal basal activation domain in CREB. Removal of any one of the CAD regions, ST3, HC, or Q3, prevents constitutive transcription activation by the CRG or CAD-G4 proteins, suggesting that the CAD may interact with...
that obtained in the absence of PKA. The results shown are the mean ± S.E. from a minimum of five independent experiments.

multiple targets in the basal transcription apparatus.

Role of the Hydrophobic Cluster—As discussed above, Phe-442 and 2 flanking leucine residues of VP16 are crucial for activation of transcription by the amphipathic helix region of VP16 (31, 32). To examine the importance of the corresponding amino acids within the hydrophobic cluster of CREB, we mutated these amino acids singly and in combination. Leu-204 in CREB is in an analogous position to Phe-442 of VP16 (31, 32). This indicates that there was no gross perturbation of protein stability or structure as a result of these mutations. Most single hydrophobic residue mutants had near wild type constitutive activity (Fig. 5). The exceptions were L193Q and L204Q, which showed reduced activity. Mutation of Leu-204 to glycine rather than glutamine had no effect on activity, indicating either that glycine is sufficiently hydrophobic to substitute in this position or that the diminished activity of the L204Q mutant is due to local disruption of structure resulting from the introduction of a charged residue. We think that the latter is more likely because these same mutants had essentially wild type constitutive activity in the CAD background (Fig. 5). These results indicate that no single hydrophobic residue is essential for CREB CAD function. This result is in marked contrast to the case of VP16, in which mutation of a crucial hydrophobic residue abolished transcriptional activation (31, 32).

To determine whether more than one hydrophobic amino acid in the CREB CAD is required for interaction with its target, multiple hydrophobic mutations were introduced. Many, although not all, of these multiply mutated CRGs and CAD-G4s exhibited reduced constitutive activity (Fig. 6). PKA-inducible activity was not greatly affected in any of these mutants. Furthermore, the pattern of slight reduction in PKA-inducible activity bore no relation to the reductions seen in constitutive activity. Unlike the single hydrophobic residue mutants, multiple hydrophobic residue mutants had reduced constitutive activity in the CAD-G4 background, as well as in the CRG background. Mutations involving Val-201 + Leu-204 tended to have the most consistent and largest effects. These residues are in positions corresponding to the critical phenylalanine Phe-442 and the preceding leucine residues required for activation by VP16 (31, 32).

Transcription activation involves interactions between several proteins. Fine-tuned regulation of transcription is likely to require multiple weak interactions that stabilize the transcription complex rather than a few strong interactions. Multiple hydrophobic contacts appear to be required for interaction of
the CREB CAD to produce constitutive activity. The involvement of multiple weak contacts is consistent with the lower transactivation potential of the CREB CAD relative to the basal level of transcription in promoters that are regulated by CAMP, and often by other hormones as well.

The requirement for three distinct structural motifs, the serine/threonine-rich ST3 region, the hydrophobic cluster, and the glutamine-rich Q3 region, suggests that the CREB CAD may make contact with multiple targets in the transcription initiation complex. Mutation of each of these regions reduced constitutive activity (Figs. 1–3), yet none of these regions is able to make contact with multiple targets in the transcription initiation complex. One target of the CAD is likely to be TAF110, because Ferreri et al. (43) showed that this region is required for interaction of the CREB CAD with its targets in the yeast two-hybrid system. A similar region with sequence similarity between CREB and SP1 is required for interaction with one of these targets. One target of the ST3 and Q3 domains remain to be identified. They are likely to be basal transcription factors, given the cis-regulatory function of the CREB CAD. Of course, the targets of the CREB CAD could be located either in a single protein or in more than one protein. It also is likely that the targets of the CAD are distinct from the targets of the kidnuc-inducible domain, based on the independence of the KID and CAD domains (9). This idea is supported by our previous study showing that the tk minimal promoter serves as a target for the CAD in JEG3 cells, whereas the SV minimal promoter does not (9) and by the lack of effect of mutations within the CAD upon PKA-inducible function in the present study. In summary, the CAD in CREB has three essential components that are distinguishable on the basis of their amino acid compositions and are all required for constitutive activation of transcription. Exon 9 of the CREB gene encodes two of these and is necessary but not sufficient for CAD function. Multiple, weak interactions of hydrophobic amino acids within HC and between ST3, HC, and Q3 are required for interaction of the CREB CAD with its targets in the transcription initiation complex.

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