Acetylation of cAMP-responsive Element-binding Protein (CREB) by CREB-binding Protein Enhances CREB-dependent Transcription*

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The coactivator function of cAMP-responsive element-binding protein (CREB)-binding protein (CBP) is partly caused by its histone acetyltransferase activity. However, it has become increasingly clear that CBP acetylates both histones and non-histone proteins, many of which are transcription factors. Here we investigate the role of CBP acetylase activity in CREB-mediated gene expression. We show that CREB is acetylated within the cell and that in vitro, CREB is acetylated by CBP, but not by another acetylase, p300/CBP-associated factor. The acetylation sites within CREB were mapped to three lysines within the CREB activation domain. Although inhibition of histone deacetylase activity results in an increase of CREB- or CBP-mediated gene expression, mutation of all three putative acetylation sites in the CREB activation domain markedly enhances the ability of CREB to activate a cAMP-responsive element-dependent reporter gene. Furthermore, these CREB lysine mutations do not increase interaction with the CRE or CBP. These data suggest that the transactivation potential of CREB may be modulated through acetylation by CBP. We propose that in addition to its functions as a bridging molecule and histone acetyltransferase, the ability of CBP to acetylate CREB may play a key role in modulating CREB-mediated gene expression.

Cyclic AMP (cAMP) is a second messenger produced in cells in response to neurotransmitters and hormones (1). Increases in cAMP levels activate a cAMP-dependent protein kinase, protein kinase A (PKA),† which in turn phosphorylates transcription factors, resulting in activation of gene transcription.

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¶ The abbreviations used are: PKA, cAMP-dependent protein kinase (protein kinase A); AD, activation domain; bZIP, DNA binding/dimerization domain; CAT, chloroformic acetyltransferase; CBD, CREB binding domain; CBP, CREB-binding protein; CRE, cAMP-responsive element; CREB, cAMP-responsive element-binding protein; CREB 5K/A, five lysine mutations within CREB; P/CAF, p300/CBP-associated factor; SRIF, somatotropin release inhibiting factor; RSV, Rous sarcoma virus; TSA, trichostatin A; WT, wild-type.

The transcription factor CREB (CRE-binding protein) is the best studied link between PKA activation and gene transcription. CREB was originally described as a transcription factor that binds to an 8-bp element known as cAMP-response element (CRE) in the somatostatin gene promoter (2). This DNA element mediates transcription in response to changes in cAMP levels. Subsequently, CREs were found in promoters of other genes activated by cAMP (3). The critical step in cAMP-induced, CREB-mediated gene expression appears to be phosphorylation of CREB by PKA at a single serine (Ser-133). CREB, when phosphorylated at Ser-133, binds to a nuclear protein, CBP (4), and a closely related protein p300 (5), a protein first identified through its ability to associate with E1A (6). p300/CBP has been shown to interact with many cellular proteins, many of which are transcription factors, supporting the concept that these coactivators may function more generally in signal integration (7).

Precisely how CBP affects gene transcription has not been resolved. One model is that CBP links DNA-bound activators to the general transcription machinery (8–11). In addition to its “bridging” function, p300/CBP (12) and its associated protein P/CAF (13) may enhance gene transcription by remodeling chromatin through the acetylation of histones. To date, several known transcriptional regulators are known to possess intrinsic histone acetyltransferase activity: GCN5 and its homologs (14, 15), P/CAF (13), p300/CBP (16), TAFII 250 (17), and the nuclear hormone receptor coactivators, SRC-1 (18) and ACTR (19). The targets of histone acetyltransferases are not restricted to histones, however (for a review, see Ref. 20). Acetylation of general transcription factors such as TFIIH and TFIIF (21) has also been demonstrated. Acetylation of factors related to transcription can either have positive or negative effects on transcriptional regulation. For example, acetylation of tumor suppressor p53 at Lys-373 and Lys-382 by CBP increases p53 DNA binding (22–24); in contrast, CBP acetylation of a Drosophila protein TCF at Lys-25 inhibits its interaction with the coactivator Armadillo, resulting in reduction of gene expression (25). These studies demonstrate that in some cases, acetylation of histones by histone acetyltransferases may not be the primary event in regulation of the activity of these transcription pathways.

