The CREB-binding protein (CBP) plays a central role in the regulation of gene expression by several different second messenger pathways including serum growth factors, cAMP and phorbol esters. CBP specifically binds to the phosphorylated forms of CREB and c-j un and is thought to activate transcription through a C-terminal activation domain. In this report, we demonstrate that the C terminus of CBP is dispensable for its ability to stimulate phospho-CREB activity, and further, that the deletion of this domain produces highly active, mutant forms of CBP. The novel N-terminal activation identified by this deletional analysis consists of the first 714 amino acids of CBP and is sufficient for high levels of transcriptional activity. This domain is also capable of stimulating the activity of a second cAMP-regulated factor, ATF-1. Surprisingly, ATF-1 activity is not significantly stimulated by full-length CBP suggesting that the C-terminal domain of CBP may also serve to regulate ATF-1/CBP activity. Additionally, the demonstration that one of our hyperactive CBP mutants is able to activate a nonphosphorylatable mutant of CREB (M1 CREB) provides the first evidence that CBP may play a role in regulating the basal transcriptional activity of CREB.

Hormonally induced changes in the levels of intracellular cyclic AMP (cAMP) regulate the expression of many cellular and viral genes through a common promoter element termed the CRE (1). The activity of the CRE is, in turn, governed by a large family of transcription factors known as CREB/ATF proteins. These proteins share several common structural features including a DNA binding and dimerization motif, known as the bZIP domain, and transcriptional activation domains located in the N terminus of the molecule. Although poorly conserved overall, these activation domains share consensus phosphorylation sites for a variety of protein kinases suggesting that their activity, like that of many bZIP factors, is highly regulated by phosphorylation (2, 3).

The transcription factor CREB (cAMP response element-binding protein) represents the prototypical member of this family and it, along with the related factors ATF-1 and CREM, mediates many aspects of cAMP-regulated gene expression (4).

The activation domains of all three of these proteins are highly homologous and can be divided into three basic elements, a kinase-inducible domain (KID), which contains a consensus phosphorylation site for the cAMP-dependent kinase (PKA), and two glutamine-rich domains (Q1 and Q2) flanking the KID. The phosphorylation of the CREB KID at a consensus PKA site (serine 133) is required for full transcriptional activity and mutation of this site effectively blocks stimulated activity (5). Although CREB is capable of interacting directly with several components of the basic transcriptional apparatus including TFIIB and TFIID, these interactions are not regulated by phosphorylation (6, 7), and it has been unclear how phosphorylation of the CREB activation domain results in enhanced transcriptional activity. The recent identification of a phosphorylation-dependent transcriptional co-activator of CREB has provided exciting new insights into this significant problem.

The CREB-binding protein (CBP) is a large nuclear phosphoprotein capable of selectively interacting with the phosphorylated form of CREB by recognizing specific residues found in the CREB KID (8–10). This interaction is required for PKA-dependent transcriptional activation as disruption of the complex by microinjection of either specific peptide fragments of the phospho-CREB binding domain or anti-CBP blocking antibodies is able to block stimulated expression of a CRE-dependent reporter gene in fibroblast cells (10, 11). CBP is highly related to the E1a-associated protein p300, and both CBP and p300 are capable of acting as CREB co-activators and binding to E1a (12, 13). The binding of E1a to CBP/p300 is believed to be responsible for mediating many aspects of E1a-dependent transformation and has recently been shown to result in a strong inhibition of transcriptional activity of CBP/p300 (14). Fusion of either the CBP or p300 cDNA to a heterologous DNA binding domain demonstrates that CBP is a potent transcriptional activator on its own (8, 12). This transcriptional activity is thought to reside in the C-terminal portion of the molecule and seems to be related to the ability of CBP to interact with the basic transcription factor TFIIB. Indeed, fusion of the C-terminal portion of CBP, including the TFIIB binding site, to a heterologous DNA binding domain produces a transcriptional activator which is more potent than the full-length CBP fusion protein (9). These results have suggested a simple model of CBP activity in which it binds selectively to the phosphorylated form of CREB proteins and activates transcription by recruiting TFIIB (14). CBP activity is not regulated by phosphorylation-dependent binding alone, however, and mutant forms of CREB have been identified which bind to CBP in a phosphorylation-dependent fashion but are not activated by CBP (15).

CBP/p300 proteins have also been implicated as potential co-activators for several other important transcriptional activators including c-j un, c-Fos, and YY-1 (11, 16, 17). Although the binding of CBP/p300 to both c-j un and CREB appears to be...
phosphorylation-dependent, the binding of CBP/p300 to c-Fos and YY-1 is not, and it remains unclear if the transcriptional activity of CBP is regulated in these cases. Because CBP/p300 is capable of interacting with a wide variety of transcription factors, it is likely that the regulation of its transcriptional activity will play a central role in the overall regulation of gene expression by second messengers. To better understand how CBP activity is regulated, we have generated a series of mutant CBP molecules and assayed them for activity in a co-transfection assay with PKA-dependent transcription factors. In this report, we demonstrate that the previously identified C-terminal activation domain of CBP is dispensable for co-activator function in F9 cells and that the N terminus of CBP is sufficient for transcriptional activation.

MATERIALS AND METHODS

Plasmids—The expression vectors coding for the full-length mouse CBP protein (Rc/RSV mCBP) has been previously described (8, 9) and was a kind gift of Dr. J. Chrizia, St. Louis University. The plasmid CBP ΔE is identical to mCBP with the exception that the sequences between the unique Scal and SmaI sites have been removed deleting amino acids 1569–1891 while retaining the open reading frame. The CBP XbaI expression vector (aa 1–1109) was constructed by completely digesting RSV mCBP with XbaI to remove the 3′ end of the cDNA and then religating the backbone. The SphI vector (aa 1–714) was constructed by subcloning a HindIII/SphI fragment from RSV mCBP into pGEM 3Z and then removing a HindIII/ XbaI fragment from this plasmid by religating it into Rc/RSV. The two internal CBP deletion mutants (ΔN and SΔP) are identical to SphI with the exception that the sequence between the SphI sites at position 1068 and 1494 (aa 357–498) have been removed in SΔP and the sequences between the DraI site and the EcoRI site at positions 160 and 1354 (aa 54–452) have been removed from ΔN. The EcoRI site at position 1494 was blunted with Klenow fragment in the ΔN construct in order to retain the correct reading frame. The ΔCBD vector (aa 1–452) was constructed by cloning the 5′-most EcoRI fragment of mCBP into pBluescript KS (Stratagene) and subsequently removing the HindIII/XbaI fragment from this plasmid into Rc/RSV. The cloning junctions of all mutant plasmids were confirmed by dideoxy sequencing using an unmodified T7 polymerase (Pharmacia Biotech Inc.). The RSV-M1 CREB expression vector was a kind gift of Dr. Richard Goodman, OHSU, Portland, OR. All other plasmids used in this work have been described previously (18).

Cell Culture and Transfection Assays—F9 cells were grown on 0.7% gelatin-coated plates in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.), penicillin (100 units/ml), streptomycin (100 μg/ml), and 1-glutamine (2 mM). Cells were plated at 5.5 × 10^6 cells per 30-cm plate 18 h prior to transfection. DNA was prepared by alkaline lysis, double-banded in CsCl gradients, phenol-extracted, and ethanol-precipitated twice prior to use. Transfections contained 4 μg of a RSV expression vector coding for the indicated transcription factor, 4 μg of RSV-PAK, 5 μg of Δ-70 SS CAT, 20 μg of a RSV expression vector coding for the indicated CBP mutant, and 2 μg of a RSV-GH expression plasmid (gift of L. Roselli-Rehfuss, ICRM, Montreal, PQ) to control for transfection efficiency. In transfections lacking PKA or CBP, an equal mass of Rc/RSV (Invitrogen) was substituted. DNA mixtures were transfected by calcium phosphate precipitation (18 h) without a glycerol shock. Cells were washed, reseeded, and allowed to grow for 30 h before harvesting.