Acetyltransferase activity is critically important for the coactivator function of CBP (26, 27). The observation that CBP and p300 may acetylate other non-histone proteins leads us to investigate whether CBP could acetylate CREB and whether acetylation of CREB influenced its activation function. Treatment with deacetylase inhibitors such as trichostatin A (TSA) and butyrate has been shown to enhance CREB-mediated gene transcription on a stably transfected CRE reporter but not on a transiently transfected CRE reporter (28). In addition, TSA
treatment prolongs the phosphorylation of CREB after forskolin stimulation, suggesting that acetylation plays a role in regulating CREB function, perhaps at the level of regulating phosphorylation of CREB. However, in contrast to these findings, we demonstrate here that TSA treatment enhances a somatostatin reporter gene activity in a transient transactivation assay. Furthermore, we show that CBP, but not P/CAF, acetylates CREB in vitro and that CREB is acetylated within the cell. We mapped the CBP-acetylated lysines to three of the five lysines within the CREB activation domain. Substitution mutations of the target lysines within the CREB activation domain which are acetylated by CBP result in enhancement of CREB-mediated gene expression. These results suggest that acetylation of CREB by CBP may modulate CREB intrinsic activity as a transcriptional activator.

**MATERIALS AND METHODS**

**Expression Vectors**—The construction of Re/RSV-FLAG-CREB341 was described by Kwok et al. (8). pDNA3-FLAG-CREB was subcloned from the HindIII and XhoI fragments of Re/RSV-FLAG-CREB, Re/RSV-FLAG-CREB lysine mutants were generated by site-directed mutagenesis (Stratagene). pET28b CREB His6 WT and its mutants were generated using PCR, and the PCR fragments were subcloned into pET28b in-frame with six copies of histidine at the carboxyl terminus. GAL4-CREB1–283 was constructed by fusing the GAL4 DNA binding domain (1–147) to the amino terminus of CREB1–283. GAL4-CBP CBD (451–652) was described by Kwok et al. (29). VP16-CREB341 and its lysine mutants were constructed by fusing CREB341 to the carboxyl terminus of the activation domain of VP16. All sequences were confirmed by sequencing.

**Recombinant Proteins**—The procedure to generate purified CBP with two copies of FLAG tag (CBP 2XFLAG) and FLAG-P/CAF was described by Kashanchi et al. (30). Baculovirus expressing FLAG-P/CAF was obtained from Rich Maurer. His-tagged CREB341 protein, the CREB activation domain (CREB1–283), and its lysine mutants were produced in bacteria and purified using nickel-nitrilotriacetic acid resin as described by the manufacturer (Qiagen).

**F9 Cell Transactivation Assay**—The F9 cell transactivation assay was described by Kwok et al. (8). F9 cells were plated at 0.15 × 10⁶ cells/60-mm plate. DNA was transfected using calcium phosphate precipitation (Invitrogen). Re/RSV vector was used to normalize the total DNA used for each sample. Procedures to determine chloramphenicol acetyltransferase (CAT) and luciferase activities were described by Kwok et al. (8).

**Cell Labeling**—COS-7 cells were seeded at 0.7 × 10⁶ cells/100-mm plate and were maintained in 10% fetal bovine serum (Invitrogen) in Dulbecco’s modified Eagle’s medium. A day later, the cells were transfected with 15 μg of pDNA3-FLAG-CREB WT using calcium phosphate precipitation (Invitrogen). Control cells were transfected with pDNA3 alone. Two days later, the cells were incubated with 1 μCi/ml sodium [3H]acetate (2.5 Ci/mmol) (ICN) for 18 h. The labeled proteins were then subjected to immunoprecipitation as described by Kwok et al. (29) using FLAG-M2 antibodies (Sigma). The precipitated proteins were separated by 10% SDS-PAGE, enhanced with Amplify (Amersham Biosciences), dried, and exposed to x-ray film (Kodak) at −70 °C for 4 weeks.

**Phosphorylation of CREB by PKA**—Purified CREB341 proteins were phosphorylated by recombinant catalytic subunit of PKA in the presence of ATP as described by Kwok et al. (8, 29).

**Acetylation of CREB by CBP**—Purified CREB341 wild-type (WT), CREB1–283, or its lysine mutant proteins (0.5 μM) were acetylated in the presence of purified full-length 50 μM FLAG-tagged CBP and [14C]-acetyl-CoA (Amersham Biosciences) (60 μCi/μmol, final concentration of acetyl-CoA, 30 μM in 20 μl). The reaction buffer contained 10 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM sodium butyrate, and 5% glycerol. The reaction was carried out at 30 °C for 1 h. After acetylation, the acetylated proteins were resolved by SDS-PAGE; the gels were stained with Coomassie Blue and destained by acetic acid/methanol, dried, and exposed to a Bio-Rad phosphorimager screen. The [14C] signal was detected using a Bio-Rad FX phosphorimager.

**Acetylation of Peptides by CBP and P/CAF**—CREB peptides and histone H3 (7–22) peptide were synthesized either by Sigma Genosys or by the Protein Core Facility at the University of Michigan. All the peptides were purified by high performance liquid chromatography to

![Fig. 1. Inhibition of deacetylases enhances CREB- and CBP-dependent transactivation.](image-url)
purified bacteriologically expressed WT CREB1–283 protein and the mutant proteins containing single lysine residue were used in the in vitro acetylation assays. Single lysine mutants were generated by mutation of 4 of the 5 lysines to alanine. As a negative control, all five lysines were mutated to alanine (5K/A). Single lysine mutant proteins and WT CREB-AD (1–283) were incubated in vitro with recombinant CBP and [3H]acetyl-CoA. In these experiments, CBP preferentially acetylates Lys-91 and Lys-136, and to a lesser extent, Lys-94 (Fig. 2C).

Although CBP acetylates CREB in vitro, CREB could also be a substrate of an alternative acetylase within the cell. Thus we also tested whether P/CAF could acetylate peptides corresponding to each of the potential CREB acetylation sites (sequences of individual peptide are shown in Fig. 2A). The results shown in Fig. 2D indicate that although CREB acetylates CREB peptides containing Lys-91/Lys-94 and Lys-136, these peptides are not substrates for P/CAF-dependent acetylation. As a positive control, both CBP and P/CAF acetylate a histone H3(7–22) peptide. These results indicate that CREB-AD is a substrate for CBP, but not P/CAF, in vitro and potentially within the cell.

**CREB Is Acetylated within the Cell**—To determine whether CREB is acetylated within the cell, we transfected COS-7 cells with a FLAG-CREB expression vector and then labeled the cells with sodium [3H]acetate. Labeled FLAG-CREB proteins were immunoprecipitated using the FLAG-M2 monoclonal antibody, separated by SDS-PAGE, and visualized by fluorography. The results shown in Fig. 3A demonstrate that CREB is acetylated in COS-7 cells. To confirm that incorporation of sodium [3H]acetate corresponds to bona fide acetylation of CREB in vivo, FLAG-CREB was immunoprecipitated from transfected COS-7 cells and subjected to Western blotting with an acetyllysine (anti-Ac-Lys)-specific monoclonal antibody (4G12, Upstate Biotechnology) (Fig. 3B).

**Substitution Mutation of Acetylation Sites Enhances the Transactivation Potential of CREB**—We next asked what role the acetylated lysines played in the transactivation potential of CREB. For these experiments, we individually mutated Lys-91, Lys-94, and Lys-136 to alanine and tested the transcriptional activity of these CREB lysine mutants for activation of the camp-responsive SRIF-CAT reporter. As controls, we also mutated Lys-123 and Lys-155, which are not acetylated by CBP, to alanine.

As we and others have demonstrated previously (8, 9), CREB WT activates the SRIF-CAT reporter in a PKA- and dose-dependent manner (Fig. 4A, black squares). The dose-response curves of CREB with single lysine mutations (K91A, K94A, and K136A) were similar, with a slight increase in activity relative to that of CREB WT (Fig. 4A). In the absence of the catalytic subunit of PKA, there were no differences between the activities of the CREB WT and the single lysine mutants (Fig. 4B). At the plateau level of activation (3.6 μg of CREB), there is a slight, but not significant, increase in transactivation by K91A, K94A, and K136A (Fig. 4C). Fig. 4D demonstrates similar levels of CREB expression in the samples used in Fig. 4C. These results suggest that single mutation of the putative CBP acetylation sites has no significant effect on the transactivation potential of CREB.