CAT activity was determined by the method of Seed and Sheen (19), and GH immunoreactivity was determined with a commercially available solid-phase RIA (Immunocorp, Montreal, PQ). Relative transcriptional activities were expressed as the ratio of CAT activity to GH activity in arbitrary units. Transfections were repeated three to six times in order to produce reproducibility and are reported as the mean ± S.E.

Western Blotting—Neuro 2A cells were grown on 60-mm plates and transfected by calcium phosphate precipitation for 18 h without a glycerol shock. Cells were washed, reseeded, and allowed to grow for 18 h before harvesting in NET (40 mM Tris (7.5), 150 mM NaCl). After pelleting, cells were lysed in 45 μl of WCE lysis buffer (50 mM Tris (8.0), 400 mM KCl, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin, and 0.6 μM leupeptin) on ice for 15 min and then cleared by centrifugation. The indicated volume of extract was denatured by adding an equal volume of 2X SDS Buffer (117 mM Tris (6.8), 3.4% SDS, 10% glycerol, 0.2 mM dithiothreitol, 2% bromphenol blue) and heating to 90°C for 5 min. Proteins were then separated by SDS-polyacrylamide gel electrophoresis on either an 8% or 10% gel and transferred to polyvinylidene difluoride (Millipore) membrane by electroblotting overnight (30 V). The transfected CBP proteins were detected by using an antibody specific for the N terminus of CBP (CBP-NT; Upstate Biotechnologies) and a chemiluminescence kit (BM Chemiluminescence; Boehringer Mannheim) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The TFIIB Binding Site of CBP Is Dispensable for Function—To determine if the ability of CBP to act as a CREB co-activator was dependent on the presence of the TFIIB binding site, we examined the transcriptional activity of a mutant form of CBP which lacks this region. To do this, sequences which comprise the TFIIB binding site (aa 1569–1891) were removed by constructing an in-frame deletion of the region (Fig. 1A) and the ability of the resulting mutant (CBP ΔE) to potentiating the activity of CREB was determined by measuring its ability to stimulate CREB-dependent transcriptional activity in F9 cells. Transient transfections contained constant amounts of a CREBdependent reporter gene (Δ-70 SS-CAT), a RSV-CREB expression vector, and a RSV expression vector coding for either mouse CBP (RSV-mCBP), the mutant CBP lacking the TFIIB binding site (RSV-CBP ΔE), or no insert. Stimulation of PKA activity was provided by co-transfecting an expression vector...
The N Terminus of CBP Acts as a Potent CREB Co-activator—To localize these additional activator sequences, we generated a series of internal and C-terminal deletions of CBP and tested each of them for their ability to stimulate phospho-CREB activity. As CBP and p300 have both been shown to act as phospho-CREB co-activators, we initially targeted the regions of CBP that were conserved with p300 (Fig. 2A). These conserved regions consist of two small (approximately 100 aa) N-terminal domains and the large (approximately 1300 aa) C-terminal region which had previously been shown to be transcriptionally active (9). To determine if the presence of this large C-terminal domain was required for CBP activity, we generated two truncation mutants which contained either the first 1109 (CBP XbaΔ) or 714 (CBP SphΔ) amino acids of CBP and assayed them for co-activator function as described previously (Fig. 2B). Surprisingly, both C-terminal truncation mutants were able to significantly stimulate CREB activity to similar levels in the presence of PKA. Indeed, both constructs stimulated phospho-CREB activity 20–30-fold over basal levels and 2–3 times better than the wild type CBP. In addition, CBP XbaΔ stimulated basal CREB activity about 5-fold, although CBP SphΔ did not.

To further investigate the nature of this novel N-terminal activation domain, we made additional mutations in the smallest active CBP mutant which were again guided by comparison with the sequence of p300. This region contains two domains which are highly conserved with p300. The first domain contains a putative zinc finger with no known function while the second appears to act as a phospho-CREB binding site. To determine if the first domain was involved in co-activation, we generated two in-frame deletion mutants of CBP SphΔ which removed either most (CBP SΔΔP) or all (CBP ΔN) of these conserved sequences. As a control, we generated a third truncation (CBP ΔCBD) which consists of the first 452 amino acids of CBP (Fig. 2A). We anticipated that this latter mutant would be transcriptionally inactive as it lacks a phospho-CREB binding domain. Transcriptional activity was again assessed by co-transfection and is shown in Fig. 2B. In the absence of PKA stimulation, neither CBP SΔΔP nor CBP ΔN were able to potentiate basal CREB activity. In the presence of PKA, both proteins were able to stimulate CREB activity 5–7-fold over basal levels and, surprisingly, are only slightly less active than the wild type CBP. As expected, deletion of the second conserved domain (ΔCBD) produced a molecule which was unable to significantly stimulate CREB activity, consistent with the idea that the CREB binding domain is required for the stimulation of phospho-CREB activity.

**Figure 2. Transcriptional activity of C-terminal deletions of CBP.** A, schematic representation of CBP mutants showing the structure of each construct. Functional regions of CBP are as described in Fig. 1A. B, relative activity of each of the C-terminal mutants in A to stimulate CREB activity in the presence or the absence of PKA stimulation. Transfections were performed as described and contained the same amount of expression vector either without an insert (Ro/RSV) or with the indicated mutant CBP co-activator. Transcriptional activities were expressed as in Fig. 1.
PKA, and co-transfection of 20 

CREB (Fig. 4). As previously reported, M1 CREB was not able 

ability of the wild type CBP and CBP Sph 

ulate the activity of a nonphosphorylatable mutant of CREB 

phosphorylated. In order to determine whether this activity 

CBP is not believed to interact with CREB unless CREB is 

ulate CREB activity in the absence of PKA was unexpected as 

result in any additional increase in transcriptional activity 

(data not shown). These results suggest that the differences in 

transcriptional activity observed between the different forms of 

CBP are a direct result of intrinsic differences in their abilities 

to activate transcription.

Taken together, our results demonstrate that the previously 

described C-terminal activation domain of CBP is dispensable 

for its ability to stimulate CREB activity and that the N-

terminal 714 amino acids alone are sufficient for full activity. 

The in-frame deletion analysis suggests that this N-terminal 

activation domain consists of at least two sub-domains; the first 

is located in the first conserved domain of CBP (aa 357–498), 

while a second is likely to be located between amino acids 498 

and 714. Because this latter region contains the second conserved 

domain of CBP, it is tempting to speculate that additional 

activator sequences might be located in this region (aa 586–679). 

However, Parker et al. (10) have recently shown that the 

second conserved domain acts as a dominant negative inhibitor of 

CREB activity in NIH 3T3 cells. Therefore, we 

conclude that these additional activator sequences reside in a 

nonconserved portion of CBP (aa 680–714). The demonstration 

that mutant forms of CBP lacking the C-terminal domain are 

significantly better co-activators of CREB suggests that the C 

terminus of CBP may play a role in regulating CBP activity. 

Although the nature of this regulation is unclear from these 

experiments, recent studies have confirmed the idea that the 

transcriptional activity of CBP is regulated independently of its 

binding to phosphorylated CREB (15, 20).