Because in vitro mapping experiments indicated that CBP acetylates as many as 3 lysine residues in the activation domain of CREB, we next tested whether multiple mutations involving Lys-91, Lys-94, and Lys-136 would affect CREB-mediated gene expression. We first generated CREB double lysine mutants and tested their ability to enhance transcription. With cotransfection of the catalytic subunit of PKA, the CREB double lysine mutants (K91A/K94A, K91A/K136A, and K94A/K136A, significantly enhance CRE-dependent transcrip-
FLAG-CREB (F-CREB). The cells were labeled with sodium $[^3]$H]acetate, and whole cell extracts were immunoprecipitated using FLAG-M2 antibodies. The precipitates were then separated by SDS-PAGE, fixed, enhanced using Amplify (Amersham Biosciences), dried, and exposed to x-ray film. 

Because a Lys → Ala mutation neutralizes the positive charge, it is possible that increases in transactivation caused by a Lys → Ala mutation is caused by an alteration in charge. To address this issue, we mutated each lysine within the CREB-AD to arginine (Lys → Arg) and tested the transactivation activity of these mutants for activation of the SRIF-CAT reporter. The results shown in Fig. 6 indicate that the double lysine (K91R/K94R) and triple-lysine (K91R/K94R/K136R) mutants enhance the activity of CREB in a dose-dependent manner (Fig. 7A) and also at plateau expression levels (Fig. 7C). Thus, the pattern of transactivation of CREB Lys → Arg mutants is similar to that described for the CREB Lys → Ala mutants (Fig. 6). These data indicate that lysine per se, not the charge, determines the function of CREB.

**Lysine Acetylation Sites within the Activation Domain of CREB Are Not Required for Its Interaction with the CRE or CBP**—One explanation of the observed enhancement of CREB-mediated gene expression is that the CREB lysine mutants may have enhanced interaction with CRE. To test this model, we fused the CREB-AD to the DNA binding domain of the activator GAL4 and tested the ability of GAL4-CREB-AD and its lysine mutants to activate a GAL4 UAS-dependent-CAT reporter (5XGAL4-CAT). We reasoned that fusion of the CREB activation domain to a heterologous DNA binding domain would distinguish between mutation-dependent alterations in intrinsic transactivation potential from influences on the DNA binding activity of CREB. However, the results shown in Fig. 8 demonstrate that mutation of these lysines alters the intrinsic activity of CREB in the absence of the CREB DNA binding domain. Like the augmentation of the activity of the triple lysine Acetylation Sites within the Activation Domain of CREB Are Not Required for Its Interaction with the CRE or CBP—One explanation of the observed enhancement of CREB-mediated gene expression is that the CREB lysine mutants may have enhanced interaction with CRE. To test this model, we fused the CREB-AD to the DNA binding domain of the activator GAL4 and tested the ability of GAL4-CREB-AD and its lysine mutants to activate a GAL4 UAS-dependent-CAT reporter (5XGAL4-CAT). We reasoned that fusion of the CREB activation domain to a heterologous DNA binding domain would distinguish between mutation-dependent alterations in intrinsic transactivation potential from influences on the DNA binding activity of CREB. However, the results shown in Fig. 8 demonstrate that mutation of these lysines alters the intrinsic activity of CREB in the absence of the CREB DNA binding domain. Like the augmentation of the activity of the triple lysine acetylation sites, the triple lysine alanine mutants (K91A/K94A/K136A) have no additional effect on CREB-mediated gene activation in F9 cells. F9 cells were transfected with the SRIF-CRE CAT reporter, RSV-luciferase, and varying amounts (1.2, 2.4, 3.6, or 4.8 µg) of either Re/RSV-FLAG-CREB WT or Re/RSV-FLAG-CREB lysine mutants, with (A) or without (B) RSV-cPKA (the catalytic subunit of PKA). The results are expressed as CAT activity after correcting for transfection efficiency with luciferase activity. In A, the experiment was repeated more than three times with similar results. The results shown are a representation of one experiment. In C, 3.6 µg of Re/RSV FLAG-CREB or its lysine mutants was used. The data are expressed as the mean ± S.E. (n = 3). Dark and white bars represent with or without the cotransfection of RSV-cPKA, respectively. D, expression of each CREB protein is shown. Equal amounts of protein were used per lane from the extracts of the experiments from C. The proteins were separated by 10% SDS-PAGE and probed with FLAG M2 antibodies and anti-β-tubulin antibodies.
lysine mutant in the context of full-length CREB, GAL4-CREB-AD K91A/K94A/K136A shows higher activity than GAL4-CREB-AD K91A, GAL4-CREB-AD K94A, or GAL4-CREB-AD WT. These results suggest that lysine mutations within CREB-AD do not alter the ability of CREB to interact with the CRE but rather alter AD function. Lysine mutations within the CREB-AD may also enhance the transactivation function of CREB by enhancing its interaction with CBP. To address this issue, we asked whether increasing CBP expression would enhance the transcriptional activity of CREB lysine mutants as one would expect if these lysines influenced the interaction of CREB with CBP. We have shown previously that in F9 cells, coexpression of CBP enhances CREB-mediated gene expression (8). However, the results shown in Fig. 9, A and B, do not support this hypothesis. In the absence of coexpressed CBP, the transactivation activity of the CREB lysine mutants is increased in a PKA-dependent manner. Cotransfection of CBP enhances CREB WT as well as its lysine mutants in a dose-dependent and parallel manner, suggesting that mutation of the lysine acetylation sites within the CREB-AD does not affect the interaction of CREB with CBP. In the absence of coexpressed catalytic subunit of PKA, CBP has no effect on the transactivation activity of CREB WT or of its lysine mutants (Fig. 9B).

To confirm the results shown in Fig. 9, A and B, we performed a mammalian two-hybrid assay using a GAL4 DNA binding domain fusion with the CREB binding domain of CBP (GAL4-CBP-CREB AD) and CREB WT and its lysine mutants fused to the carboxyl terminus of the activation domain of VP16. The results shown in Fig. 9, C and D, suggest that VP16 CREB WT and its lysine mutants activate GAL4-CBP CBP in a dose-dependent manner with the cotransfection of the catalytic subunit of PKA. As a control, CREB M1, in which the PKA phosphorylation site (Ser-133) is mutated to alanine, does not activate the GAL4-CBP CBP. Without the cotransfection of the catalytic subunit of PKA, VP16 CREB WT as well as its lysine mutants do not activate GAL4-CBP CBP (Fig. 9D).
**DISCUSSION**

It has become increasingly clear that in addition to its bridging function and its histone acetylase activity, p300/CBP regulates the activity of transcription factors and other nuclear proteins by acetylation (20). Although a previous report demonstrated that inhibition of deacetylases enhances CREB-mediated transcription from a stably transfected reporter but not from a transiently transfected reporter (28), our results show that TSA treatment significantly enhances the SRIF-CAT reporter activity transiently transfected cells. The differences between our results and this previous study may be the result of differences in the cell lines used: we used F9 cells in contrast to NIH 3T3 cell line D5 used in their study. Nevertheless, our results suggest that acetylation of non-histone proteins may be responsible for the activation of CREB-mediated expression. We find that CREB is acetylated at 3 lysines within its activation domain by both CBP and p300. Mutation of these lysines significantly enhances CREB-mediated gene expression, suggesting that regardless of the acetylation state of chromatin proteins (transiently transfected templates *versus* stably transfected templates), acetylation of CREB augments the transactivation potential of CREB. We propose a model in which when CREB is activated by PKA phosphorylation, it recruits p300/CBP, and p300/CBP in turn acetylates CREB.