CBP XbaΔ Stimulates the Activity of a Nonphosphorylatable CREB Mutant—The ability of the CBP XbaΔ mutation to stimu-

late CREB activity in the absence of PKA was unexpected as 

CBP is not believed to interact with CREB unless CREB is 

phosphorylated. In order to determine whether this activity 

was the result of CBP XbaΔ binding to trace amounts of phos-

phorylated CREB, we tested the ability of CBP XbaΔ to stimu-

late the activity of a nonphosphorylatable mutant of CREB 

termed M1 CREB (5). As a control, we also determined the 

ability of the wild type CBP and CBP SphΔ to activate M1 

CREB (Fig. 4). As previously reported, M1 CREB was not able 

to stimulate a CRE-dependent reporter gene in response to 

PKA, and co-transfection of 20 μg of either the RSV-CBP or 

RSV-CBP SphΔ expression vector did not enhance its tran-

scriptional activity. In contrast, co-transfection of an RSV-CBP 

XbaΔ expression vector stimulated M1 CREB activity about 

4-fold in both the presence and the absence of PKA stimulation. 

Control transfections (data not shown) demonstrate that this 
effect is specific for the CREB transcription factor as CBP XbaΔ 
is unable to stimulate the transcriptional activity of either a 

Gal4-VP16 transcriptional activator or the estrogen receptor 

under the same conditions. This result suggests that while CBP 
is clearly an important component of phospho-CREB activity, 
it may also play a role in regulating the basal activity of CREB. 

The hypothesis that CBP binds exclusively to the phosphoryl-

ated form of CREB is based on an analysis of the N-terminal 

700 amino acids of CBP (8) and does not rule out the possibility 

of a second CREB interaction site elsewhere on the mole-

cule. Indeed, studies of the role of CBP in the regulation of both c-Fos 

and YY-1 have concluded that both these proteins interact in a 

non-phosphorylation-dependent manner with unique binding 
sites located in the C terminus of CBP (16, 17). Alternatively, 
it is possible that the ability of CBP XbaΔ to activate M1 CREB is 
the result of an indirect interaction through a second adaptor 

protein. Because both CBP SphΔ and CBP XbaΔ are able to 
activate phospho-CREB activity to the same extent and only 

CBP XbaΔ stimulates basal CREB activity, it seems likely that 
this M1 CREB interaction site may be located between amino 

acids 714 and 1109. Preliminary experiments suggest that this 

region does not directly interact with M1 CREB in a yeast 
two-hybrid system (data not shown) supporting the idea of an 
indirect interaction between CBP and M1 CREB.

Wild Type CBP Is Not a Potent Co-activator of ATF-1 in F9 Cells—To determine if the N-terminal activation domain of 

CBP would function in potentiating the activity of a second 
cAMP-regulated transcription factor, we tested the ability of 

the wild type and mutant CBPs to co-activate ATF-1 in F9 cells. 

To do this, co-transfection assays utilizing an ATF-1 expression 

vector were performed as described for CREB. As shown in 

Fig. 5, ATF-1 was 2-fold more active at stimulating the CRE-
dependent reporter gene in the presence of PKA as compared to 

the unstimulated activity. This level of PKA-dependent stimu-

lation is less robust than that observed with CREB; however, 

ATF-1 is known to be less PKA-responsive under these condi-

tions (21). With the exception of CBP SphΔ and CBP XbaΔ, 

none of CBP constructs we tested were able to significantly 

stimulate ATF-1 activity in the presence or absence of PKA. 

Additional co-transfection assays performed with either twice 

the amount of ATF-1 or 1.5 times the amount of CBP confirm 
this result (data not shown) and suggest that the poor tran-

scriptional activity we observed with CBP and ATF-1 is not the
The requirement for CBP to adopt a specific conformation in transactivation events, recruitment of additional regulatory proteins, or these additional signals might include additional phosphorylation of CREB phosphorylation. For the case of the wild type CBP, additional regulatory signals, although the correct positioning to assume this more active conformation in the absence of any signal, deletion of the C terminus would allow the N-terminal activation domain to interact with the phosphorylated form of CREB. In this scenario, deletion of the C terminus would again allow CBP to be transcriptionally active when bound to phosphorylated ATF-1. Further characterization of the events which regulate CBP activity will be required in order to fully understand the role CBP plays in control of gene expression.

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