**p300/CBP Specifically Acetylates the Activation Domain of CREB**—In this study we demonstrate that CREB is specifically acetylated by CBP and p300, but not by P/CAF. Bannister et al. (34) have suggested that a glycine or a serine residue immediately before the acetylated lysine is important for CBP acetylation. However, the sequences surrounding the 5 lysines within the CREB-AD do not fit this pattern (Fig. 2A). Thompson et al. (35) reported that a positively charged residue (either lysine or arginine) at either the −3 or +4 position relative to the acetylated lysine is required for CBP acetylation. Although not all CBP acetylation sites fit this profile, the sequences surrounding 2 of the 5 lysines (Lys-91 and Lys-94) within the CREB-AD fit the pattern described by Thompson et al. (35) (Fig. 2A). Furthermore, Lys-91 and Lys-94 locate within the α-peptide region of the CREB molecule, and in some isoforms of CREB, such as CREBα (also known as CREB327), this region is deleted by alternative splicing (36, 37). Studies have shown that CREB341 and CREB327 are uniformly expressed in most tissues (38) and that CREB327, like CREB341, acts as a transcriptional activator of cAMP-mediated gene expression (37, 38). However, one study has suggested that CREB327 may act as an inhibitor of CREB341 (39). In our experiments, mutation of Lys-122 (equivalent to Lys-136 of CREB341), like that of CREB341, markedly increases the ability of CREB327 to activate the SRIF-CRE CAT reporter in the F9 transactivation assay (data not shown).

Nevertheless, the sequence surrounding Lys-136 does not fit the CBP consensus acetylation sequence described by either Bannister et al. (34) or Thompson et al. (35). Moreover, the levels of acetylation by CBP of Lys-91, Lys-94, and Lys-136 differ: Lys-136 has the highest and Lys-94 the lowest level *in vitro* (Fig. 2). The lower acetylation level of Lys-91 and Lys-94

**FIG. 9.** CREB lysine mutations do not affect the interaction with CBP. In A and B, F9 cells were transfected with the SRIF-CAT reporter, RSV-luciferase, Ro/RSV FLAG-CREB, or its lysine mutants (3.6 μg) with (A) or without (B) the cotransfection of the catalytic subunit of PKA and various amounts of Ro/RSV-CBP-HA-RK as indicated. Results are expressed as CAT activity after correcting for transfection efficiency with luciferase activity, and the experiments were repeated more than three times with similar results. In C and D, F9 cells were transfected with a 5XGAL4-CAT reporter, RSV-luciferase, and Ro/RSV GAL4-CREB 451–682 (0.5 μg), with C or without D the cotransfection of the catalytic subunit of PKA, and various amounts of Ro/RSV VP16 CREB341 or its lysine mutants, as indicated. Results are expressed as the mean relative CAT activity after correcting for transfection efficiency with luciferase activity, and the experiments were repeated more than three times with similar results.
may be because the CREB1–283 mutant that contains only Lys-91 has a K94A (+4 lysine) mutation, which disrupts the putative consensus acetylation site described by Thompson et al. Likewise, the CREB1–283 mutant that contains only Lys-94 has a K91A mutation (–3 lysine). Determination of the true relative degree of acetylation by CBP awaits more detailed kinetic measurements.

**Acetylation Alters the Activity of the CREB Transactivation Domain**—In the simplest model, the mechanism by which CREB acetylation might augment CREB activation of gene expression is that these lysines participate in restraining the conformation of the CREB molecule, allowing CREB to enhance gene expression by increasing its 1) interaction with CRE, 2) interaction with CBP, 3) sensitivity to phosphorylation by PKA, and 4) prolonging the dephosphorylation rate of CREB.

Several transcription factors, when acetylated by CBP, increase their binding to DNA (20). CREB has been shown to bind to the CRE with high affinity, but the role of phosphorylation in regulating DNA association remains controversial (40–42). Studies have shown that Tax-1, a human T-cell leukemia virus type 1 protein, facilitates the binding of CREB to the Tax-response element (which is also a CRE) by a direct interaction with CREB and flanking DNA (43, 44). These studies suggest that the binding of CREB to CRE may be altered by other proteins. It is possible that lysine mutation within the CREB-AD may allow CREB to interact with other proteins, resulting in an increase in interaction with CRE. Our results however indicate that CREB acetylation alters transactivation potential independent of the CREB DNA binding domain and interaction with the CRE (Fig. 8).

CREB acetylation or mutation of these lysines may enhance the interaction with CBP. Recent studies have shown that the recruitment of CBP to CREB is a critical factor in CREB-activated gene expression. Cardinaux et al. (45) produced a constitutively active CREB by substituting the CREB kinase-inducible domain with the CBP-interacting sequence of SREBP (DIEDML) (46). This chimeric CREB protein activates the somatostatin CRE reporter independently of PKA by constitutively binding CBP. Mutation of Tyr-134 to phenylalanine (Y134F) increases the phosphorylation of CREB by PKA and permits CREB to interact with CBP in the absence of PKA in vivo (47). Conversely, Shaywitz et al. (48) have shown that altering 1 amino acid (L607F) within the CREB binding domain of CBP increases the binding strength of the kinase-inducible domain of CREB, even in the absence of PKA phosphorylation. These results suggest that the transactivation potential of CREB is a function of the interaction between CREB and CBP. However, using the F9 transactivation assay (Fig. 9, A and B) and the mammalian two-hybrid assay (Fig. 9, C and D), we show that CREB lysine mutation does not affect the ability of CREB to interact with CBP, suggesting that, although one of the acetylated lysines is located within the kinase-inducible domain, a region of CREB that is necessary for the interaction of CREB with CBP, these lysines do not play a substantial role in the interaction between CREB and CBP.

The observation that Lys-136 is acetylated by CBP is intriguing because of its proximity to the PKA phosphorylation site, a site that is conserved in ATF-1 (49) and CREM (50), both of which are activated by phosphorylation. The basic residues surrounding the PKA phosphoacceptor site are important for recognition by PKA. Arginines at –3 and –2 positions relative to the phosphorylation site are preferred for phosphorylation by PKA (51). However, the necessity for basic residues carboxy-terminal to the PKA phosphorylation site is unclear. Du et al. (47) demonstrated that simultaneous mutation of Arg-135 and Lys-136 to glutamine converts CREB to a higher affinity substrate for PKA, resulting in a constitutively active form of CREB. However, it is not known whether mutations of both Arg-135 and Lys-136 are required because the contribution of each residue was not tested individually. Nevertheless, these results suggest that acetylation of Lys-136 may affect the phosphorylation of CREB by PKA. In the absence of the coexpressed catalytic subunit of PKA, CREB lysine mutants show a slight increase in basal activity, perhaps because of an increase in its affinity for PKA as suggested by Du et al. (47). If mutation of these lysines increased phosphorylation, we would expect an enhanced interaction of CREB with CBP in the mammalian two-hybrid assay; however, results shown in Fig. 9D do not support this model. Our results suggest that Lys-91, Lys-94, and Lys-136 restrain the transactivation potential of CREB in a manner separable from effects on phosphorylation, CBP binding, or interaction with DNA.

**What Is the Role of Acetylation in Modulating the Transactivation Potential of CREB?**—An important factor regulating CREB-dependent transactivation is the duration of the phosphorylation of CREB. Studies have shown that after cAMP stimulation, the transcriptional response follows so-called “burst-attenuation” kinetics with maximum rates 30–60 min after stimulation followed by a gradual attenuation phase that may last as long as several hours (52). The attenuation phase is not dependent on a loss of PKA activity but rather results from dephosphorylation of CREB (52, 53). Inhibition of phosphatases prolongs the attenuation phase, resulting in the increase in PKA-stimulated gene expression (52–55). How dephosphorylation of CREB is regulated is not clear, however. Michael et al. (28) demonstrated that inhibition of deacetylase activity, without affecting phosphatase activity, prolongs the phosphorylation of CREB after forskolin stimulation. These results suggest that acetylation of components of the PKA signaling pathway may regulate the dephosphorylation of CREB. Our results are consistent with this observation.

Collectively, our results demonstrate that in addition to acting as a bridging factor as well as a histone acetyltransferase, CBP may regulate the intrinsic transactivation potential of CREB by directly acetylating the activation domain of CREB. The precise mechanism of this alteration in CREB activity is unclear. The three acetylated lysines within the activation domain are important for the transactivation function of CREB. Mutation of these lysines to either alanine or arginine was equally effective in enhancing the activity of CREB, suggesting that a lysine residue at this position rather than a charged residue per se restrains the activity of CREB. We postulate that acetylation, like mutation, increases the activity of CREB, perhaps through an alteration in the structure of CREB to a more active conformation. The structural changes induced by acetylation may prolong CREB phosphorylation by diminishing phosphatase-dependent attenuation of CREB activity, either by directly interfering with recruitment of phosphatases or by altering phosphatase recognition of CREB as a substrate.

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